Epidemiological Significance of the Domestic Black Pig (*Sus scrofa*) in Maintenance of Bovine Tuberculosis in Sicily

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Bovine tuberculosis (bTB) is an emerging disease among wild animals in many parts of the world. Wildlife reservoir hosts may thus represent a potential source of infection for livestock and humans. We investigated the role played by the Sicilian black pig, an autochthonous free- or semi-free-ranging domestic pig breed, as a potential source of bTB infection in an area where bTB prevalence in cattle is high. We initially performed a preliminary field study to assess the occurrence of bTB in such animals. We sampled 119 pigs at abattoir and found 6.7% and 3.4% of them to be affected by gross tuberculous-like lesions (TBL) and *Mycobacterium bovis* culture positive, respectively. We then proceeded to investigate the dissemination and characteristics of lesions in a second field study performed on 100 animals sampled from infected herds. Here, tissues collected at the abattoir were examined macroscopically, microscopically, and by culture tests. Most pigs with TBL showed generalized lesions in both gross and histological examinations (53% and 65.5%, respectively). Head lymph nodes were the most frequently affected in both localized and generalized TB cases observed macroscopically and microscopically. *M. bovis* was the most frequently isolated etiologic agent. The molecular characterization of isolates from both field studies by spoligotyping and analysis of 12 mycobacterial interspersed repetitive-unit–variable number tandem repeat (MIRU-VNTR) loci, followed by their comparison to isolates of cattle origin, suggested a potential transmission of mycobacteria from domestic animals to black pigs and vice versa. Our findings, along with ethological, ecological, and management considerations, suggest that the black pig might act as a bTB reservoir in the ecosystem under study. However, additional studies will be necessary to establish the true epidemiological significance of the Sicilian black pig.

Bovine tuberculosis (bTB) is an important zoonosis worldwide that has been reemerging in different ecological scenarios. In industrialized countries, animal bTB eradication programs and the practice of milk pasteurization have drastically reduced the incidence of disease caused by *Mycobacterium bovis* in both cattle and humans, but the presence of wildlife reservoirs hampers eradication. In nonindustrialized countries, on the other hand, control measures are either absent or applied sporadically, and pasteurization is limited. Animal bTB is, therefore, still widely distributed and spreading to new territories (25).

Mycobacterium bovis, the main etiological agent of bTB, has the widest host range of any member of the *M. tuberculosis* complex, and an intricate epidemiological pattern of infection involving domestic, farmed, free-ranging, and wild animals, as well as humans (18, 32). As noted, in industrialized countries, a major obstacle to bTB eradication in livestock is the fact that wildlife animals, such as deer, badgers, feral pigs, and wild boars, may act as reservoir hosts, contributing to the spread and persistence of disease (1, 5, 24, 28, 31, 35, 41). This is of particular concern since infected animals often show no clinical signs, even in cases where lesions are well developed, and since no preventive measures or practicable treatment options exist for free-ranging wildlife. The identification of wildlife reservoir hosts and the assessment of risk factors associated with their presence are, therefore, crucial for the implementation of effective control measures.

In Italy, a national eradication program resulted in a gradual reduction of bTB prevalence in most regions. In Sicily, an insular region, bTB is still a major concern, however. Recently, cases of tuberculous-like lesions (TBL) have been reported in a population of Sicilian black pig, an autochthonous variety of domestic pig (unpublished data). Those animals live mostly in the woods of the Natural Parks of Nebrodi and Madonie, two rural nature reserves in northeastern Sicily, and are reared in free or semifree roaming conditions, frequently sharing pastures with cattle. According to the latest Italian Ministry of Health annual report on bTB in cattle, submitted to the European Commission in Bruxelles (Decision 2008/940/EC) in 2010, the proportions of infected cattle herds around Catania and Messina, the two major areas neighboring the parks, were 8.58% and 5.36% of cattle herds, respectively, that tested positive for TB by purified protein derivative (PPD) skin tests (unpublished data).

Evidence regarding the role of wild suids in bTB epidemiology is somewhat conflicting. In Australia and New Zealand, the low prevalence of generalized lesions in feral pigs, as well as management and genetic considerations, has led to the conclusion that
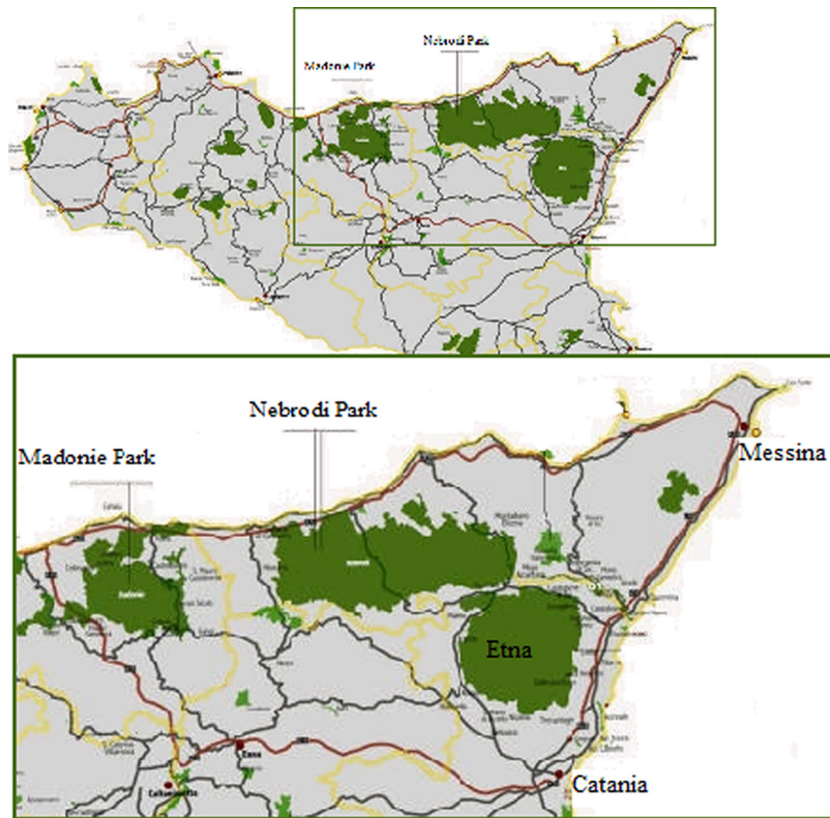
FIG 1 The geographic area under study: the Nebrodi and Madonie Natural Parks. (Modified from a map available at www.siciliainmoto.it with permission.)

