Mycobacterium caprae *in a dairy farm in Northeast Italy*

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Bovine tuberculosis, Dairy cattle, *M. caprae*, *Mycobacterium* spp., Surveillance, Zoonoses.

Summary
Veneto region, Northeast Italy, has been declared officially free from bovine tuberculosis since 2008, although the disease is sporadically detected in association with cattle trade. In September 2015, bovine tuberculosis was detected in a dairy cattle farm of the region, in a holding with 69 animals. The herd underwent single intradermal tuberculin testing as part of the regional surveillance plan, and 24 animals resulted positive. *Mycobacterium caprae* was evidenced in 22 samples, further genotyped by PCR-based assays as Allgäu type. Epidemiological investigation reported that sixteen animals were introduced from an officially tuberculosis free Member State in previous years. Nevertheless, spoligotyping and multilocus variable tandem repeat analysis (MLVA) indicated that *M. caprae* was strictly related to the strain circulating in 2007-2009 in Trento province (Trentino-Alto Adige region, Northeast Italy), although no at-risk contacts were described. *M. caprae* is a zoonotic pathogen and further analyses are warranted in order to control its spread and impact on public health and animal trade.

Descrizione di un focolaio di Mycobacterium caprae *in un allevamento da latte della Regione Veneto*

Parole chiave
Bovini da latte, *Mycobacterium* spp., Sorveglianza, Tubercolosi bovina, Zoonosi.

Riassunto
Con Decisione 2008/404/CE, la Regione Veneto è stata dichiarata Ufficialmente Indenne (UI) da tubercolosi bovina ai sensi della Direttiva 64/432/CEE. A settembre 2015 è stato identificato un focolaio di tubercolosi bovina in un allevamento di 69 bovini da latte della provincia di Padova. Sottoposti a controlli ufficiali, 24 di essi (34.78%) hanno reagito positivamente alla prova intradermica. *Mycobacterium caprae* è stato isolato in campioni appartenenti a 22 capi e identificato attraverso tecniche biomolecolari. Le PCR utilizzate hanno rilevato la presenza del sottotipo Allgäu, e non è stata riscontrata nessuna variazione tra i diversi isolati. L'indagine epidemiologica ha evidenziato che 16 bovine erano state introdotte dall'Austria tra la fine del 2011 e l'inizio del 2015. Tuttavia le analisi biomolecolari hanno evidenziato una stretta correlazione tra il *M. caprae* isolato in questo studio e il ceppo identificato negli anni 2007-2009 nella Provincia di Trento, sebbene nessun contatto a rischio sia stato individuato. Il *M. caprae* è un agente zoonotico che rappresenta un grave pericolo per la salute pubblica e risulta indispensabile che i piani di controllo si basino su un'attenta valutazione del rischio di possibile introduzione anche nei territori dichiarati ufficialmente indenni da tubercolosi bovina .

Introduction

Bovine tuberculosis (bTB) is a well-known infectious disease of cattle that may pose a serious threat to public and animal health. The etiological agents of the disease are members of Mycobacterium Tuberculosis Complex (MTC), including *Mycobacterium bovis* and *Mycobacterium caprae*. While *M. bovis* has been widely studied and is acknowledgedly associated with bTB, *M. caprae* has only recently been classified as a separate species (Aranaz *et al.* 2003), and included in the MTC (Pérez-Lago *et al.* 2014). The main hosts for bTB are cattle and ruminants, although the disease can affect a broad range of domestic and wild animals (Pérez-Lago *et al.* 2014). Bovine TB represents a threat for human health in developing, and industrialized nations (Pérez-Lago *et al.* 2014), although the main agent of human TB is considered *M. tuberculosis*. However, a substantial number of zoonotic TB human cases are characterized by extra-pulmonary manifestation (Prodinger *et al.* 2014).

In Italy, bTB is subjected to national control activities since the 1960s (Zanardi *et al.* 2013). Since 2008 Veneto Region has been declared officially tuberculosis free (OTF) (Decision n. 2008/404/EC), and a multiannual regional control plan has been implemented, in concert with risk-based surveillance activities, to maintain the OTF status. In the last four years, few cases were identified, at the slaughterhouse, in nearly all animals introduced from non-OTF Member States or Italian regions. In 2015, surveillance activities revealed a single farm, in Veneto region, positive for bTB. In this manuscript, we describe an autochthonous bTB case case and provide data obtained by the regional surveillance plan for bTB.

