

# Human exposure to piroplasms in Central and Northern Italy

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## Keywords

*Babesia*,  
Italy,  
Risk exposure,  
Serosurvey,  
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Tick-borne zoonoses.

## Summary

A serosurvey has been conducted in Northern and Central Italy to investigate the presence in humans of antibodies against zoonotic *Babesia* and *Theileria* species. The study focused on a total of 432 volunteers, of which 290 were persistently exposed to tick bites because of their jobs (forester employees, livestock keepers, veterinary practitioners, farmers and hunters) and 142 resident in the same area less frequently exposed. An indirect fluorescent antibody test (IFAT) for humans was used to detect antibodies to *Babesia microti*, IFAT tests for veterinary use were modified to detect reactivity to *Babesia bovis*, *Babesia canis* and *Theileria equi*. A laboratory-derived ELISA was employed to detect antibodies to *Babesia divergens*. Both reactive and 10 negative sera were analysed against plasmodial antigens to evaluate possible aspecificity.

A high reactivity to piroplasm antigens was found, showing significant difference between the sera of the two groups of volunteers (24% vs 7.0%;  $p < 0.001$ ). No cross-reactivity was observed, while each professional group showed reactivity that would fit with the professional risk exposure. In particular, a high reactivity to *B. microti* and *B. divergens* antigens was observed in foresters and hunters (32% and 12%, respectively).

This is the first report on the human seroreactivity to piroplasms in Italy; it also provides additional epidemiological information on these tick-borne zoonoses in Europe. Our findings suggest the possible occurrence of piroplasm infections in Italy and alert physicians to consider these otherwise neglected parasitic diseases when dealing with any febrile illness, especially in subjects exposed to tick bites.

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## Sieroprevalenza delle piroplasmosi nella popolazione umana del Centro e Nord Italia

### Parole chiave

*Babesia*,  
Fattore di rischio,  
Italia,  
Piroplasmosi,  
Sieroprevalenza,  
*Theileria*,  
Zecca,  
Zoonosi.

### Riassunto

Il lavoro descrive i risultati di un'indagine sierologica effettuata nel 2009 nel Nord e Centro Italia per valutare, in campioni di siero umano, l'eventuale presenza di anticorpi verso specie di *Babesia* e *Theileria*, capaci di trasmettere zoonosi. Lo studio ha riguardato 432 volontari, di cui 290 esposti con maggiore frequenza al morso di zecca a causa della loro professione (allevatori, agricoltori, guardie forestali, medici veterinari, cacciatori) e 142 individui residenti nella stessa area ma non esposti allo stesso fattore di rischio. La ricerca di anticorpi verso *Babesia microti* è stata condotta utilizzando kit di immunofluorescenza indiretta (IFI) per la diagnosi nell'uomo. La reattività verso gli antigeni di *Babesia bovis*, *Babesia canis* e *Theileria equi* è stata valutata mediante test IFI per uso veterinario opportunamente modificati. Per la ricerca di anticorpi anti-*Babesia divergens* è stato impiegato un saggio sperimentale (ELISA). I sieri positivi e i 10 sieri negativi sono stati analizzati anche per la reattività ai plasmodi per valutare possibili reazioni aspecifiche.

Lo studio ha rivelato un numero elevato di sieri umani reattivi agli antigeni dei piroplasma, con differenze significative tra i soggetti più esposti e meno esposti alla puntura da zecca (24% vs 7,0%;  $p < 0,001$ ). Non è stata osservata cross-reattività ad antigeni di specie diverse dai piroplasma né a plasmodi. Ogni gruppo professionale ha mostrato una reattività coerente con il rischio di esposizione relativo al lavoro svolto. In particolare, nelle guardie forestali e nei cacciatori è stata osservata un'elevata reattività agli antigeni di *Babesia microti* e *Babesia divergens* (fino a 32% e 12%, rispettivamente).

