Molecular epidemiology of Mycobacterium tuberculosis complex strains isolated from livestock and wild animals in Italy suggests the need for a different eradication strategy for bovine tuberculosis

Molecular epidemiology of Mycobacterium tuberculosis complex strains isolated from livestock and wild animals in Italy suggests the need for a different eradication strategy for bovine tuberculosis

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Summary

Bovine tuberculosis (bTB) is an important zoonosis, which has been re-emerging in different ecological scenarios. In Sicily, Italy, from 2004 to 2014, an anatomopathological survey for tuberculosis-like lesions both in farmed and wild animals was performed. The isolates were genotyped using spoligotyping and Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) techniques. High prevalence of lesions was observed for cattle (4%), pigs (4.9%) and wild boars (6.8%), and a total of 625 *Mycobacterium bovis* isolates were identified. Genotyping analysis showed the presence of 37 different spoligotypes including fifteen spoligotypes not present in other Italian regions and 266 MIRU-VNTR profiles. Spoligotype SB0120 exhibited the highest prevalence in cattle (50%) and pigs (56%) and the highest genetic variety with 126 different MIRU-VNTR profiles. The isolation of *M. bovis* in a farmer underlines the importance of *M. bovis* identification during the human TB diagnostic processes. This study supported the use of the genotyping analysis as a valuable tool for the evaluation of the epidemiological role of pigs and other domestic reservoirs such as goats and the role of wildlife in the maintenance of bTB infection.

INTRODUCTION

Mycobacterium bovis is the main causative agent of bovine tuberculosis (bTB) and, among all the members of the *Mycobacterium tuberculosis Complex* (MTBC), has the broadest host range (Pesciaroli et al., 2014; Rodriguez et al., 2010). It can infect farm animals such as cattle, farmed buffalo, goats, farmed deer, pigs, horses and sheep but also cats and dogs, (Buick, 2006; Rodriguez et al., 2010). Furthermore, *M. bovis* has been found in various wildlife species like red deer, fallow deer, badger and wild boars (Delahay et al., 2007; Krajewska, Lipiec, Zabost, Augustynowicz-Kopeć, & Szulowski, 2014; Scantlebury, Hutchings, Allcrof, & Harris, 2004; Schoepf et al., 2012) and sporadically in more than 40 free-ranging wild animal species including a pinniped (grey seal, *Halichoerus grypus*) (Barnett et al., 2013; OIE, 2010). Human beings are also susceptible to *Mycobacterium bovis* infection mainly through the consumption of unpasteurized milk and dairy products from infected herds (Cosivi et al., 1998; Lo Bue, Enarson, & Thoen, 2010). Within MTBC, *M. bovis* subsp. *caprae* is now considered as a new species *Mycobacterium caprae* comb. nov., sp. nov (Aranaz, Cousins, Mateos, & Domínguez, 2003). *M. caprae* is evolutionarily older, and it is involved in a lower number of zoonotic TB outbreaks restricted to European countries (Prodinger, Indra, Koksalan, Kilicaslan, & Richter, 2014; Rodriguez, et al., 2011; Rodriguez, Smith, Boniotti, & Aranaz, 2014). *M. caprae* is responsible for over 10% of TB outbreaks in cattle herds in Italy (Boniotti et al., 2009; unpublished results).

The surveillance of bTB is challenging due to its complex epidemiology, which involves multiple hosts in domestic and wild animal populations (Gortazar et al., 2011). Thanks to control measures and bTB eradication campaigns, many nations are currently classified as bovine tuberculosis-free (EFSA scientific report, 2016). Eradication programmes are in progress in a few European countries, as well as in Japan, New Zealand, the United States and some countries of Central and South America (Humphrey, Orloski, & Olea-Popelka, 2014; Livingstone, Hancox, Nugent, Mackereth, & Hutchings, 2015; Max, Paredes, Rivera, & Ternicier, 2011; Reviriego Gordejo & Vermeersch, 2006; Shimao, 2010).