feral pigs are spillover hosts rather than sources of bTB infection (8, 9, 36, 37). Similarly, European wild boar (*Sus scrofa*) in northwestern Italy has been deemed unlikely to be a reservoir for bTB (47). In Spain and Portugal, however, the wild boar is believed to act as a bTB reservoir (2, 27, 31, 46).

To gain insight into the role played by the Sicilian black pig population in the epidemiology of bTB infection in its habitat, we carried out two field studies. We sought to obtain (i) an assessment of the presence of bTB among slaughtered black pigs, (ii) a pathological characterization of TBL at the macroscopic and microscopic levels, and (iii) molecular typing of *M. bovis* isolates from the free- or semi-free-ranging black pigs and a comparison of these to genetic profiles of isolates previously obtained from local cattle.

MATERIALS AND METHODS

The area under study and its black pig population. Our study was carried out in two neighboring areas in northeastern Sicily, the Nebrodi and Madonie Natural Parks, comprising about 86,000 ha and 40,000 ha, respectively (Fig. 1). These are mostly mountainous areas (700 m to 1,800 m above sea level), covered by wide pastures and woods (beech, oak, chestnut, and hazel) giving shelter to numerous species of wild mammals, birds, reptiles, and invertebrates. Wild boars are absent in the Nebrodi Park. Instead, few examples are present in Madonie Park, although no data are available about their exact number and distribution. Both parks also host grazing cattle and sheep. The sharing of pasture lands is a traditional animal husbandry practice among local herders.

The Sicilian black pig (*Sus scrofa*) (Fig. 2), an autochthonous domestic pig breed, inhabits these areas, where it reproduces naturally. Through a long process of selection, this pig developed specific morphological and functional characteristics—such as a strong maternal instinct and the ability to survive food scarcity or make do with food of low nutritional value—that allow it to thrive despite difficult environmental conditions.

There are more than 2,500 pig herds in these mountains, with a medium density of 20.6 black pigs/ha. This domestic porcine variety, physically resembling the wild boar, to which it is related, is hardy and disease resistant. The Sicilian black pig is bred under free or semifree conditions in the woods, where it forages for plant material, such as roots, bulbs, tubers, mushrooms, fruit, and berries. Breeders sometimes supplement its diet with small amounts of grain and bran, especially around the time of slaughter, which normally occurs before adulthood. Its meat is in high demand and is used to produce local specialty food products.

Field studies. We carried out the following two field studies.

(i) FS1. For field study no. 1 (FS1), in 2009, 119 Sicilian black pigs from the area under study, transported to the abattoir Frigomacello C.P.C. Mirto in the city of Messina for slaughtering, were randomly selected for postmortem inspection aimed to detect macroscopic lesions suggestive of TB. Culture tests on one lymph node pool per animal were also performed. Lymph nodes affected by gross lesions and those with no visible lesions were pooled per animal.

(ii) FS2. For field study no. 2 (FS2), in 2010, 100 pigs were randomly selected from two bTB-infected herds detected during FS1, with herd sizes of 300 and 400 animals, respectively. Both herds have an estimated TB prevalence rate of 7%. The animals breed under semifree conditions.

Animals were transported to the above mentioned abattoir for slaughtering and postmortem inspection. Tissues were examined at the macroscopic and microscopic levels. Culture tests were performed on each collected tissue sample.

Inspections at abattoir were carried out according to national laws, and no permission from the owners of the abattoir was needed.

M. bovis isolates from both field studies were genotyped by spoligo-

FIG 2 Morphological characteristics of the Sicilian black pig. The pig has black skin entirely covered by strong bristles, a long head, a thin and lengthened snout, a small trunk with a compressed thorax, a short abdomen, a curly tail, strong and medium-length limbs, thin joints, and hard and resistant hooves.

typing and mycobacterial interspersed repetitive-unit–variable number tandem repeat (MIRU-VNTR) analysis as described below.

The sex and age of all animals were recorded. Animals were classified as juvenile (<1 year old), subadult (1 to 2 years old), or adult (>2 years old).

Anatomical examination and lymph node collection. In both field studies, at the abattoir, slaughtered pigs underwent routine meat inspection procedures. Lymph nodes from the head (mandibular, retropharyngeal, and parotid lymph nodes), the thorax (bronchial lymph nodes), and the abdomen (hepatic, gastric, and intestinal lymph nodes) of each pig were sectioned, inspected, and then collected for laboratory analysis.

Based on macroscopic appearance, lesions were recorded as either TBL or atypical tuberculous lesions (ATL). TBL were, in turn, classified as either caseous-necrotic-calcified, caseous-necrotic, or miliary lesions, as well as by size, as either <1 cm in diameter or ≥1 cm in diameter. The diameters of ATL (i.e., whitish and/or fibrous lesions) were also measured.

Pigs from both studies were thus divided into the following three groups: TBL (animals with TBL in at least one anatomical region), ATL (animals with ATL only), and animals with no visible lesions.

Cases of TB were classified as either localized, with lesions in a single anatomical region (head, thorax, or abdomen) or generalized, with lesions in at least two different anatomical regions.

