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Brucella spp. in Wildlife of the Lombardy Region, Northern Italy

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ABSTRACT: Surveillance data collected in the period 2017–20 for *Brucella* spp. in wildlife of the Lombardy Region in northern Italy were used to describe the exposure of the wildlife species to *Brucella* spp. in wild boar (*Sus scrofa*), European brown hare (*Lepus europaeus*), fallow deer (*Dama dama*), red deer (*Cervus elaphus*), and roe deer (*Capreolus capreolus*). Among the tested species, wild boar (n=6,440) showed the highest percentage of seropositive samples (5.9%). Notably, wild boars of perifluvial area of the Po River showed higher percentages of positivity than those of the pre-Alpine district. In addition, during the hunting season in 2018, 95 organs (uterus or testes, spleen, and submandibular lymph nodes) from wild boar of the perifluvial area of the Po River were collected for bacteriological examination. *Brucella suis* was isolated in culture from 18.9% of tested lymph nodes. These serological and microbiological results highlight the presence of *B. suis* in wild boar and suggest the importance of wild boar as a reservoir for *B. suis*. Comparison of the spatial distribution of *Brucella*-seropositive wild boars with the location of backyard swine farms revealed a higher chance of contact between the two populations only in the areas where the lower percentage of seropositive samples was observed. Conversely, the high percentage of seropositive samples observed in the Po River area coupled with positive microbiological cultures suggest a greater risk of infection for the humans directly or indirectly involved in wild boar hunting activity. These results may serve as a basis to establish sound wildlife management and to adopt education campaigns aimed at reducing the risk of human infection in people involved in wild boar hunting related activities.

Key words: Brucellosis, ELISA, hunting, One-Health, wild boar.

INTRODUCTION

Wildlife disease surveillance is crucial to identify changes in wildlife disease occurrence and epidemiology, and it is an essential part of the One-Health approach (Yon et al. 2019). Surveillance is required to identify new and reemerging pathogens to recognize possible changes in disease occurrence in host and vectors species, adopt appropriate measures to protect domestic animals and human health, and safeguard the wildlife ecosystem. A wildlife surveillance program targeting several pathogens has been running in the Lombardy Region of northern Italy for several years. The populations of wild boar (*Sus scrofa*), European brown hare (*Lepus europaeus*), fallow deer (*Dama dama*), red deer (*Cervus elaphus*), and roe deer (*Capreolus capreolus*) are sampled for the presence of antibodies against *Brucella* spp. Brucellosis is a worldwide zoonosis caused by a gram-negative facultative intracellular bacterium belonging to the genus *Brucella*. To date, 12 *Brucella* species have been identified from a wide spectrum of hosts (Khurana et al. 2021). Some species infect terrestrial mammals: *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, *Brucella neotomae*, and *Brucella microti*. *Brucella ceti* and *Brucella pinnipedialis* affect marine mammals. Novel species named *Brucella papionis*, *Brucella vulpis*, and *Brucella inopinata* were respectively isolated from baboons (*Papio* spp.), red foxes (*Vulpes vulpes*), and human breast implant infection, although the natural reservoir of these species remains uncertain (Scholz et al. 2010, 2016). Brucellosis causes significant economic losses in animal production, due to diminished milk yield, abortion, infertility, and other reproductive disorders. Considering its impact on human and animal health, brucellosis is a notifiable disease in

many countries, including Italy (Khurana et al. 2021). Within the European Union (EU), livestock brucellosis caused by *B. abortus*, *B. melitensis*, and *B. suis* has been eradicated in many European Member States. Croatia and Spain are close to achieving eradication; conversely, Greece, Italy, and Portugal, although with declining incidence rates, still report the infection in their livestock populations. In the Lombardy, as well as in some other Italian regions, bovine, ovine, and caprine brucellosis have been eradicated for several years (European Food Safety Authority and European Centre for Disease Prevention 2021).