In Europe, the epidemiological situation partially reflects differences in bovine husbandry systems and environmental conditions (Martinez-Lopez et al., 2014; Vial, Miguel, Johnston, Mitchell, & Donnelly, 2015). Surveillance strategies are adopted differently in various countries and regions, making the comparisons within Europe and other countries difficult (Reviriego Gordejo & Vermeersch, 2006; Rivière, Carabin, Le Strat, Hendrikx, & Dufour, 2014). In Italy, bTB prevalence is variable: regions such as Lombardia, Toscana, Trentino

Alto Adige, Emilia Romagna, Friuli Venezia Giulia are officially declared "bTB-free," while the highest prevalence is recorded in Southern Italy and, above all, in Sicily (about 4%) (ANON, 2015a). Sicily is divided into nine provinces (Messina, Palermo, Agrigento, Catania, Trapani, Caltanissetta, Enna, Ragusa, Siracusa), each one presenting different ecological scenarios, natural reserves and farming traditions. In Sicily, the national eradication programme in cattle and susceptible animals is based on "test and cull" method, using both single and comparative skin test and, when required, IFN-gamma assay (ANON, 2015b). However, wildlife is not included in official surveillance plans. When an outbreak is identified, epidemiological surveys are performed to track the origin and possible spread of the infection. The infected animals are slaughtered, and samples of organs are submitted to bacteriological analysis to confirm the presence of the mycobacterium. Generally, the risk factors involved in the spread of *M. bovis* infection depend mainly on livestock systems (extensive, intensive) and animals (e.g., local, exotic, cross-breed animals) (Green & Cornell, 2005; Humblet et al., 2010; Kaneene, Bruning-Fann, Granger, Miller, & Porter-Spalding, 2002; Tschopp, Schelling, Hattendorf, Aseffa, & Zinsstag, 2009). Important factors for the maintenance of infection have been found to be the animal husbandry system, animal movements, the presence of wildlife reservoirs and the sharing of pastures and water sources (Barasona et al., 2016; Fink et al., 2015; Gates, Volkova, & Woolhouse, 2013; Hardstaff, Marion, Hutchings, & White, 2014; Humblet, Boschiroli, & Saegerman, 2009; Vial et al., 2015). Recent studies showed that environmental factors such as natural habitat and herd management are important predictors of bTB spread in high-prevalence areas suggesting that the environment plays a role in the maintenance and short-distance diffusion of the infection (Brooks-Pollock, Roberts, & Keeling, 2014; Courtenay et al., 2006; Winkler & Mathews, 2015). In the Nebrodi ecosystem (Messina province, Sicily), farming practises are based on extensive livestock systems with pastures and water sources being shared among several animal species (Di Marco et al., 2012). Especially during the dry season, high animal density and transhumance increase the possibility of contact between susceptible species such as cattle and the autochthonous black Nebrodi pigs, which might act as reservoir of the infection (Di Marco et al., 2012). In this area, a case of bovine tuberculosis with generalized lesions in a fallow deer was caused by a strain of *M. bovis* with a spoligotype and MIRU-VNTR profile identical to an isolate obtained from a wild boar of the same area, suggesting the spreading of the disease in wildlife (Amato et al., 2016). Molecular epidemiology is very useful, especially in those countries where the classical eradication programmes and movement restrictions are still in progress (Boniotti et al., 2009; Durr, Clifton-Hadley, & Hewinson, 2000; Hauer et al., 2015). Spoligotyping is the most common molecular method used to type *Mycobacterium bovis* strain evolution (Aranaz et al., 1996; Gormley, Corner, Costello, & Rodriguez-Campos, 2014; Haddad, Masselot, & Durand, 2004; Milian-Suazo et al., 2008; Smith et al., 2003). The combination of spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) is a high discriminatory technique to differentiate *Mycobacterium bovis* isolates and could be used to provide clues regarding the source of infection, to trace transmission routes, understanding of epidemiological links and the identification of wild animal reservoirs (Allix et al., 2006; Aranaz et al., 1996; Boniotti et al., 2009; Gormley et al., 2014; Hauer et al., 2015; Skuce et al., 2005).