Histopathological examination. All lymph nodes collected from FS2 were submitted to histopathological examination. Collected tissue samples were fixed in 10% neutral buffered formalin and routinely processed to obtain 4-μm paraffin-embedded histological sections, which were then stained with hematoxylin and eosin and with Ziehl-Neelsen stain and examined by light microscopy.

Based on microscopic appearance, lesions were classified as granulomatous or atypical. Granulomatous lesions were, in turn, subdivided into five histopathological types, applying the criteria reported in the literature (3, 27) with slight modifications: "granuloma type 1," initial granuloma composed of mononuclear inflammatory cells (especially lymphocytes) without a necrotic center or aggregate of polymorphonuclear cells (neutrophils); "granuloma type 2," typical tuberculous granuloma with a necrotic center surrounded by epithelioid cells and a variable number of multinucleated giant cells, macrophages, and lymphocytes; "granuloma type 3," necrotic with minimal calcifications at the center; "granuloma type 4," granuloma with a necrotic calcified center and peripheral fibro-

plasia; and "granuloma type 5," granuloma with abundant calcifications occupying the entire necrotic area.

Pigs were thus divided into the following three groups based on whether they had granulomatous lesions (animals with granulomatous lesions in at least one anatomical region), atypical lesions (animals showing atypical lesions only), or no lesions (animals showing no microscopic lesions).

Culture examination and molecular identification of isolates. (i) FS1. For each animal, lymph nodes were pooled and cultured. Briefly, about 2-g samples were homogenized, decontaminated with 1 volume of 4% NaOH for 30 min at 37°C, neutralized with 0.067 M phosphate-buffered saline (PBS) at pH 7.2, and centrifuged for 15 min at 3,000 × g. Pellets were suspended in PBS, inoculated into the Middlebrook 7H9 medium (Biolife, Italy) with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson and Company), and then incubated in CO_2 for 8 weeks at 37°C for bacteriological examination according to the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (52).

To test for the presence of *M. bovis* in culture media, DNA was extracted from all cultures using Wizard SV genomic DNA purification system (Promega Corporation, Madison, WI). It was then screened for the presence of *M. tuberculosis* complex (MTC) genome by a specific PCR assay able to amplify a 194-bp fragment of IS6110 (26). All amplifications were performed in a total volume of 25 μl using GoTaq Green master mix (Promega Corporation, Madison, WI) following the manufacturer's instructions. The amplification profile was as follows: 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. The *M. bovis* ATCC 19210 genome, kindly provided by the Department of Infectious, Parasitic and Immune-Mediated Diseases of the Istituto Superiore di Sanità, was used as control. After amplification, all reaction mixtures were analyzed by electrophoresis in a 2% agarose gel, stained with Gel Red nucleic acid stain (Biotium, Inc., Hayward, CA), and photographed. The presence of MTC genome was confirmed through nucleotide sequence analysis of PCR products.

PCR-positive samples were examined further. Isolates were identified as *M. bovis* by staining for acid and alcohol fastness, colony morphology, and molecular typing by PCR-restriction fragment length polymorphism (RFLP) analysis of the *gyrB* gene (33) and by RD1-PCR (40).

(ii) FS2. Cultures from each collected tissue sample were performed for a more in-depth microbiological examination. Samples, from 2 to 15

g, were homogenized in filter stomacher bags. Two decontamination methods and three media were used. Briefly, the filtered material was divided into two aliquots: one part was decontaminated with 1 volume of 2% NaOH for 15 min at 37°C and then neutralized with 20% HCl; the other part was decontaminated with 1 volume of 1.5% hexadecylpyridinium (HPC) for 30 min at room temperature. Specimens were then centrifuged at 3,000 × g for 20 min. Pellets were seeded on the following media: Stonebrink (Heipha Diagnostika, Germany), Lowenstein-Jensen (Biolife, Italy), and Lowenstein-Jensen without glycerol (Biolife, Italy), following the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (52). Cultures were incubated for the first 15 days at 37°C in a 5 to 10% CO_2-enriched atmosphere and then at 37°C in normal atmosphere. They were monitored weekly for 90 days.

Colonies suggestive of mycobacteria were Ziehl-Neelsen stained. Alcohol- and acid-resistant bacilli underwent DNA extraction by boiling and then underwent molecular testing. The multiplex PCR assay described by Kulski et al., with some modifications, was used for the detection and identification of members of the genus *Mycobacterium* as well as for the differentiation between members of the MTC, *Mycobacterium avium*, and *Mycobacterium intracellulare* (22). Briefly, reactions were performed in a total volume of 50 μl containing 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates (dNTP), 10 pmol of primers, 2.5 U of *Taq* polymerase (Promega Corporation, Madison, WI), and 5 μl of DNA. The cycling conditions were denaturation at 94°C for 5 min, followed by 35 cycles of 20 s at 94°C, 30 s at 57°C, and 30 s at 72°C and a final extension cycle at 72°C for 7 min. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Expected fragment lengths were 1,030 bp, 372 bp, 180 bp, and 850 bp for *Mycobacterium*, MTC, *M. avium*, and *M. intracellulare*, respectively.

Colonies identified as MTC were then PCR typed as described by Huard and colleagues (20) in order to detect *M. bovis* genome.

Nontuberculous mycobacteria (NTM) were further characterized using the MicroSeq 500 16S rRNA-based bacterial ID system (Applied Biosystems) following the manufacturer's recommendations. Sequencing was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems). Data were analyzed by Microseq ID Microbial Identification software v.1.0 (Applied Biosystems).

Suspected *Rhodococcus equi* colonies were identified by both standard bacteriological methods (43) and the biochemical API Coryne test (bioMérieux, France).