In addition, brucellosis represents a human health problem because *Brucella* spp. can infect humans as an incidental host. Generally, *Brucella* is transmitted to humans through the consumption of contaminated animal products, especially unpasteurized milk and cheeses, or through direct contact with infected tissues or secretions (Moreno 2014). Certain occupations, such as slaughterhouse workers, meat-packing employees, veterinarians, hunters, and laboratory workers are characterized by a higher risk of brucellosis (Pereira et al. 2020). The most pathogenic and invasive species for humans is *B. melitensis*, followed by *B. abortus*, *B. suis*, and *B. canis* (Khurana et al. 2021). Human brucellosis cases are rarely reported in Europe (Bagheri Nejad et al. 2020).

Despite their respective host preferences, various *Brucella* species have been reported in several wild animal species. In Europe, *B. suis* biovar 2 has been reported in wild boars and hares; *B. melitensis* and *B. abortus* (rarely reported in wildlife) have been reported in Alpine ibex (*Capra ibex*), chamois (*Rupicapra* sp.), Spanish ibex (*Capra pyrenaica*), and red deer (European Food Safety Authority and European Centre for Disease Prevention 2021). Native wild ruminants are mostly considered as dead-end hosts rather than as true reservoirs for *Brucella* spp. (Ferroglio et al. 1998; Muñoz et al. 2010) and may serve as an epidemiological sentinel for the presence of *B. melitensis* in domestic livestock (Godfroid et al. 2013).

The presence of *B. suis* has been reported in wild boar and hares in some EU states for decades, and these two species have been identified as reservoir of *B. suis* in Europe (European Food Safety Authority 2009). Data on *B. suis* infection in European brown hares are limited and reported seroprevalences range from 0 to 17% in different parts of Europe (Winkelmayer et al. 2005; Tsokana et al. 2020). Seroprevalence of *Brucella* spp. infection in wild boars in Europe spans from 0 to 60% (Cvetnic et al. 2004; Wu et al. 2011; Grégoire et al. 2012; Hälli et al. 2012; Risco et al. 2014). There are no available official data suggesting that *B. suis* is currently present in any of the indoor commercial pig holdings in the EU (European Food Safety Authority 2009). Nevertheless, the prevalence observed in wild boar suggests that this species might act as a potential source of transmission of *B. suis* biovar 2 to domestic pigs in outdoor farming systems or backyard herds. *Brucella suis* biovar 2 has been isolated from a semi–free-range pig farm in Italy (Barlozzari et al. 2015).

The aim of our study was, through the analysis of the data collected during the wildlife surveillance program, to describe *Brucella* exposure and diffusion in the sampled wildlife, information useful for risk analysts to carry out a rapid qualitative-quantitative risk assessment, and to estimate the risk of livestock and human exposure to *Brucella* spp., to inform consistent and effective control measures against brucellosis in a One Health perspective.

MATERIALS AND METHODS

Study area

We collected samples in the provinces of Cremona, Mantova, Brescia, Lodi, Pavia, Bergamo, Como, and Milano, Lombardy Region, northern Italy. The study area covered various ecosystems from riparian forest along the Po River to deciduous forest, coniferous forest, and tundra of the Alps.

Data collection

This retrospective study covered a 4-yr period (2017–20). Blood and organs were collected from animals hunted or found dead under the frame of the regional wildlife surveillance program by

using nonprobabilistic convenience sampling, and they were used to estimate *Brucella* spp. presence in the studied territory. As part of this study, 8,129 serum samples from wild boar (6,440), European brown hare (1,502), fallow deer (91), red deer (58), and roe deer (38) were sampled through hunting activity or through passive surveillance. Blood samples from European brown hares were collected when the animals were released for repopulation purposes in view of the onset of the hunting season. In the wild boar, sex and age classes were also recorded; the age of each individual was estimated using tooth eruption and tooth replacement, and the animals were divided in three classes: juveniles (<1 yr old), yearlings (1–2 yr old), and adults (>2 yr old) (Matschke 1967).