This study describes a molecular epidemiological investigation based on spoligotyping and MIRU-VNTR profiles of *Mycobacterium bovis* and *Mycobacterium caprae* strains circulating in both livestock and wildlife population in Sicily during the period January 2004-December 2014.

MATERIAL AND METHODS

2.1 Gross pathological lesions survey

In accordance with Italian and EU legislation for TB eradication program (DM 592/95 and DLgs 196/99, and Council Directive 64/432/EEC, Annexes A, I and B, respectively), an anatomopathological survey was carried out both in an abattoir and in the diagnostic laboratory of the Istituto Zooprofilattico Sperimentale della Sicilia A. Mirri Area di Barcellona P.G. This anatomopathological survey was completed for diagnostic and epidemiological purposes on carcasses of different species (livestock and wildlife) coming from routine

livestock slaughtering and hunting activities, from January 2004 to December 2014 (Table 1). Thoracic, abdominal organs and lymph nodes were carefully dissected following a standardized protocol of gross examination (Gormley et al., 2014). Tissue samples showing tubercle-like lesions were collected for further investigations.

Table 1. Results of the inspection of animal carcasses and relative prevalence

	Number of tested animals	Tuberculosis-like lesions	Gross lesion/animal inspected (%)
Cattle	412.975	16.519	4
Buffalos	35.000	35	0.1
Pigs	6.714	330	4.9
Goats	8.000	9	0.11
Sheep	10.000	12	0.12
Wild boars	280	19	6.79
Fallow deera	47	12	25.5

- [a] All deer were coming from the same herd (Amato et al., 2016).

2.2 *Mycobacterium bovis* strain isolation

Tissue samples were processed according to the official international protocol (OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2010): one to four grams of tissue sample was homogenized with 3 mL of phosphate-buffered saline (PBS [pH 6.8], 30 mM phosphate buffer [pH 7.2], 150 mM NaCl, 2 mM EDTA), for 120 s in a Stomacher 80 laboratory blender. The homogenate was decontaminated using NaOH (sodium hydroxide) 1 mol/L, centrifuged at 3,000 *g* for 15 min at 4°C, and the obtained sediment was suspended in PBS. Then, it was cultured at 37°C using the solid media Löwenstein-Jensen (L-J) medium (Heipha, GmbH, Eppelheim, Germany), Stonebrink (St) medium (Heipha, GmbH, Eppelheim, Germany) plus pyruvate and the liquid medium Middlebrook 7H9 broth (BBL MGIT, Becton, Dickinson) in the Bactec MGIT 960 system (Becton, Dickinson, Sparks, MD). The isolates were confirmed as members of the MBTC by acid alcohol-fast staining by the method described by Kulski et al. (Kulski & Pryce, 1996), and by PCR restriction fragment length polymorphism (RFLP) analysis of the gyrB gene through RsaI and SacII restriction enzymes (Huard et al., 2003; Kasai, Ezaki, & Harayama, 2000; Niemann, Harmsen, Ru¨sch-Gerdes, & Richter, 2000). Isolates were mainly obtained from farm animals (cattle and pigs), but some isolates from wildlife were available (fallow deer (Amato et al., 2016) and wild boars) (Tables 2 and 3), were also included in this study. A *M. bovis* isolate was obtained from a person, the owner of a TB infected herd, who showed suspected clinical symptoms (pneumonia).