Spoligotyping and MIRU-VNTR analysis of *M. bovis* isolates. All *M. bovis* strains isolated in the two field studies were then further typed by both spoligotyping (21) and MIRU-VNTR typing of 12 genomic loci. In spoligotyping, the spacer sequences contained in the direct repeat locus were detected by hybridization onto a spoligotyping membrane (Isogen Bioscience BV, Maarssen, The Netherlands). For MIRU-VNTR typing, 12 genomic loci were selected and amplified individually: VNTR loci 2165, 2461, 0577, 580, and 3192 (i.e., ETR-A to -E) (15); VNTR locus 2996 (i.e., MIRU26) (50); VNTR loci 2163a, 2163b, 3155, and 4052 (49); and VNTR loci 1895 and 3232 (45). PCRs were performed following the method of Boniotti and colleagues (4). *M. tuberculosis* H37Rv was used as a reference strain. Allele assignment was performed on the basis of PCR fragment size compared to a 50-bp ladder (molecular weight marker).

The resulting genetic profiles, obtained by combining spoligotyping and MIRU-VNTR, were compared to a database collection of *M. bovis* genotypes of isolates originating from Italian cattle from the geographic area under study. This database comprises more than 100 spoligotype patterns from about 4,000 Italian *M. bovis* isolates representing more than 2,000 outbreaks.

Statistical analysis. The Chi-square (χ^2) test according to Mantel-Haenszel was used to analyze the relationship between occurrence of tuberculous lesions and sex or age classes. Differences were considered significant at $P \leq 0.05$.

RESULTS

FS1. Of the 119 pigs enrolled, 61 were males and 58 females. Forty-two animals were juveniles (19 males and 23 females), 73 were subadults (39 males and 34 females), and the remaining 4 were adults (3 males and 1 female). Animals showed no clinical signs of disease upon antemortem inspection.

Upon anatomical inspection, TBL were detected in 8 (6.7%) Sicilian black pigs. All lesions were caseous necrotic calcified, and all but one were large (>1 cm in diameter). TBL affected the head in 5 animals (5/8 [62.5%]), the abdomen in one animal (1/8 [12.5%]), and both head and thorax in 2 animals (2/8 [25%]). Head lymph nodes, particularly mandibular and retropharyngeal lymph nodes, were the most frequently affected (7/8 [87.5%]). Macroscopic TBL were observed almost exclusively in subadult males (7/8). No statistically significant difference was found by the χ^2 test ($P > 0.05$).

M. bovis was isolated from 4 pigs (3.4%): 3 animals were affected by macroscopic TBL, and 1 had no visible lesions. All *M. bovis* culture-positive animals were male subadults.

FS2. Of the 100 Sicilian black pigs originating from bTB-infected herds identified in FS1, 55 were males and 45 were females. Thirty-seven animals were juveniles (14 males and 23 females), and 63 were subadults (41 males and 22 females). All animals appeared clinically healthy. Results of anatomical inspection and of histopathological examination are reported in Tables 1 and 2, respectively.

Gross pathology. Macroscopic TBL were observed in 32 black pigs (32%). As shown in Table 1, such lesions most frequently affected head lymph nodes (22/32 [69%]), followed by thoracic (19/32 [59%]) and abdominal lymph nodes (18/32 [56%]). An example of affected head lymph nodes is shown in Fig. 3.

A total of 15 pigs had localized lesions (15/32 [47%]). The "head-only" group was the largest, with 8 cases (8/15 [53%]), followed by the "thorax-only" group, with 5 cases (5/15 [33%]), and the "abdomen-only" group, with 2 cases (2/15 [13%]). Localized lesions appeared most frequently as caseous necrotic-calcified tubercles <1 cm in diameter (10/15 [66.7%]). Larger lesions (>1 cm in diameter) were not macroscopically observed in this group.

Generalized TBL were observed in 17 pigs (17/32 [53%]). In this group, the head was almost always affected (14/17 [82.4%]). Lesions affecting all three anatomical regions in the same animal were observed in 59% of generalized TB cases (10/17). Lesions appeared most frequently as caseous necrotic-calcified tubercles <1 cm in diameter (8/17 [47%]) and as miliary lesions with sclerosis and calcification (10/17 [58.8%]). Larger lesions (>1 cm in diameter) were encased in a thick fibrotic capsule and were macroscopically observed in 4 pigs with generalized TB (4/17 [27%]).

As in FS1, the age group most frequently affected by TBL was that of the subadults (25/32 [78%]; $\chi^2 = 4.57$, df = 1, $P = 0.03$). ATL were detected in 16 black pigs (16%). The ATL appeared most frequently as whitish nodules 1 mm in diameter and tended to affect the head lymph nodes (11/16 [69%]). No ATL were observed in TBL-affected animals in any anatomical region. Fifty-two percent of enrolled animals showed no visible macroscopic lesions.

Microscopic pathology. Granulomatous lesions were observed in 29 black pigs (29%). Head lymph nodes, particularly mandibular and retropharyngeal lymph nodes, were the most fre-

TABLE 1 Gross pathology results by macroscopic examination from FS2[a]

Group and type of lesions (n)		Lesion distribution (n)	Lesion description (n)
TBL (32)			
	Localized	Head only (8)	<1-cm caseous necrotic-calcified lesions (10)
		Thorax only (5)	<1-cm caseous-necrotic lesions (2)
		Abdomen only (2)	1-mm miliary lesions (3)
	Generalized	Head and thorax (1)	>1-cm encapsulated caseous necrotic-calcified lesions (4)
		Head and abdomen (3)	<1-cm caseous necrotic-calcified lesions (8)
		Thorax and abdomen (3)	<1-cm caseous-necrotic lesions (2)
		Head, thorax, and abdomen (10)	1-mm miliary lesions (10)
ATL (16)			
	Localized	Head only (9)	
		Thorax only (5)	>1-mm whitish/fibrous nodules (14)
	Generalized	Thorax and abdomen (1)	>1-mm whitish/fibrous nodules (2)
		Head, thorax, and abdomen (1)	1-mm whitish nodules (2)

[a] n = 100. Throughout, n represents the number of animals. Animals with more than one type of lesion are counted more than once. TBL, tuberculous-like lesions; ATL, atypical tuberculous lesions.

quently involved (22/29 [76%]), followed by thoracic lymph nodes (16/29 [55%]) (Table 2).