At the end of 2017, as a result of the high percentage of *Brucella*-seropositive samples detected in wild boar of Cremona Province (Po River area), hunters and gamekeepers were asked to collect tissue samples during the 2018 hunting season. In 2018, 95 wild boars were sampled: 92 spleens, 92 submandibular lymph nodes, 50 testicles, and 42 uteri, from 92 animals; a further 3 submandibular lymph nodes from 3 animals were submitted to the laboratory for pathological examinations. *Brucella* microbiological culture was carried out from all the lymph nodes (n=95) and from other organs with macroscopic pathological lesions (two testes and one uterus).

Serological analyses

Serum samples were analyzed using a competitive multispecies ELISA test to detect antibodies directed against *B. abortus*, *B. melitensis*, and *B. suis* (SVANOVIR Brucella-Ab C-ELISA kit, INDICAL Bioscience, Uppsala, Sweden). The test was performed according to the manufacturer's instructions by using recommended cut-off values. The presence of antibodies was evaluated by reading the optical density at a wavelength of 450 nm on a spectrophotometer (Tecan, Männedorf, Switzerland).

Detection and identification of *Brucella* spp. by culture and real-time PCR

Isolation of *Brucella* spp. was performed according to Office International des Epizooties (OIE) (now the World Organisation for Animal Health) Terrestrial Manual (OIE, 2016). In brief, animal tissues were cultured directly on both Farrell medium and modified Thayer-Martin medium and incubated for 10 d at 37 C in the presence of 5–10% CO_2. Suspected colonies were subcultured on brain heart infusion agar and tested for urease, catalase, and oxidase production. *Brucella* isolates were characterized by real-time PCR (qPCR). The DNeasy Blood and Tissue kit (Qiagen, Leipzig, Germany) was used for the extraction of DNA. The target IS711 was amplified to ascertain the genus *Brucella* (Bounaadja et al. 2009). We used NCTC 10502 DNA as a positive control. Real-time PCRs based on single-nucleotide polymorphism analysis were performed for the identification of *Brucella* species such as *B. suis*, as described previously (Gopaul et al. 2008). Two isolates were sent to the National Reference Centre for Brucellosis (Teramo, Italy) for biovars identification by PCR-restriction fragment length polymorphism (OIE 2016).

Data analysis

Considering the nonprobabilistic nature of sampling for data generation, which exposes it to an unpredictable selection bias in magnitude and direction, only the overall proportion of positive samples and the proportions in the different factors of interest were calculated and are expressed as percentages. A map (Fig. 1) was drawn using leaflet (Cheng et al. 2023) and sf (Pebesma 2018) packages in R (R Core Team 2023).

RESULTS

During 2017–20, 8,129 serum samples were tested for the presence of *B. abortus*, *B. melitensis*, and *B. suis* antibodies. Wild boar and hare were species most commonly represented in the samples, with 6,440 and 1,502 tested animals, respectively. The geographical distribution of the samples of these two species is summarized in Table 1. Except for wild boar, all the tested wild ungulates were seronegative (data not shown). We found three seropositive European brown hares (0.2%). In the wild boar, 2,304 animals were male (1,098 adults; 585 yearlings; 470 juveniles; 151 age unknown) and 2,541 were female (1,130 adults; 740 yearlings; 515 juveniles; 156 age unknown); sex information was not available