Table 2. Isolates and spoligotypes in the period 2004–2014 in Sicily

Spoligotype	Cattle	Pigs	Goats	Variants of MIRU-VNTR profiles
SB0120	268	20		126
SB0121	17	1		12
SB0133	2			1
SB0134	123	2		51
SB0418 *(M. caprae)*	1			1
SB0818	1			1
SB0822	1			1
SB0828	4			4
SB0833	2	2		2
SB0841	90	12	2	18
SB0866 *(M. caprae)*	3	1		2
SB0897	8	1		3
SB0950	4			4
SB0961	3			1
SB1169	1			1
SB1550	9			7
SB1554	1			1
SB1564	4			3
SB1567	6			2
SB1569	1			1
SB1945	4			3

Spoligotype	Cattle	Pigs	Goats	Variants of MIRU-VNTR profiles
SB1305	11			1
Total	564	39	2	246

Table 3. Sicilian unique spoligotypes (2004–2014)

Sicilian spoligotypes	Cattle	Pig	Wild boar	Prevalence	Previous isolation in European or Extra European countries
SB0942			1	0.16	France
SB2018		1		0.16	NR
SB0162	1			0.16	Belgium, Spain, Africa
SB0807	3			0.47	Mexico, Spain
SB0868	1			0.16	Spain
SB0875	1			0.16	Spain
SB1335	1			0.16	Spain
SB1355	1			0.16	Spain
SB1388	1			0.16	Spain, France, Africa
SB0850	3			0.48	Africa
SB1643a	2			0.32	New isolate
SB1946	1			0.16	NR
SB1999	1			0.16	NR
SB2061	1			0.16	NR
SB2063	1			0.16	NR

Sicilian spoligotypes	Cattle	Pig	Wild boar	Prevalence	Previous isolation in European or Extra European countries
Total	18	1	1		

- NR, Not Reported isolates present in the Mycobacteria database but without any details or reference to publication.
- [a] The only spoligotype never reported before and presenting two MIRU-VNTR profiles.

2.3 Spoligotyping and MIRU-VNTR analysis

Molecular identification and genotyping of strains were performed by spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) analysis.

Spoligotyping was performed as described by Kamerbeek et al. (Kamerbeek et al., 1997). The spacer sequences contained in the direct repeat locus were detected by hybridization onto a spoligotyping membrane (Isogen Bioscience BV, Maarssen, the Netherlands).

MIRU-VNTR analyses were carried out by amplifying 12 individual VNTR loci: ETRA, ETRB, ETRC, ETRD, ETRE, VNTR 2163a, 2163b, 4052, 3155, 1895, 3232 and MIRU 26 (Boniotti et al., 2009). The resulting genetic profiles, obtained by combining spoligotyping and MIRU-VNTR, were compared to the national database collection of *M. bovis* genotypes. The database included 163 spoligotype patterns from about 4,000 Italian *M. bovis* isolates representing more than 2,000 outbreaks (Pacciarini, personal communication).

In this study, isolates from fallow deer and feral pigs (black Nebrodi pigs) describe in Amato et al. (Amato et al., 2016) and Di Marco et al. (Di Marco et al., 2012) were also considered.

Statistical analysis based on chi-square test was performed on the most representative spoligotypes to evaluate a possible correlation among the most frequent spoligotypes (SB0120, SB0841, SB0134) and host species (cattle and pigs). The result is considered significant at p-value <.05.

3 RESULTS

3.1 TB survey

The province of Messina recorded the highest number of outbreaks followed by Enna and Palermo during the entire period (Figure 1). These provinces in Sicily include the harshest mountainous regions where rural and traditional farming is highly diffused. In these areas, the highest genetic variability was also detected (Figure 1). The lowest genetic variability was found in the Ragusa province, a territory characterized by a plateau in which a semi-intensive breeding of dairy cattle is performed. In this province, the three main spoligotypes were detected, but few rare spoligotypes were found (others in Figure 1).