Localized lesions were found in 10 animals (10/29 [34.5%]) and affected the head in 5 cases, the thorax in 3 cases, and the abdomen in 2 cases. Lesions appeared mostly as initial granulomas or small neutrophil aggregates (type 1, 4/10 [40%]) or as classical fibronecrotic granulomas with calcified center (type 4, 5/10 [50%]). Only in one case were type 5 granulomas found affecting the head.

Generalized lesions were observed in 19 animals (19/29 [65.5%]). The head was almost always affected (17/19 [89.5%]). In 8 cases (8/19 [42%]), all three anatomical regions were involved in the same animal. The presence of a necrotic center was observed more frequently in generalized than in localized cases. Lesions in fact appeared mostly as type 4 granulomas (11/19 [58%]) and as granulomas with abundant calcifications (type 5, 8/19 [42%]). Several neutrophils in and around the necrotic center were observed in 36% of cases positive for *M. bovis*. An example of a type 3 granuloma is shown in Fig. 4.

Pigs affected by generalized lesions had more advanced granulomatous lesions in the head (i.e., histological type 4 or 5). The most severe lesions were found in the mandibular and retropharyngeal lymph nodes. Here too, no statistically significant differences in the frequency of lesions were observed between males and females, and subadults were the age group most likely to be affected (24/29 [83%]; $\chi^2 = 6.77$, df = 1, $P < 0.01$).

Heterogeneous atypical lesions were observed microscopically in 21 pigs (21%): 11 animals showed ATL only, and 10 animals showed ATL as well as granulomatous lesions. Atypical lesions were recorded mostly as hemorrhagic foci and affected all anatomical regions. Most enrolled pigs showed no lesions (60%).

Comparing gross pathology to histology results, of the 32 pigs with macroscopic TBL, 25 also showed microscopic lesions. Specifically, six animals with localized TBL observed macroscopically were also affected by generalized microscopic lesions, invariably involving the head region. Seven pigs with gross TBL consisting in

TABLE 2 Histopathology results from FS2[a]

Group (n)		Lesion distribution (n)	Lesion description (n)
Granulomas (29)			
	Localized	Head only (5)	Type 1 (4)
		Thorax only (3)	Type 4 (5)
		Abdomen only (2)	Type 5 (1)
	Generalized	Head and thorax (3)	Type 1 (6)
		Head and abdomen (6)	Type 2 (6)
		Thorax and abdomen (2)	Type 3 (3)
		Head, thorax, and abdomen (8)	Type 4 (11)
			Type 5 (8)
Atypical lesions (11)			
	Localized	Head only (2)	Hemorrhagic foci (5)
		Thorax only (3)	Neutrophil aggregates (2)
		Abdomen only (4)	Necrotic foci (2)
	Generalized	Head and abdomen (2)	Hemorrhagic foci (3)
		Thorax and abdomen (1)	Neutrophil aggregates (1)
			Lymphoid rarefaction (2)

[a] n = 100. Throughout, n represents the number of animals. Animals with more than one type of lesion are counted more than once.

FIG 3 Gross pathology. Shown are caseous necrotic-calcified tuberculous-like lesions in the mandibular, retropharyngeal, and parotid lymph nodes of a black pig.
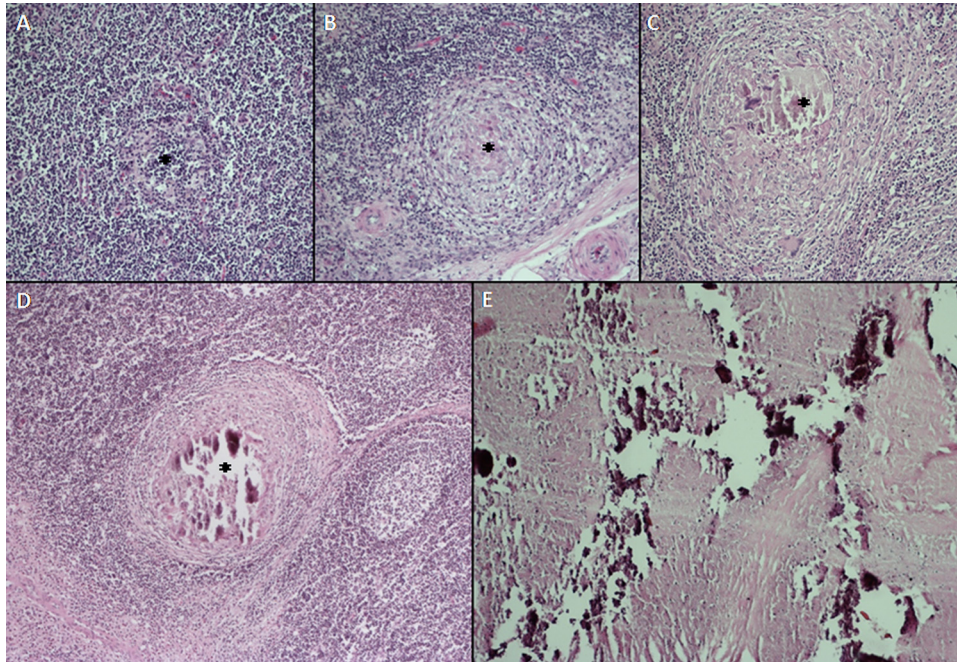
FIG 4 Histopathology. Panels A to E show granulomatous lesions by type. (A) Type 1: initial granuloma composed by mononuclear inflammatory cells (*). Magnification, 20×. (B) Type 2: granuloma with a necrotic center (*) surrounded by epitheliod cells, a variable number of giant cells, macrophages, and lymphocytes. Magnification, 20×. (C) Type 3: necrotic granuloma with minimal calcifications in the center (*). Magnification, 20×. (D) Type 4: granuloma with necrotic calcified center (*) and peripheral fibroplasias. Magnification, 10×. (E) Type 5: granuloma with abundant calcifications occupying all of the necrotic area. Magnification, 10×. Hematoxylin and eosin.