Questo è il primo studio sulla sieroprevalenza delle piroplasmosi nella popolazione italiana. Lo studio aggiunge ai dati europei nuove informazioni epidemiologiche relative alle zoonosi trasmesse da zecche. I risultati della ricerca evidenziano la possibile insorgenza di casi di piroplasmosi in Italia e suggeriscono ai medici di considerare queste parassitosi, generalmente trascurate, nei casi di sintomatologia febbrile in soggetti a rischio di infezione.

### Introduction

Piroplasmoses are tick-borne emerging zoonoses that can induce malaria-like syndromes. The etiological agents of these diseases are haemoprotozoa of the genus *Babesia* and *Theileria* (Apicomplexa: Piroplasmida), which both infect a wide range of domestic and wild animals and constitute an obstacle to livestock production in farming areas. These parasites are transmitted from specific ticks that feed on several hosts. Humans are included among the parasites' blood sources and, once bitten, can develop diseases of different degree of severity. Human infections were first described in 1957 in Europe in a splenectomised cattle farmer (Skrabalo and Deanovic 1957) and, in 1968, in the US in an asplenic man (Scholtens et al. 1968). To this day, more than 100 *Babesia* species have been described worldwide, but human infection has been associated with only few of them, i.e. *Babesia microti* and *Babesia duncani* in North America and *Babesia divergens* in Europe (Conrad 2006, Leiby 2006, Meliani et al. 2006), where the occurrence of human subclinical and persistent infections due to *B. microti* has also been recently documented (Hildebrandt, 2007). It is noteworthy that over the past few years, further zoonotic *B. divergens*-like pathogens have been identified using molecular techniques both in the United States (the WA1, CA1

and MO1 parasites) and in Europe (the EU1, proposed by Herwaldt et al., 2003 as *Babesia venatorum*) (Herwaldt 2003) and have been shown to cause a significant number of human infections (Centeno-Lima et al. 2003, Herwaldt et al. 2004, Gray 2006, Holman 2006, Vannier and Krause 2009). Although not well-documented, a few reports have also been provided on human infections with other species such as *Babesia bovis* and *Babesia canis s.l.* (Homer 2000).

In Italy, piroplasms affect livestock, pets and wild animals (Pietrobelli et al. 2007, Cassini et al. 2009, Tampieri et al. 2008, Moretti et al. 2010, Cassini et al. 2012) and the first case of human infection has been reported in 2003 (Herwaldt 2003). The main aim of this article is to report the preliminary results of a serological screening performed in Central and Northern Italy to evaluate the seroreactivity to piroplasm antigens in people resident/working in areas where *Babesia/Theileria* species have been detected in animals.

### Materials and methods

#### Population study

During the 2009, 432 blood samples were collected from volunteers living and/or working in

Northern and Central Italy, in areas included within 46°1'33"N-11°54'44"E and 42°24'29"N-12°51'36"E. *Babesia EU1*, *B. microti*, *B. microti*-like, *B. divergens*, *B. canis*, and *Theileria equi* have been previously found in animals living in these areas (Pietrobelli et al. 2007), and ticks carriers of *B. microti*-like, *B. bovis*, *B. canis canis*, *B. canis vogeli* and *T. equi* have also been reported (Iori et al. 2010).

Informative meetings to explain motivation, aims and plan of the research were organized with volunteer blood donors, and no children were involved in the research. All study protocols followed the principles of the Helsinki Declaration<sup>1</sup> and its subsequent modification, as well as those of the Italian rule (Ministerial Decree, 18.03.98)<sup>2</sup> and the Italian National Law no. 675.1996<sup>3</sup> concerning the protection of personal data. Relevant data concerning age, place of residence (urban or rural), recreational pursuits (e.g. camping, hiking, fishing, hunting, gardening, and walking dogs) as well as occupational activities (involving or not job in brushy or grassy areas that might be inhabited by ticks), and awareness of tick bite were collected from each subject by means of a semi-structured form.

All volunteer blood donors signed an informed consent stating to be fully conscious about procedures described by the physician. Moreover, each individual authorized us to carry out the diagnosis of babesiosis/theileriosis, as well as the publication of the results and the storage of his/her blood sample. Results of serological investigations remained anonymous and were directly communicated exclusively to each individual who was interested in knowing them. Positive subjects had access to further specific medical checks.