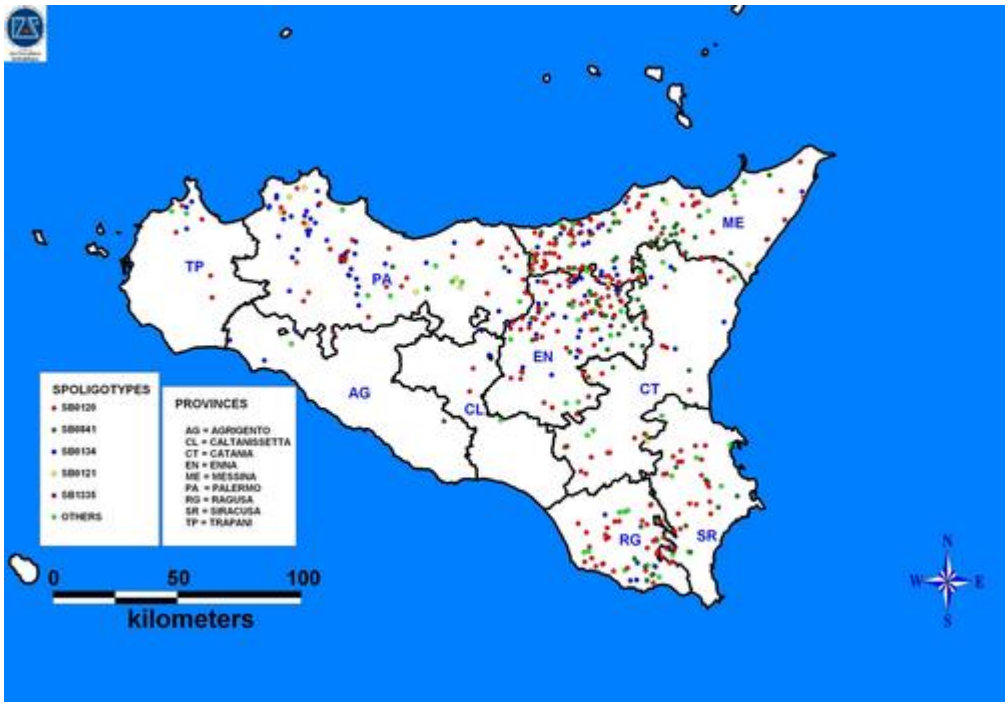

Figure 1

Spoligotypes distribution in the nine provinces of Sicily

3.2 Tuberculosis inspection on livestock carcasses

The lymph nodes of the head and internal organs (lungs, liver, spleen, stomach, intestine with their respective lymph nodes) were examined for macroscopic lesions. The gross pathology examinations revealed that the most frequent organs affected by bTB in cattle were the lungs, pleura and related lymph nodes with various degrees of calcification and caseous/colliquative necrosis in relation to the development of the disease and the immune reaction of the host. Isolation of *M. bovis* from the supramammary lymph nodes was performed, and a high prevalence of mycobacterium was detected also in samples which did not have any evidence of macroscopic lesion (unpublished data). Moreover, lesions were observed at the intestinal and hepatic lymph nodes in young subjects suggesting the spread of the pathogen through the milk or during pregnancy. Generalized lesions involving lymph nodes and organs as well some evidences of tuberculosis affecting the intestinal wall were also detected mainly in pigs. bTB lesions were also found in mammary glands and bones (Amato et al., 2017). Colliquative necrosis phenomena are rare and almost observed in the mammary glands. The bTb lesions in goats were generalized and had a chalky appearance. The prevalence of granulomatous lesions (TBL) was about 4% in cattle, 4.9 in pigs, 6.8% in wild boar and 0.1% in buffalo, goat and sheep (Table 1).

Mycobacterium bovis and *M. caprae* strains were isolated in farmed animals (cattle, pigs and goats) (Table 2), in a wild boar, but no isolates were obtained from buffalo and sheep. Single cases of *M. bovis* were also previously reported in fallow deer and wild boars (Amato et al., 2016). The human isolate included in this study was identified as *M. bovis*. Environmental mycobacteria (MOTT, Mycobacteria other than tuberculosis) were prevalent in wild boars (data not shown).