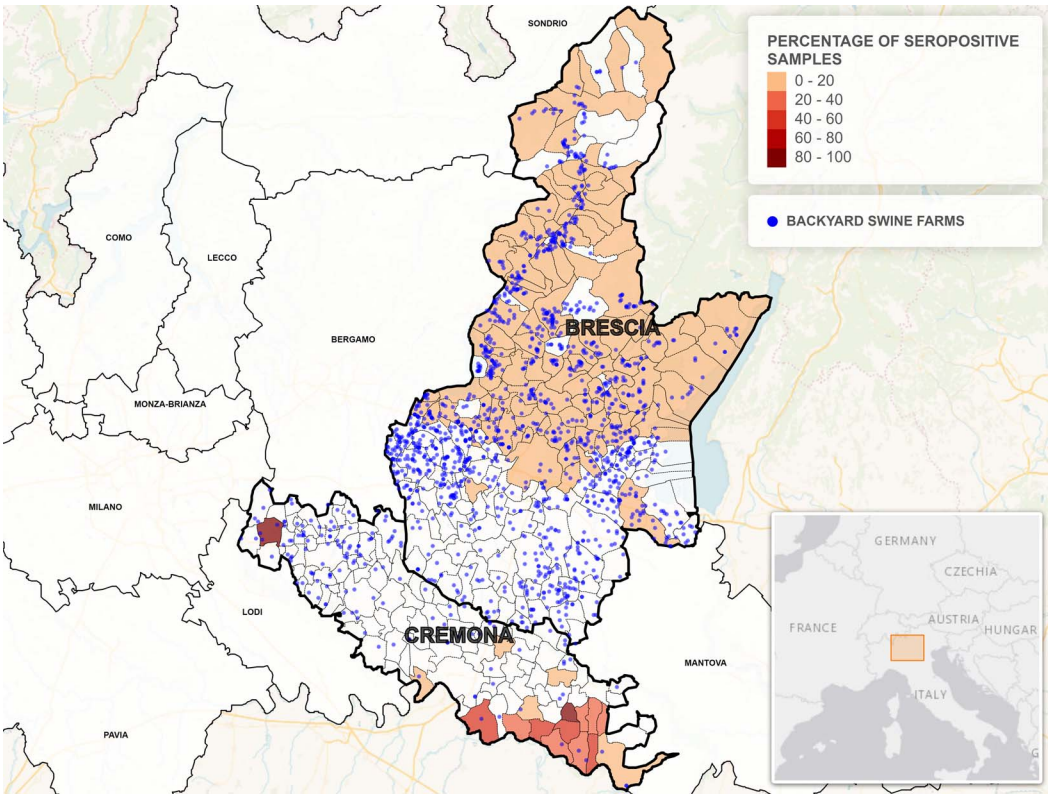

FIGURE 1. Map showing the spatial distribution of backyard swine farms and *Brucella* seropositivity percentages in wild boar (*Sus scrofa*) in Cremona and Brescia provinces, Lombardy Region, northern Italy, 2017–20. The inset shows the position of the study region within Italy.

for 1,595 carcasses (42 adults; 25 yearlings; 25 juveniles; 1,503 age unknown). The overall percentage positivity was 5.9%, ranging from a low of 0% to a maximum of 13.9% (Table 2).

TABLE 1. Sample size by province of wild boar (*Sus scrofa*) and European brown hare (*Lepus europaeus*) populations in the Lombardy Region of northern Italy, 2017–20, tested for the presence of antibodies against *Brucella* spp.

Province	Wild boar	Hare
Brescia	5,580	156
Cremona	774	1,014
Bergamo	0	178
Lodi	19	40
Pavia	23	35
Mantova	1	47
Como	39	0
Milano	4	32

The number of tested and positive samples for each class is shown in Table 2. Regarding the sex classes, the positivity observed was 171/2,304 males, 119/2,541 females, and 88/1595 animals for which sex was unknown. By age classes, positivity was observed in 125/2,270 adults, 90/1,350 yearlings, 48/1,010 juveniles, and 115/1,810 animals with undefined age. The differences observed in the different sex and age groups were considered not to be epidemiologically relevant.

Results on *Brucella* serological tests of wild boars are shown by area in Table 3. Higher percentages of positivity were observed in samples of the southern provinces of Lombardy Region (Cremona, Pavia, Lodi) that are located near the course of the Po River. Conversely, wild boars of the pre-Alpine area of Brescia and Como provinces showed lower percentages of positive results. The spatial distribution of the

TABLE 2. Number of tested (tot) and *Brucella* spp.–seropositive (pos) wild boars (*Sus scrofa*) and respective percentage of seropositive samples in Lombardy Region, northern Italy, 2017–20, separated by age and sex.