Enrolled volunteers were grouped in subjects potentially more exposed to an infection risk due to their job (n=290: foresters, veterinary practitioners, livestock keepers, hunters and farmers) and individual resident in the same areas (n=142) but less exposed to the risk of an infection as their job or life do not occur in wildlife microenvironments.

### Serological analyses

The indirect fluorescent antibody (IFA) kit (Fuller Laboratories, Fullerton, CA, USA) was used to detect antibodies to *B. microti*. Human IgG antibodies against *B. bovis*, *B. canis* and *T. equi* were captured on

IFAT slides carrying pre-fixed infected erythrocytes available for diagnosis in animals (*B. bovis*-IFA IgG, *B. canis*-IFA IgG, *T. equi*-IFA IgG Antibody kit, Fuller Laboratories, Fullerton, CA, USA) by means of a specific anti-human secondary antibody.

As recommended in the data sheet of *B. microti* IFAT, sera were diluted serially in phosphate buffered saline (PBS) solution from 1:32 to 1:256 and then added on the slides. The cut-off value was fixed at 1:64, as suggested by the manufacturer. After incubation in a moist chamber for 30 min at 37°C and 3 wash cycles with PBS solution, the fluorescein-conjugated goat anti-human IgG antibody (Sigma Aldrich, St. Louis, MO, USA) was added for incubation at 37°C for 30 min. Wells were counterstained with 0.02% Evans Blue containing 30% glycerol (Fuller Laboratories, Fullerton, CA, USA), and were submitted to fluorescence microscopic analysis at 40X magnification. Positive and negative controls, supplied by each kit, were used to ensure accurate test performance.

In order to highlight the presence of antibodies to *B. divergens*, a laboratory-derived Enzyme-Linked Immunosorbent Assay (ELISA) based on metabolic antigens of the cultured parasite recently developed (Gabrielli et al. 2012) was applied. Briefly, *B. divergens* antigens, obtained from the culture supernatant and quantified by Bradford assay as 17.3 µg/µL, were adsorbed onto the ELISA wells. Sera (diluted 1:50 in PBS-Tween 20) were tested following standard ELISA procedures by using anti-human IgG secondary antibody HRP conjugated (diluted 1:2000 in PBS-Tween 20) (Sigma, St Louis, MO, USA). Orthophenylene-diamine (Sigma, St Louis, USA, USA) in 0.05M citrate buffer (pH=4.0) with 0.04% (v/v) H<sub>2</sub>O<sub>2</sub> was added as substrate, and the enzymatic reaction was stopped with 0.5M H<sub>2</sub>SO<sub>4</sub>. Optical Density (O.D.) was measured at 492nm (Bio-Rad, Life Sciences, Hercules, CA, USA). The cut-off value was calculated as O.D. arithmetical mean plus 3 times standard deviation for 50 sera from healthy people with PCR-negative blood samples (Gabrielli et al. 2012). A positive reaction corresponded to an Ab index ≥1.0.

To evaluate the specificity of serological assays applied, all reactive sera and 10 negative ones were further tested to evidence antibodies to plasmodia (protozoa closely related to piroplasms) with the DiaMed ELISA malaria antibody test (DiaMed-Italiana S.r.l., Milan, Italy).

<sup>1</sup> Declaration of Helsinki. Ethical Principles for Medical Research Involving Human Subjects. Adopted by the 18<sup>th</sup> World Medical Association (WMA) General Assembly, Helsinki, Finland, June 1964; amended by the 29<sup>th</sup> WMA General Assembly, Tokyo, Japan, October 1975; 35<sup>th</sup> WMA General Assembly, Venice, Italy, October 1983; 41<sup>st</sup> WMA General Assembly, Hong Kong, September 1989; 48<sup>th</sup> WMA General Assembly, Somerset West, Republic of South Africa, October 1996, and the 52<sup>nd</sup> WMA General Assembly, Edinburgh, Scotland, October 2000.