Materials and methods

The Regional control programme for bTB includes active and passive activities. Cattle are screened by the Local Veterinary Services at the slaughterhouse, and notification of lesions consistent with bTB is mandatory. Risk-based surveillance activities consist in testing cattle originating from areas considered at high risk for bTB, sampled-based tests on beef cattle, and cadenced tests on residential dairy cattle. All animals older than six weeks have to be inspected with single intradermal tuberculin test (SIT), as indicated in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016 (Chapter 2.4.6) and in the Annex B of EU Directive 64/432/EEC. The site of injection has to be examined after 72 hours and positive reactions identified. In case of positive tests, retropharyngeal, tracheobronchial, mediastinal, mandibular, mesenteric, and hepatic lymph nodes and tonsils need to be collected at the slaughterhouse. In lactating cows, also supramammary lymph nodes need to be collected. Anatomo-pathological inspections of the lymph nodes and tonsils, histological investigations, and Ziehl-Neelsen staining are performed at the local territorial laboratory (Istituto Zooprofilattico Sperimentale delle Venezie - IZSVe). Bacteriological culture and PCR for IS6110, an insertion element found exclusively within the members of the *Mycobacterium tuberculosis* complex, are performed at the National Reference Laboratory (NRL) at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), followed by molecular test for identifying, genotyping and differencing Mycobacteria subtypes (Domogalla *et al.* 2013, Boniotti *et al.* 2014). Genotyping is performed through spoligotyping and Mycobacterial Interspersed Repetitive Units (MIRU) - Variable Number of Tandem Repeats (VNTR) analyses, as described by Boniotti and colleagues (Boniotti *et al.* 2014).

When positive SIT reactors are detected, investigations need to be also carried out on animal handlers. Latent infections are routinely identified by sensitization to TB antigens (Mantoux test), while pulmonary lesions can be detected by X-ray body scans.

Results

A total of 305,969 residential dairy cattle were examined through SIT in Veneto Region in 2015 (source: National Animal Database). In September, a single farm tested positive to SIT, and further epidemiological and diagnostic investigations were performed (Table I). The holding was a family-run dairy farm located in Padua province. A total of 69 dairy cattle of all ages were present in the farm, 30 of which were currently lactating. The examination of the site of SIT injections allowed the detection of 24 positive animals. Nine animals (37.50%) had increased skin thickness (> 4 mm) only, eight (33.33%) showed increased skin thickness (> 4 mm) and pain at palpation, and seven (29.17%) presented also at least one other sign including: extensive oedema, necrosis at the injection site, or inflammation of the regional lymph nodes.

Following the high intra-herd prevalence of SIT positive animals (P = 34.78%), the local veterinary services decided to cull all the cattle present in the farm. Two SIT-negative calves underwent on-farm emergency slaughter due to health issues not directly related to bTB, and no further analyses were performed. At the slaughterhouse, lymph nodes and tonsils were collected as indicated in Chapter 2.4.6 of OIE Terrestrial Manual, 2016, and in the Annex B of EU Directive 64/432/EEC.

Twenty-four out of 67 inspected animals (35.82%) showed lesions consistent with bTB, of these nineteen tested also positive to SIT (Table I). Seven animals (7/24, 29.17%) had multifocal, coalescing granulomatous pneumonia involving most part of the lungs. In other six cases (6/24, 25.00%), multifocal to confluent caseating granulomas were identified in tracheobronchial and mediastinal lymph nodes. The remaining eleven heads (11/24, 45.83%) had non-coalescent granulomas in the tracheobronchial, mediastinal, mesenteric and/or retropharyngeal lymph nodes. Further histological examinations revealed nodular lesions with a necrotic mineralized center surrounded by macrophagic and lymphocytic infiltration, with rare giant multinucleated cells and fibro-connective host reaction, consistent with bTB. The Ziehl-Neelsen staining confirmed the presence of acid-resistant bacteria in six animals with histological lesions (6/24, 25.00%) (Table I); five of these (83.46%) resulted positive for *M. caprae* also by culture. However, the acid-resistant bacteria are hardly identified in old lesions because of the presence of cells and fibrosis around the center of the lesion, de facto enclosing the microorganisms and preventing the staining (McGavin and Zachary 2007).

Of the five animals which tested positive by SIT with no evidence of gross lesions, one presented histological lesions and resulted positive to the Ziehl-Neelsen test; one had histological findings in addition to the positivity to SIT, and one was positive by microbiological culture (Table I). The last two SIT positive individuals were negative to all further tests performed.

Organ samples were delivered to the National Reference Laboratory (NRL) at the Istituto Zooprofilattico Sperimentale of Lombardy and Emilia Romagna (IZSLER) for bacteriological culture and molecular test. *M. caprae* was isolated from 22 samples, and identified by molecular tests. The panel of PCRs described by Domogalla and colleagues (Domogalla *et al.* 2013) revealed the presence of subtype Allgäu containing the complete RD4 region sequence. The combined spoligotyping and MLVA with 12 MIRU-VNTR markers (order of markers: ETRA, ETRB, ETRC, ETRD, ETRE, MIRU26, VNTR2163a, VNTR2163b, 3155, 4052, 1895, 3232) was: SB0418, 4, 3, 5, 3, 4, 5, 5, 2, 3, 2, 4, 15. No variation was found in the isolates of the reported case. With exception of VNTR3232, the profile reliably matched with the genotype isolated in 2007-2009 in the outbreaks of Trento province (Chin *et al.* 2009).