miliary lesions <1 cm in diameter, on the other hand, showed no lesions microscopically. Of the 16 pigs with macroscopic ATL, 2 animals were affected by initial microscopic lesions (type 1) and 3 animals had atypical lesions consisting of hemorrhagic foci. Of 52 pigs with no macroscopically visible lesions, 2 animals showed type 1 granulomas, and 8 showed atypical lesions only at histopathology.

Microbiology. Mycobacterial colonies were isolated from 34 animals, and *R. equi* was isolated from 1 pig. Molecular tests allowed us to identify *M. bovis* (in 27 pigs), *M. avium* (in 1 pig), and NTM other than *M. avium* (in 6 pigs). Results are shown in Table 3.

The pig infected with *R. equi* had ATL, namely, whitish nodules, at macroscopic examination and atypical lesions at histopathology, such as colliquative necrotic areas and lymphoid rarefaction.

M. bovis was isolated from animals showing both macroscopic and microscopic lesions (23 pigs), animals with macroscopic TBL only (1 pig), initial microscopic lesions only (2 pigs), or necrotic foci only at histopathology (1 pig). The head lymph nodes, specifically the mandibular and retropharyngeal lymph nodes, were found to be the most frequently infected tissue. Here, too, no association was found between sex and infection. *M. bovis* was isolated more frequently in the subadult age group (24/27 [89%]).

M. avium was isolated from one pig with hemorrhagic foci at histopathology and macroscopically visible TBL. Other NTM were isolated from six animals. According to the 16S rRNA gene sequence analysis, one NTM isolate was 100% identical to *M. nonchromogenicum*, and the other five were 99% identical to *M. genavense*/*simiae* (n = 3), *M. colombiense* (n = 1), and *M. nonchromogenicum* (n = 1). Further tests are required to better identify these NTM species. This is, however, beyond the scope of this report.

Typing of *M. bovis* isolates and comparison to cattle database. A total of 31 *M. bovis* isolates from both field studies were genotyped by both spoligotyping and 12 MIRU-VNTR loci. Results are shown in Table 4.

Four spoligotypes were identified: SB0120 (23 isolates), SB0841 (5 isolates), SB0833 (2 isolates), and a new spoligotype (1 isolate), lacking spacers 6, 7, 14, 15, and 17. This genetic profile has not been described in Italy before nor found in the *Mycobacterium bovis* Spoligotype Database, available at http://www.mbovis.org/, where, following our report, it was registered as SB2018. SB0120 is the predominant spoligotype in Italy as well as in Sicily, account-

TABLE 3 Microbiology results from FS2[a]

Positive culture (n)	Macroscopic/microscopic pathological group (n)
M. bovis (27)	TBL/granulomas (23)
	TBL/no lesions (1)
	No visible lesions/granulomas (2)
	No visible lesions/atypical lesions (1)
M. avium (1)	TBL/atypical lesions (1)
Other NTM (6)	TBL/atypical lesions (1)
	ATL/granulomas (2)
	ATL/atypical lesions (1)
	No visible lesions/atypical lesions (2)
R. equi (1)	ATL/atypical lesions (1)

[a] n = 100. Throughout, n represents the number of animals. "Other NTM," nontuberculous mycobacteria other than *M. avium*.

TABLE 4 Genetic profiles of *M. bovis* isolates[a]

Spoligotype (*n*)	MIRU-VNTR type												Profile (*n*)	Genotype from combined typing results (*n*)[c]
	2165[b]	2461[b]	0577[b]	580[b]	3192[b]	2163a[b]	2163b[b]	4052[b]	1895[b]	3155[b]	3232[b]	2996[b]		
SB0120 (23)	4	5	5	3	3	10	4	3	4	3	6	5	a (12)	SB0120/a (10)
	4	5	5	3	3	10	4	4	4	3	6	5	b (8)	SB0120/b (8)
SB0841 (5)	4	5	5	3	3	10	4	4	4	3	6	4	c (1)	SB0120/c (1)
SB0833 (2)	4	5	5	3	3	10	3	4	4	3	8	5	d (1)	SB0120/d (1)
	5	5	5	3	4	10	4	5	4	3	6	5	e (1)	SB0120/e (1)
SB2018 (1)	5	5	5	3	3	10	4	4	4	3	6	5	f (4)	SB0120/f (1)
	3	3	5	3	3	10	4	4	4	3	6	5	g (1)	SB0120/g (1)
	5	5	5	3	3	10	4	4	4	3	5	5	h (2)	SB0841/f (3)
	5	5	5	3	3	5	4	4	4	3	6	5	i (1)	SB0841/h (1)
														SB0841/i (1)
														SB0833/a (2)
														SB2018/h (1)

[a] *n* = 31. Throughout, *n* represents the number of isolates.
[b] The repeat copy number at each locus is indicated.
[c] Genotypes obtained by combining spoligotyping and MIRU-VNTR results.

ing for 55% and 33% of *M. bovis* spoligotypes, respectively. SB0841 is also common both in Sicily (9%) and in Italy in general (4.8%), while SB0833 is rare.