<sup>2</sup> Decreto ministeriale of March the 18<sup>th</sup> 1998 'Linee guida di riferimento per l'istituzione e il funzionamento dei Comitati etici'. Off J, 122, 28/05/1998.

<sup>3</sup> Legge n. 675 of December the 31<sup>st</sup> 1996 'Tutela delle persone e di altri soggetti rispetto al trattamento dei dati personali'. Off J (Suppl. n. 3), 5, 8/01/1997.

## Statistical analyses

The Chi-square test was used to compare differences between the overall seroprevalence of more and less exposed groups of individuals and between Northern and Central population. Whereas the Fischer-Yates exact test was used to compare seroprevalence for each piroplasm species, P values <0.05 were considered to be statistically significant (Sullivan *et al.* 2009).

## Results

Eighty-one subjects showed to be reactive to *Babesia/Theileria* antigens, accounting for an overall positivity of 18.7%. No cross-reactivity to different babesial nor to plasmodial antigens was found. Antibodies to *B. microti*, *B. bovis*, *B. divergens*, *B. canis* and *T. equi* antigens were detected in 4.6%, 4.3%, 3.9%, 3.4%, and 2.3% of the tested sera, respectively. Table I summarizes reactivity to each piroplasm species per each study group. The prevalence of antibodies was significantly higher in the more exposed subjects (71/290; 24.4%) than in less exposed ones (10/142; 7.0%) ( $\chi^2=19.03$ ;  $p<0.001$ ).

Seroreactivity against *B. microti* antigens was the most frequently found, with IgG titres from 1:64 to 1:256, and it was mainly detected in people highly exposed to tick bites as foresters and hunters (32.0% and 11.7%, respectively). Two foresters and 4 hunters reported a tick infestation in their anamnesis. In addition, 3 (3.7%) out of 80 livestock keepers, and 6 (4.2%) out of 142 subjects exposed to a recreational risk of infection turned out positive to *B. microti* IFAT (at 1:64 serum dilution).

Similarly, antibodies against *B. bovis* (from 1:64 to 1:128 screening dilutions) were detected only in sera of people exposed to cattle farm environment such as livestock keepers (6.2%) and veterinary practitioners (9.3%); therefore its seroprevalence

was strongly correlated to the professional risk of infection ( $p<0.001$ ).

Antibodies to *B. canis* antigens (at 1:64 screening dilution) were found in 3.4% of the sera tested, mostly obtained from veterinary practitioners (8.0%), hunters (5.8%) and livestock keepers (1.2%). A less exposed subject also turned out to be positive (0.7%), however seroprevalence was significantly higher ( $p=0.02$ ) in the more exposed group.

About 6% of the veterinary practitioners and only 1 subject resident in Central Italy (0.7%) showed IgG reactivity against *T. equi* antigen (at 1:64 screening dilution), suggesting a significant professional and recreational risk of infection ( $p<0.001$ ). Finally, anti-*B. divergens* antibodies were detected in 17 (3.9%) of the 432 subjects, who were mainly livestock keepers, but also foresters, veterinary practitioners and less exposed people. It is important to stress that the infection risk was significantly correlated with the job exposure ( $p=0.04$ ), livestock keepers being the most seroreactive group.

As for professional groups, foresters showed the highest number of positivities (39.2% of them showed antibodies mostly to *B. microti* and to *B. divergens* antigens), followed by veterinary practitioners (25.3%, most of them showed to be reactive to *B. bovis*, *B. canis* and *T. equi* antigens), livestock keepers (23.7%, mainly reactive to *B. divergens* and *B. bovis* antigens), and hunters (17.6%, reactive to *B. microti* and *B. canis* antigens). Concerning the study area, the overall serological reactivity detected in Northern Italy (66/279; 23.6%) was higher ( $p<0.05$ ) than the one recorded in Central Italy (15/152; 9.8%) (Table II).

## Discussion

This study aimed to evaluate the possible involvement of people living in Italy in an emerging

**Table I.** Seroreactivity to piroplasm antigens classified by professional risk of infection in the samples collected in North and Central Italy in 2009.