	Male		Female		Unknown		Total	
Age class[a]	Pos/Tot	%	Pos/Tot	%	Pos/Tot	%	Pos/Tot	%
Adult	67/1,098	6.1	53/1,130	4.7	5/42	11.9	125/2,270	5.5
Yearling	57/585	9.7	31/740	4.2	2/25	8.0	90/1,350	6.7
Juvenile	26/470	5.5	22/515	4.3	0/25	0.0	48/1,010	4.8
Unknown	21/151	13.9	13/156	8.3	81/1,503	5.4	115/1,810	6.4
Total	171/2,304	8.4	119/2,541	4.7	88/1,595	5.5	378/6,440	5.9

[a] Age determined by tooth eruption patterns.

backyard pig farms in the province of Brescia and Cremona and the *Brucella* serological results observed in wild boar in the municipalities of these two provinces are both depicted in Figure 1. Backyard pig farming is rare in the municipalities of Cremona where a high percentage of positive samples was recorded. Conversely, in the northern area of the province of Brescia, backyard farms are common in municipalities where low seropositivity was detected in wild boar.

Of 95 animals tested for *Brucella* spp. by culture of submandibular lymph nodes, 18 resulted positive for *Brucella* spp. (18.9%). All the isolates were confirmed as *B. suis* by qPCR; two were analyzed by PCR-RFLP and characterized as *B. suis* biovar 2 by the National Reference Centre for Brucellosis.

No lesions related to brucellosis were identified in any spleens examined. Conversely, of 92 wild boars' reproductive organs, 3 showed macroscopic pathological findings. Specifically, severe unilateral chronic orchitis was observed in two male yearlings (1–2 yr old) and miliary metritis, characterized by the presence of 2–3-mm nodules seeded on the uterine mucosa, was found in one female of the same age class (Fig. 2). Microbiological culture of the organs with pathological lesions and of the submandibular lymph nodes from the same individuals were negative for *Brucella* spp., whereas qPCR from testes and uterus with lesions was positive for *B. suis*. For a summary of the pathological, microbiological, and PCR findings, see Table 4.

DISCUSSION

We used serological data collected during the wildlife monitoring plan of the Lombardy Region, Italy, to describe the exposure of the

TABLE 3. Number of the tested and positive wild boars (*Sus scrofa*) and respective percentage of *Brucella*-seropositive samples by province within the Lombardy Region, northern Italy, 2017–20.

Province	Seropositive samples	Tested samples	% seropositive samples
Brescia	110	5,580	2
Cremona	253	774	32.7
Como	0	39	0
Pavia	6	23	26.1
Lodi	8	19	42.1
Milano	1	4	25
Mantova	0	1	0
Total	378	6,440	5.9

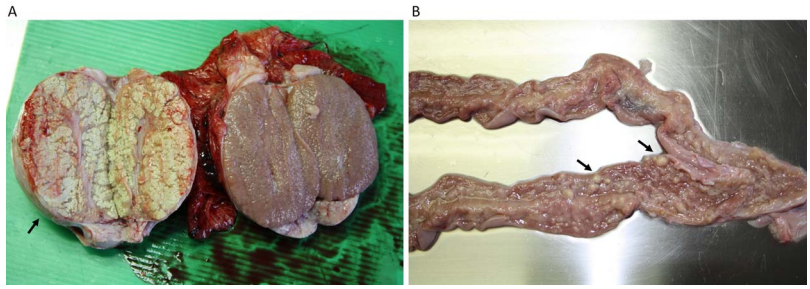

FIGURE 2. Severe unilateral chronic orchitis (A) and miliary metritis with 2–3-mm nodules (indicated by arrows) seeded on the uterine mucosa (B) caused by *Brucella suis* in 1–2-yr-old wild boars (*Sus scrofa*) from the Lombardy Region, northern Italy.

wildlife species to *Brucella* spp. Given the nonprobabilistic nature of sampling characterizing this retrospective study, our results are biased and therefore should be evaluated with caution when interpreted as seroprevalence estimation.