3.3 Genotypes distribution

A total of 625 isolates were analysed using spoligotyping and MIRU-VNTR techniques. Tables 2 and 3 summarize the strains detected during the period 2004–2014 considering spoligotypes and MIRU-VNTR profiles. Molecular characterization of *M. bovis* isolates showed the presence of 37 different spoligotypes and 262 MIRU-VNTR profiles (Tables 2 and 3). SB0120 was the most frequent spoligotype

circulating in farmed animals in Sicily (cattle, pigs), with a percentage of about 46% (268/582) in cattle and 50% (20/40) in pigs followed by spoligotypes SB0134 and SB0841 (Table 4). The uneven distribution in cattle and pigs of diffused spoligotypes SB0841 and SB0134 had a statistical significance ($p < .05$ by chi-square test). SB0841 is present at double percentage in pigs, and SB0134 is present mainly in cattle, whereas SB0120 showed almost the same prevalence in the two animal species. Spoligotypes SB0120 and SB0134 showed the highest variability with respectively 126 and 51 MIRU-VNTR profiles, in contrast, 11 strains identified as spoligotype SB1305, exhibited only one MIRU-VNTR profile indicating a single clone circulating in cattle only. Four further spoligotypes were shared among different species: SB0121 (17 of 582 bovines, one of 40 pigs), SB0833 (two of 582 bovines, two of 40 pigs) and SB0897 (eight of 582 bovines, one of 40 pigs) (Table 2). The spoligotype SB0866 (three of 582 bovines, one of 40 pigs) was identified as *M. caprae*. In some cases pigs, cattle and goats shared the same spoligotype and MIRU-VNTR profile, suggesting a clonal diffusion of some isolates in different animal species as observed in wildlife (Fink et al., 2015; Schoepf et al., 2012). However, 30 spoligotypes were isolated from a single animal species: 28 in cattle, one in pig and one in wild boar. Fifteen spoligotypes were detected in Sicily and not in other Italian regions. Sicilian spoligotypes have a unique MIRU-VNTR profile except for SB1643 (Table 3), which was isolated twice from cattle in the province of Messina. The first one isolated in 2013 showed MIRU-VNTR profiles 5,4,3,3,3,10,2,5,4,3,7,2, and the second one isolated in 2015 have MIRU-VNTR profiles 6,4,3,3,3,10, 2,5,4,3,7,2. Three other previously described new Sicilian spoligotypes SB2018, SB2061 and SB2063 (Acevedo et al., 2013; Di Marco et al., 2012), never isolated before were recorded onto the International *M. bovis* database (www.mbovis.org, Smith & Upton, 2012). Two further Sicilian unique spoligotypes SB1946, SB1999 were detected (Acevedo et al., 2013). The remaining spoligotypes (SB0942, SB0162, SB0807, SB0868, SB0850, SB1388, SB0875, SB1335, SB1355) were recorded in countries within and outwith Europe except for SB1643. The overall prevalence of each rare spoligotype calculated over the total 625(Tables 2 and 3), ranged from 0.16 to 0.48 (Table 3). The human isolate showed a spoligotype SB0120 and MIRU-VNTR profile 4,5,5,3,3,10,4,4,4,3,5,5; the same profile was isolated from bovine herds in the Centre and South of Italy (Umbria, Campania, Basilicata, Puglia), suggesting its widespread spatial diffusion in cattle in Italy (unpublished results).