Examined subjects	No.	No. seroreactive subjects (%)					Total
		<i>B. canis</i>	<i>T. equi</i>	<i>B. bovis</i>	<i>B. divergens</i>	<i>B. microti</i>	
<b>More exposed</b>	290	14 (4.8)	9 (3.1)	19 (6.5)	15 (5.1)	14 (4.8)	71 (24.4)
Foresters	28	0 (0)	0 (0)	0 (0)	2 (7.1)	9 (32.0)	11 (39.2)
Livestock keepers	80	1 (1.2)	0 (0)	5 (6.2)	10 (12.5)	3 (3.7)	19 (23.7)
Veterinary practitioners	150	12 (8.0)	9 (6.0)	14 (9.3)	3 (2)	0 (0)	38 (25.3)
Hunters	17	1 (5.8)	0 (0)	0 (0)	0 (0)	2 (11.7)	3 (17.6)
Farmers	15	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<b>Less exposed</b>	142	1 (0.7)	1 (0.7)	0 (0)	2 (1.4)	6 (4.2)	10 (7.0)
<b>Total</b>	432	15 (3.4)	10 (2.3)	19 (4.3)	17 (3.9)	20 (4.6)	81 (18.7)
<b>p</b>		0.02	<0.001	<0.001	0.04	>0.05	<0.001 $\chi^2=19.03$



**Table II.** Seroreactivity to piroplasm antigens of subjects exposed to different risk level. The data refer to the samples collected in North and Central Italy in 2009 and categorised by study area.

Examined subjects	No.	No. seroreactive subjects (%)					Total
		<i>B. canis</i>	<i>T. equi</i>	<i>B. bovis</i>	<i>B. divergens</i>	<i>B. microti</i>	
<b>Northern Italy</b>	279	12 (4.3)	9 (3.2)	16 (5.7)	13 (4.6)	16 (5.7)	66 (23.6)
More exposed (foresters, livestock keepers, veterinary practitioners)	207	12 (5.8)	9 (4.3)	16 (7.7)	13 (6.3)	12 (5.8)	62 (29.9)
Less exposed	72	0 (0)	0 (0)	0 (0)	0 (0)	4 (5.5)	4 (5.5)
<b>Central Italy</b>	153	3 (1.9)	1 (0.6)	3 (1.9)	4 (2.6)	4 (2.6)	15 (9.8)
More exposed (livestock keepers, hunters, farmers)	83	2 (2.4)	0 (0)	3 (3.6)	2 (2.4)	2 (2.4)	9 (10.8)
Less exposed	70	1 (1.4)	1 (1.4)	0 (0)	2 (2.8)	2 (2.8)	6 (8.5)
<b>P</b>							<0.001 $\chi^2=12.45$

sanitary problem as zoonotic piroplasmoses. Serological tests, widely accepted as important surveillance tools, were therefore applied to samples of the local population, which were deemed representative of the categories exposed to different degrees to several piroplasm species. To our knowledge, this is the first published data on the piroplasm seroprevalence in Italy. To overcome the lack of commercial assays to diagnose in humans *B. bovis*, *B. canis* and *T. equi* infections, we applied an adapted protocol to kits available for the diagnosis in animals. The reliability of the results is supported by the absence of reactivity to plasmodial antigens (the human protozoa most closely related to piroplasms) and to different babesial species (no mixed infections were found). This result was expected in view of the fact that we classified as positive only specific reactions indicated by the manufacturer as distinct apple-green inclusion bodies within the infected erythrocytes.

Notwithstanding the need for a prudential interpretation of these results because of the human diagnostic adaptation and the limited sample size tested for some groups, these preliminary data are quite alarming.

Seroreactivity to piroplasm species was detected in more than 18% of the samples tested, showing an overall mean seroprevalence higher than expected. The possible piroplasm transmission to people is likely a consequence of the high presence of these parasites in animal populations of the same area (where infection rates range from 17% to 56%, according to the animal species) (Pietrobelli et al. 2007). The second important aspect suggesting an easy involvement of people among the hosts was the molecular identification, in unselective feeder ticks of piroplasms closely related to zoonotic species, as *B. microti*-like, *B. rodhaini* and *B. canis*, detected in *Rhipicephalus sanguineus*, *Ixodes ricinus* and *R. turanicus*, respectively (Iori et al. 2010).