MIRU-VNTR analysis, on the other hand, differentiated nine profiles (MIRU-VNTR-a to MIRU-VNTR-i) (Table 4). The most frequent genetic profiles were MIRU-VNTR-a (12 isolates), MIRU-VNTR-b (8 isolates), and MIRU-VNTR-f (4 isolates). The combination of both typing methods increased the number of genetic profiles to 12 (Table 4). The most frequent profile was genotype SB0120/a, with 10 isolates.

The combined genetic profiles were then run against the national database collection of *M. bovis* isolates from cattle. With the exception of genotypes SB0833/a and SB2018/h, all profiles matched genotypes in the database. Genetic profiles SB0120/b, SB0120/e, SB0120/g, SB0841/f, and SB0841/i were previously reported to have occurred in Sicily. According to available information on management practices, genotypes SB0120/b, SB0120/e, SB0120/g, and SB0841/i matched profiles isolated from cattle herds that practice semifree grazing and seasonal moves (twice a year) on lands neighboring the Natural Parks of Nebrodi and Madonie.

DISCUSSION

The present study aimed to assess the epidemiological role of the black pig in bTB in the ecosystem under study. To the best of our knowledge, this is the first study to have analyzed culture assays together with granulomatous and atypical lesions in *Sus scrofa* both macroscopically and microscopically. We found that *M. bovis* infects pigs reared under free and semifree conditions and that the characteristics of the lesions, their localization, and the genetic profiles of *M. bovis* isolates all support the hypothesis that Sicilian black pigs, in this ecological setting, might act as a reservoir of bTB infection.

Our findings provide evidence that bTB—both gross TBL (6.7%) and *M. bovis* infection (3.4%)—is present among black pigs in the area under study.

We found no association between bTB and sex in the population studied. Some studies reported a higher prevalence in males

(38, 51) and others in females (46), while no association between gender and *M. bovis* infection was found elsewhere (53).

The occurrence of bTB differed substantially by age. Subadults were more likely than juveniles to be affected by both macroscopic and microscopic tuberculous lesions. We were unable to estimate the occurrence of bTB in the older age group because of the small number of adults in our samples (4 in FS1 and none in FS2). As such, our data probably underestimate the true prevalence of the disease in this population. It should be remembered, however, that adult pigs are, indeed, a small minority in Sicilian black pig populations, as these animals are normally slaughtered relatively early in life. Our findings are not surprising in view of the fact that bTB is usually a chronic and not highly fatal disease. The proportions of infected animals and animals with gross lesions are, thus, expected to increase with age. This trend has been observed for wild boars (51) and red deer (53). Similar levels of gross lesions and infections across ages were found in wild boars by others (27, 46, 53). In our population, infection in juveniles may have still been unapparent or different behavioral characteristics may have contributed to a higher exposure of subadults to *M. bovis*.

Few studies have addressed the macro- and microscopic characteristics of bTB in *Sus scrofa*, often with conflicting results. Corner et al. (9) found most Australian bTB-affected feral pigs to have TBL localized in the mandibular lymph nodes. Martin-Hernando and colleagues (27), on the other hand, reported a majority of infected European wild boars to have generalized, rather than localized bTB. In our population, most black pigs with TBL had generalized lesions at both gross and histological examinations (53% and 65.5%, respectively). Combining gross pathology and histology results, the proportion of animals with generalized lesions increased, suggesting that the prevalence of the disease in black pig populations could be underestimated if based solely on macroscopic examinations.

Histologically, initial granulomas of type 1 and advanced granulomas of type 4 were predominant in cases of localized bTB, whereas advanced granulomas of types 4 and 5 were more prevalent in cases of generalized bTB. This pattern is consistent with

disease progression and thus also with the presence of active infection among black pigs.

Head lymph nodes, particularly mandibular and retropharyngeal lymph nodes, were the most frequently affected in both localized and generalized cases of bTB infection, as observed both macroscopically and microscopically. They were also the tissue most frequently infected with *M. bovis*, and thus probably the most suitable for pathogen isolation for diagnostic purposes. The frequent involvement of mandibular lymph nodes in bTB pathology observed is in accordance with previous reports on *Sus scrofa* (9, 17, 27). Martin-Hernando et al. observed lesions in the retropharyngeal and parotid lymph nodes only in the presence of lesions in the mandibular lymph nodes (27). In our study too, lesions in the retropharyngeal lymph nodes tended to appear in the presence of lesions in the mandibular lymph nodes. In some cases, however, the retropharyngeal lymph nodes were the only lymph nodes affected in the head region. The frequent involvement of those lymph nodes, whose afferents drain the nasal cavities, the nasopharynx, and the auditory tubes, suggests that both respiratory and food-borne transmission may occur.

Thoracic lymph nodes were also frequently affected in both localized and generalized cases of bTB infection, as observed both macroscopically (59.4% of cases with gross pathology) and microscopically (55.2% of cases with histopathology). Gross lesions in thoracic lymph nodes were also observed with a similar prevalence (57%) in the wild boar (27).

We observed that, in cases of generalized bTB, head lymph nodes tended to show lesions of more advanced histological types (types 4 and 5) than other anatomical regions. It has been demonstrated that granuloma formation and maturation involve both the cooperation of many different host cell types and complex interactions between the host and mycobacteria (6). Previous studies have shown that granuloma formation and structure are influenced by numerous factors, such as bacterial load, susceptibility to infection, disease progression, and granulomatous inflammatory tissue-specific response (14, 44). According to these studies, more advanced stages of granuloma development, such as those observed here in the head region, may suggest that head lymph nodes are more susceptible to injuries than other lymph nodes or that they are more likely to be the primary sites of infection, and, therefore, sites where infection is likely to be more established. The most frequent involvement of mandibular and retropharyngeal lymph nodes in this study, however, suggests that mycobacteria commonly enter both the oral and nasal mucosae through food, water, and air. Behaviors such as the sharing of water and food, as well as direct oro-nasal contact between animals, known to occur in suids, are likely to contribute to such transmission.