Table 4. The most frequent spoligotypes isolated in cattle and pigs

Spoligotype	Cattle prevalence (%)	Pig prevalence (%)
SB0120	46	50
SB0134	21	5
SB0841	15	30

4 DISCUSSION

Genotyping of *M. bovis* and *M. caprae* using spoligotyping and MIRU-VNTR can provide a valuable epidemiological tool to understand the diversity, spread, geographical localization, host preference and worldwide dispersion of this important pathogen (Allix et al., 2006; Aranaz et al., 1996; Boniotti et al., 2009; Drewe & Smith, 2014; Gormley et al., 2014; Hauer et al., 2015; Skuce et al., 2005).

This study evidenced a wide circulation of *M. bovis* spoligotypes and MIRU-VNTR profiles in different species of domesticated animals (goats, cattle and pigs), while the diffusion of *M. caprae* was limited to few isolates found in cattle and pigs (SB0866, SB0418), (Table 2). In Sicily, TB infection rate in cattle population is particularly high, compared to the rest of Italy, especially in the province of Messina, Enna and Palermo (ANON, 2015a). This high prevalence might be due to some failings in the application of the national

eradication programme related to the geography of the territory and the livestock management. The lack of bTB controls of livestock animals (not only cattle), that are reared in a freeroaming condition, the use of common pastures and spring water, the consequent promiscuity of herds with different sanitary status and/or feral livestock not officially registered may contribute to the maintenance of the infection in the territory.

The highest prevalence observed in pigs could be associated with the observation of severe diffuse lesions in this species (Di Marco et al., 2012), even in the mammary gland (Amato et al., 2017), supporting the hypothesis that feral pigs act as a maintenance host of bTB infection and not as a spillover host (Di Marco et al., 2012). In addition, the detection of *M. bovis* DNA in swine faecal samples suggests the active excretion of the pathogen through faeces (unpublished data) highlighting the swine role in environmental contamination.

Bovine tuberculosis is well documented in buffaloes (De Garine-Wichatitsky et al., 2010; Michel, de Klerk, Gey van Pittius, Warren, & van Helden, 2007) and goats (Cadmus, Adesokan, Jenkins, & van Soolingen, 2009; Napp et al., 2013), whereas ovine TB is considered uncommon and poorly studied (Malone, Wilson, Pollock, & Skuce, 2003; Muñoz Mendoza et al., 2012; Muñoz-Mendoza et al., 2016). However, a case of bTB in a sheep was previously described in Sicily, the strain was spoligotype SB0841 and had MIRU-VNTR profile 5,4,5,3,3,10,4,4,4,3,6,5 (Marianelli et al., 2010). In the present study, *M. bovis* was not isolated from any sheep and buffalo showing tuberculosis-like lesions, and a low prevalence of bTB infection was detected in goats and sheep, probably due to a scarce surveillance at the abattoir and in farms.

Genotyping analysis showed eight spoligotypes were shared among bovines and pigs and spoligotype SB841 was shared among pigs, cattle and goats, suggesting circulation of some isolates in different animal species. In this study, the spoligotype SB0942, never found before in Italy, was isolated in a wild boar. This isolation reveals the possibility that TB might also circulate in wildlife independently of what occurs in farm animals as already suggested from the previously reported isolate in fallow deer and wild boar (Amato et al., 2016).

The genotyping analysis revealed the presence of 37 different spoligotypes including twelve (SB1643, SB1388, SB0162, SB0807, SB0850, SB0868, SB0875, SB1335, SB1355, SB1946, SB1999 and SB0942), never isolated before in the national territory, and three completely new spoligotypes (SB2018, SB2061, SB2063), recently recorded onto the international *M. bovis* database. Moreover, 262 MIRU-VNTR profiles were characterized, confirming the circulation of a great genetic variety of isolates on the island. The number of genotypes, relative to the total number of strains typed per year, did not vary dramatically during the period of study (Table 5).