Serology is an effective and inexpensive tool to carry out surveillance in wildlife and especially wild ungulates. Some wild ungulate species, such as wild boar, roe deer, and red deer, have the potential to colonize new territories of southern Europe (Vingada et al. 2010; Linnell et al. 2020) and thus may potentially carry zoonoses into new areas. All the wild ungulates tested in the current study, with the exception of wild boar, were seronegative for *Brucella* spp. Low *Brucella* seroprevalence in wild ungulates (0.4%), with the exception of wild boar, has also been reported in Spain (Muñoz et al. 2010).

The hare population included in the Lombardy Region wildlife surveillance program showed 0.2% seropositive samples. Similarly, low values were reported in other investigations: 3.54% in Austria with considerable regional variations (Winkelmayer et al. 2005); 0% in the Czech Republic in the period 1986–91 (Hubálek et al. 1993) and in 2003 (Winkelmayer et al. 2005); 0% in a German Region (Schleswig-Holstein) during 1998–2000 (Frölich et al. 2003); and 1.6% in the Czech Republic during 2004–06 (Treml et al. 2007).

Notwithstanding their low seroprevalence, hares are known to be a reservoir of *B. suis* (Godfroid 2018). In Italy in 1995, *B. suis* biovar 2 was detected in a male hare imported from Hungary (Quaranta et al. 1995). Over the time, several studies have proposed that *B. suis* was introduced in Italy through the importation of hares, for repopulating hunting areas, from Eastern Europe, where the infection was endemic in wild species (Ebani et al. 2003; Gennero et al. 2004; Bergagna et al. 2009; De Massis et al. 2012).

The higher detection rate of anti-*Brucella* immunoglobulins in wild boar (5.9%) than in

TABLE 4. Pathological, microbiological, and PCR findings in organs from wild boar (*Sus scrofa*) collected in the Cremona Province (Lombardy Region) of northern Italy in the 2018 hunter season.

	Pathological lesions		Microbiological analyses for *Brucella* spp.		
Tissue	Presence	Total	Positive	Total	PCR
Lymph-node	NA[a]	95	18	95	*Brucella suis*
Testicle	2	50	0	2	*B. suis*
Uterus	1	42	0	1	*B. suis*
Spleen	0	92	NA	NA	NA

[a] NA = not applicable.

other wildlife species is not surprising considering that this species is a well-known animal *B. suis* reservoir (Godfroid et al. 2013). The similar *Brucella* serological results observed in male and female wild boar are in accordance with those of previous studies (Montagnaro et al. 2010; Pilo et al. 2015). In contrast with previously reported data (Risco et al. 2014), we found that percentages of seropositive samples did not increase with the age of the animals; this difference may be attributed to the low number of positives samples for each age class. Notably, when considering data disaggregated by area, the southern provinces of Lombardy Region (Cremona, Pavia, Lodi), along the Po River, presented the highest values. This might be explained by considering the land morphology of this territory, characterized by a dense network of natural watercourses and canals bordered by a riparian forest. These natural areas are surrounded by rich and specialized crops, mainly intended for production of cereals and fodder for livestock. The availability of shelter, the abundance of fresh water and food, make this relatively small area particularly attractive for wild boar. Family groups are closely linked to these resource-rich environments, favoring potential infectious contacts between individuals, which may explain the higher exposure observed compared to the other investigated area.

The seropositive results observed in wild boar in our study are lower than those reported in other European countries such as Switzerland (35.8%; Wu et al. 2011) and various regions of Spain (25–46%; Muñoz et al. 2010). Conversely, our data are mostly in agreement with previous Italian records: antibodies against *Brucella* were found in wild boar in a range from 5.74% to 19.76% in different regions of Italy (Bergagna et al. 2009; Montagnaro et al. 2010; Pilo et al. 2015; Cilia et al. 2021; Fabbri et al. 2022; Jamil et al. 2022). However, the comparison of values obtained in different studies and areas should be interpreted with caution due to the different sampling strategies and characteristics of the diagnostic tests used.