In previous studies, most gross TBL observed in wild boars were encapsulated by a fibrous layer (3, 17, 46). Here, localized and generalized macroscopic lesions appeared most frequently as miliary lesions and as caseous necrotic-calcified tubercles. Encapsulated TBL were rarely observed (27%). Histologically, granulomas with peripheral fibroplasia and central necrosis, with different degrees of calcification (types 4 and 5) were the most prevalent. Numerous granulomas without peripheral fibroplasia, such as types 1, 2, and 3, however, were also frequently detected. While the fibrous layer of encapsulated TBL is thought to reduce the likelihood of excretion (46), caseous necrosis contributes to the creation of infectious material that might be spread to other

individuals (6). Lesions not contained by a fibrous layer, such as those observed in this study, suggest that these pigs are likely to excrete mycobacteria present in their sputum, mucus, or stool by way of coughing, sneezing, or defecation.

The distribution and characteristics of the lesions observed in the Sicilian black pig are, thus, consistent with active infection, the shedding of mycobacteria, and the resulting contamination of food, pastures, and water. Mycobacteria, therefore, can be transmitted to livestock at shared pastures. Humans can also become infected by the contact with infected animals and through the handling of infected tissues, carcasses, or by-products. Thus, farmers, slaughterhouse workers, animal transporters, persons who process animal products, and veterinarians all constitute high-risk groups for bTB.

The *M. bovis* culture is regarded as the "gold standard" for diagnosis. In our study, not all animals with lesions were positive for *M. bovis*. Conversely, *M. bovis* was isolated from two animals with no lesions. It has been observed that *M. bovis* infection may not be accompanied by the presence of macroscopic lesions (16). Discordance between bacterial culture and gross and microscopic pathology in wild boars has been attributed either to latency making mycobacteria difficult to culture (48), to the protocol applied (10, 11), or to sampling error in cases of intralesional heterogeneity (19). Moreover, fibrotic and calcified granulomas may not contain viable bacteria (39). Here, the use of several diagnostic methods applied to the same animal, such as cultures on different media, gross pathology, and histopathology, allowed us to achieve the highest sensitivity in the detection of mycobacterial infection in black pigs.

In addition to *M. bovis*, other potential zoonotic pathogens, such as *R. equi* and *M. avium*, were isolated from pigs presenting both atypical microscopic lesions (1 pig each for *R. equi* and *M. avium*) and either ATL (*R. equi*-infected pig) or TBL (*M. avium*-infected pig). NTM other than *M. avium* were isolated in this study from animals showing TBL, ATL, or no visible lesions at necropsy. Microscopically, most of them presented neither granulomatous lesions nor atypical lesions. Our results are in accordance with recent studies on swine in which environmental mycobacteria were identified in lymph nodes in the absence of lesions (23) and in the presence of suspected tuberculous lesions (30). However, we have not sufficient data to exclude that these NTM may be responsible of disease in this animal since the low occurrence observed here.

To investigate the origin of bTB in black pigs, we used molecular typing. Spoligotyping and MIRU-VNTR typing have become valuable tools in the study of *M. bovis* epidemiology, allowing investigators to better identify the sources of bTB infection and its transmission routes. Spoligotyping alone confirmed SB0120 as the predominant spoligotype in Italy and in Sicily. The presence in pigs of spoligotypes never detected in the Sicilian cattle population nor described in the international scientific literature suggests that bTB circulates among pigs and that new variants could be generated with the potential of infecting cattle and humans. Due to the limited collection of strains isolated in Sicily, however, we cannot exclude the possibility that these genotypes circulate in cattle populations as well. To increase the discriminatory power of the molecular typing, we combined spoligotyping with analysis of 12 MIRU-VNTR loci. Most of the genetic profiles thus obtained were identical to *M. bovis* cattle profiles already observed in Sicily. Moreover, 4 of them matched profiles from nearby cattle herds

that practice seasonal moves from neighboring lands to the Natural Parks of Nebrodi and Madonie. This is a common farming practice in Sicily that increases the possibility of contact between domestic ruminants and black pigs in this ecosystem. It is likely, therefore, that the infection was originally transmitted from cattle, the most abundant and natural host of *M. bovis*, to black pigs sharing the same pastures and subsequently spread within the black pig population. It is likely that horizontal transmission of *M. bovis* may also occur from pigs to cattle. It has recently been demonstrated that *M. bovis* strains isolated from wild boars may be pathogenic for cattle, producing TB lesions (29). Direct or indirect contact between bTB wild hosts and livestock may occur at shared pastures, water holes, and feeding sites. The presence of bTB and of new *M. bovis* genetic profiles among Sicilian black pigs suggests that the disease is self-maintaining in this population. Moreover, the high density of this population and its social behavior, common farm practices, irregular food availability, and the Mediterranean climate together promote the aggregation of animals at waterholes, especially in summer. The above are all considered risk factors likely to facilitate pathogen transmission directly and/or indirectly, thus hindering disease control (31).

Bovine tuberculosis is an important emerging disease in many parts of the world where wildlife reservoir hosts for *M. bovis* have been identified (7, 12, 13, 34, 42). Although the role played by wild ungulates in the epidemiology of bTB is somewhat controversial in the literature, our findings—namely, that the animals are infected, that the disease is active and self-maintaining, and that the conditions exist for black pigs to transmit it to cattle and humans—support the hypothesis that the Sicilian black pig might act as a bTB reservoir in its ecosystem. However, additional studies will be necessary to establish unequivocally the host status of the Sicilian black pig.

ACKNOWLEDGMENTS

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