Table 5. Number of Genotypes relative to total strains per year

	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
N° GENOTYPES	1	1	–	4	18	13	41	29	44	58	42
N° ISOLATES	1	1	–	7	37	27	81	49	95	95	72
Ratio	//	//	//	0.57	0.48	0.49	0.50	0.51	0.46	0.61	0.58

The isolation of SB0807, SB0868, SB0875, SB1335, SB1355, SB1388, SB0162 in other countries such as Spain (Rodriguez-Campos et al., 2012; Smith & Upton, 2012), the fact that SB0162 was the predominant spoligotype in Belgium (Humblet et al., 2010), and SB0850 was identified in Africa (Müller, 2010), might suggest that these strains were imported from other countries. Other Sicilian isolates reported in the

Table 3 were previously described (Acevedo et al., 2013 and Di Marco et al., 2012), except for SB1643 that we isolated from cattle and SB0942 isolated from a wild boar only. This last isolate was previously detected in France and recorded as a rare spoligotype in the *M.bovis* database. The high number of VNTR profiles observed in some spoligotypes such as SB0120 and SB0134, in contrast to others such as SB1305, suggests an ancient appearance of these most frequent spoligotypes and their evolution into many different variants.

The isolation of *M. bovis* in a clinical case of pneumonia observed in an adult male, the owner of a bTB infected herd, underlines the risk of bovine tuberculosis for humans. The genetic profile of this isolate was the same as from five Italian cattle outbreaks. In Sicily, an average of 4.4 human cases/100,000 inhabitants was reported until 2013, and the proportion of cases was higher in foreign-born individuals (Mammina et al., 2014). A genetic study on MTBC isolates from native and foreign population in the province of Palermo showed a wide genetic heterogeneity of the isolates and four *M. bovis* strains (3.7%) was identified only from Sicilian-born patients (Bonura et al., 2014). The province of Palermo together with Messina and Enna is the ones with the highest prevalence of bTB in cattle so it is possible that human cases are also present. The prevalence of bTB in humans might be underestimated because differentiation between *M. tuberculosis* and *M. bovis/M. caprae* isolates is not routinely performed (Perez-Lago, Navarro, & Garcia-de-Viedma, 2014). In Europe, human tuberculosis caused by *M. bovis* has a low prevalence (EFSA scientific report 2015; Perez-Lago et al., 2014) and is not always directly correlated to the consumption of unpasteurized milk products (Lo Bue et al., 2010; Perez-Lago et al., 2014). However, it is more prevalent in developing countries where the contact between animals and humans is more frequent and the consumption of dairy products from unpasteurized milk is much more common (Thoen, Steele, & Kaneene, 2014). Considering the high prevalence recorded in bovines and the zoonotic implication due to the consumption of traditional dairy products from unpasteurized milk, new strategies for surveillance need to be considered to control the infection in rural areas where several bTB susceptible animal species (cattle, sheep, goats, buffalo and pigs) share the same pastures. The high numbers of positive subjects recorded in wild boar population during the gross examination were not confirmed by the bacterial isolation (Table 1). Probably most of them were infected by MOTT (Mycobacteria other than tuberculosis) unlike feral pigs, which support the hypothesis of their different epidemiological role. In other Italian regions, a high prevalence of *M. microti* was detected in wild boar (Boniotti et al., 2014), but a longer incubation time is necessary to growth this mycobacterium and under detection might occur.

In the author's opinion, the application of stringent control measures both in farm and at abattoir is fundamental for the success of the bTB eradication plan in rural areas. The use of epidemiological tools to identify the main risk factors involved, track animal movements associated with infected herds, genotype of *M. bovis* isolates and conduct surveillance at the abattoir in different domestic animals, is necessary to monitor the prevalence of the disease in the territory and the effectiveness of the veterinary activities. Moreover, the epidemiological role played in this kind of ecosystem by wildlife, including cervids and wild boars, as potential reservoirs for *M.bovis* needs to be considered and studied in depth to obtain the complete eradication of bovine tuberculosis (Gates et al., 2013).

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CONFLICT OF INTEREST

Authors declare that they have no financial and personal relationships with other people or organizations that may compromise or inappropriately influence their work.

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