Finally, the increase of tick habitat used by humans for recreational and occupational purposes, the spread of these arthropods towards upper altitudes and new habitats in relation to available hosts and climatic conditions, are all factors favouring human infection. Therefore, the results presented in this study are surprising but nearly expected.

It is important to remark that the unexpected high reactivity to *B. microti* antigens (an average of 4.6%, which rises 11.7% in hunters and 32% in foresters) overcomes the one recently reported in Poland (4.4% in foresters) (Pancewicz et al. 2010), and the one found in Northern Italy (3.4% in foresters) [Sambri 2003, unpublished data, cited by Genchi (Genchi 2007)]. The findings described in this article support the data reported in Switzerland, where residents in the Eastern part of the country showed a 1.5% seroreactivity to *B. microti* (Foppa et al. 2002), and in Germany, where this value was 1.7% (Hunfeld et al. 2002).

As for *B. divergens*, despite most of the reported human cases of babesiosis in Europe being attributed to this species, no human cases have been yet reported in Italy. Nevertheless, this study found reactivity to *B. divergens* antigens in 3.9% of the examined sample taken in areas where the seroprevalence in bovines ranges from 8% to 14% and the parasite has been molecularly detected in the examined cattle (Tampieri et al. 2008). Furthermore, different *B. divergens*-like strains are highly present in wild animals of the same area (Cancrini et al. 2008), a factor that increases the infection risk. However, it is useful to underline that contacts with *B. divergens*-like species, such as *B. venatorum*, could remain undetected by the laboratory-derived ELISA applied in this study, which proved very specific (more than the conventional IFA and ELISA home-made using corpuscular antigen) because based on metabolic antigens of the cultured *B. divergens* Rouen strain.

The other piroplasm species (*T. equi*, *B. canis* and *B. bovis*), not documented but suspected as possible agent of zoonosis, proved their possible contact to humans, especially for those subjects more exposed to the infection risk. This result is of concern not only because it is indicative of a more wide number of possible zoonotic species, but also because it includes, among them, species that usually infect animals strongly associated to humans, particularly *B. canis*. For example, in the studied areas 3.4% of the people showed reactivity to *B. canis*, which has been found (as *B. c. canis* and *B. c. vogeli*) in 4.9% of the ticks collected (Cassini *et al.* 2009) and in 6.0-20% of the examined dogs (Pietrobelli *et al.* 2007, Solano-Gallego *et al.* 2008).

Statistical analyses showed a high association between job (and therefore environments more often frequented) and specific reactivity for piroplasms. As for less exposed subjects, those living in Northern Italy (mostly near natural protected areas) proved strongly exposed to *B. microti*, whereas those living in Central Italy (where natural recreational areas are available for people, and breeding, farming, and hunting are largely practised) proved at risk of infection with any species. Only farmers (a very small sample) proved negative to all tested antigens. Different seroreactivity found in Northern and Central Italy may be determined by distinct environmental features as well as by tick species that act as piroplasm vector/carrier, but it may also be biased by the different characteristics of the sampled population.

Finally, a positive anamnesis to tick bite was reported in 6 cases, it is useful to remember that tick bite often

remains unnoticed and, therefore, asymptomatic infections may occur.

## Conclusions

This study documented the human seroreactivity to piroplasms in Italy and provides new epidemiological information on these zoonoses in Europe. It highlighted significant differences between more exposed and less exposed subjects. It also showed species-specific reactivity that fits in with the risk exposure to tick bite in different environmental conditions due to the different professional groups examined. Our findings suggest that infections due to several species, but overall to *B. microti* and *B. divergens*, may occur in Italy. Physician should indeed consider piroplasmoses when dealing with any febrile illness, especially in people who are exposed to infection risk for work-related reasons.

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