As a result of microbiological analyses from wild boars of Cremona Province, *B. suis* was isolated from 18.9% of submandibular lymph nodes. Other authors have previously reported similar prevalence: 17.7% in the Iberian Peninsula (from cranial and iliac lymph nodes, spleen, and sexual organs; Muñoz et al. 2010) and 17% in Croatia (from uteri; Cvetnic et al. 2004), 10.8% in the Regional Park of Piedmont, northwest Italy (from uteri, spleen, and testicles; Bergagna et al. 2009). Isolation of *B. suis* biovar 2 from lymph nodes of a female wild boar has been reported also in the Abruzzo Region of central Italy (De Massis et al. 2012).

Our bacteriology data confirm the specificity of serological results and therefore the circulation of the infection in this species. Moreover, the isolation of *B. suis* from submandibular lymph nodes suggests the importance of the oral transmission route in the pathogenesis of *B. suis* infection, for example, through the ingestion of heavily contaminated aborted fetuses, fetal membranes, or contaminated foodstuffs (European Food Safety Authority 2009), especially in animals that have not reached sexual maturity and for which the venereal transmission route is unlikely (Elmonir et al. 2022). The presence of *B. suis* in reproductive organs confirms the possible contribution of the venereal transmission. The negative microbiological result yielded from the three reproductive organs with macroscopic lesions and positive PCR might indicate chronic infection associated with low number of bacteria in the analyzed tissues.

The characterization of *B. suis* as biovar 2 agrees with the situation recently depicted in Italy. Analysis of *Brucella* field strains submitted for typing to the Italian National Reference Laboratory for Brucellosis in 2007–15 revealed that *B. suis* biovar 2 was the main strain isolated from wild boars (De Massis et al. 2019). Likewise, in Europe *B. suis* biovar 2 has previously been identified as the main etiological agent of brucellosis in wild boar (Jamil et al. 2022).

Although the serological and bacteriologic findings suggest a potential risk of transmission of *B. suis* to domestic pigs, to date no

spillover from wild boar to domestic pigs has been reported. A significant number of large commercial pig farms are present in the Cremona Province; however, the consistent application of biosecurity measures reduces the risk of introduction of infectious agents in this kind of farms. Noncommercial farms, also known as backyard farms, are generally recognized as being at greater risk of contact between pig and wild boar population due to lower biosecurity levels and because backyard farming often provides outdoor access to domestic animals. Previous studies on *B. suis* have identified population density and spatial overlapping of wild boar and domestic pig farms, together with fence characteristics, as risk factors for disease transmission (Wu et al. 2011; Risco et al. 2014).

In this light, the low density of backyard farms (Fig. 1, blue dots) in Cremona province, suggests that spillover from wild board to domestic pigs is unlikely to occur, even with the high percentages of *Brucella* seropositive results in wild boar, but attention should still be paid to identifying possible spillover to livestock.

Higher risk for human *Brucella* exposure is connected to hunting activities of wild animals in infected areas (Kmetiuk et al. 2021). Despite being considered low pathogenic for humans, human infections with *B. suis* are increasingly reported (Centers for Disease Control and Prevention 2009; Carrington et al. 2012; Franco-Paredes et al. 2017; Mailles et al. 2017; Gowe et al. 2022). Being involved in wild boar hunting–related activities such as field dressing or preparation of raw meat have been shown as risk factors for this infection. The consequences of *B. suis* infection cannot be underestimated, particularly considering the rising average age of the Italian hunters' population (Istituto Nazionale di Statistica 2003). Hunters also need to be aware that hunting dogs may become infected by *B. suis* while playing with the animal carcass or eating raw meat (Centers for Disease Control and Prevention 2017). Training on safe handling of carcasses, coordinated surveillance, and research activities may help to control zoonotic foci and minimize public health risks (Martin et al. 2011).

Despite the inevitable limitations inherent in the studies of wildlife surveillance, nonrandom samples as used in this study nevertheless may be useful for monitoring the spread of brucellosis in wildlife (Boyd et al. 2023). Thus, this descriptive epidemiological analysis provides useful data on reservoir species of *Brucella* for developing risk assessments and adopting preventive measures to limit transmission of the disease to domestic animals and humans.

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