Table I. *Comparison between the results of the single intradermal tuberculin test (SIT) and other tests.*

		Single Intradermal Tuberculin Test[1]		
		+	-	Total
Gross lesions	+	19	5	67
	-	5	38	
Ziehl-Neelsen	+	5	1	67
	-	14	4	
	n.a.[2]	5	38	
PCR	+	17	5	67
		7	38	

[1] SIT was carried out on all of the animals present in the farm (n = 69); two SIT-negative calves undergone emergency on-farm slaughter and no further analyses were performed.
[2] Ziehl-Neelsen test is performed only on samples that resulted positive to the gross lesions and histological examinations (n = 24).

Five people were tested twice with the human SIT (Mantoux) in September and November 2015; the other two were subjected to thoracic X-rays scans. Laboratory investigations did not reveal any positive results.

Epidemiological investigations on the affected farm allowed to track back cattle that were introduced up to the identification of bTB. Sixteen animals were introduced from Austria between the last quarter of 2011 and the first quarter of 2015, 14 of which were first introduced in lairage facilities in Trento province. Of the two remaining cattle, one was moved to a dairy farm in Bolzano province and then to the lairage facility in Trento, before arriving in Padua. The last cattle remained in a dairy farm in Trento between November 2009 and October 2013 before being introduced in the affected farm in Padua. Of the remaining 53 heads present at the time of *M. caprae* detection, 44 were born in the farm, while nine were introduced from other farms located in Padua province where *M. caprae* was never detected.

Discussion

In past years only sporadic cases of *M. caprae* were detected in individual animals in Veneto region; this pathogen also circulated in other northern Italy areas in late 2000s (Chin *et al.* 2009). Tracing activities of animal movements permitted to attribute most of the previous *M. caprae* detections to the introduction of cattle from Austria and Germany, which at that time were already classified as OTF.

Epidemiological investigations on the case we reported, revealed that 16 animals were introduced from Austria between the last quarter of 2011 and the first quarter of 2015. Nine of the lactating cows testing positive to SIT originated from Austria, and seven of them had lesions consistent with bTB, with isolation of *M. caprae*. Austria has been an OTF member state since 1999 (Decision n. 1999/467/EC), although nowadays bTB has been evidenced in some areas of the Country. *M. caprae* was previously evidenced in wild deer (*Cervus*

elaphus) populations in the Austrian Alps (Fink *et al.* 2015). Furthermore, *M. caprae*- related tuberculosis cases had been repeatedly notified in Austria in recent years (Prodinger *et al.* 2002, ProMed 2016, Schoepf *et al.* 2012). The Allgäu subtype has been detected in cattle and red deer of specific Austrian alpine regions (Rettinger *et al.* 2017). However, by genotyping, it was not possible to connect *M. caprae* detected in this study with that circulating in Austria. In fact, the spoligotyping indicated that the *M. caprae* identified in 2015 was related to the strain circulating in 2007-2009 in Trento province (Chin *et al.* 2009). Nevertheless, no at-risk contacts was highlighted between the farm in Padua province and the one resulted positive in Trento. Moreover, the epidemiological scenario is further complicated by the fact that the cattle introduced from Austria, which was temporarily located in a lairage facility in Trento province before being moved to the farm in Padua, tested negative to all screening tests. Overall, more data are needed to assess the origin of this *M. caprae* strain. More recent methods, such as Whole Genome Sequencing (WGS), could help identifying the actual origin of the pathogen.

Since its first identification in 1999 in Spain (Pérez-Lago *et al.* 2014) *M. caprae* has been detected in domestic and wild animals also in other EU Countries (Pérez-Lago *et al.* 2014). Cases in humans were also reported in Austria and Germany, stressing the potential zoonotic risk of *M. caprae* (Kubica *et al.* 2003, Prodinger *et al.* 2002). In particular, in Germany, the pathogen had been reported as the cause of more than 30% of the human TB cases in 1999-2001 (Kubica *et al.* 2003). Furthermore, as diagnostic tools able to discriminate *M. bovis* and *M. caprae* have been developed only recently, early *M. caprae* cases might have been underestimated (Kubica *et al.* 2003). SITs have been indeed developed specifically for *M. bovis* and underestimation of *M. caprae* could also be related to the fact that the Directive n. 64/432/EEC[1] provides mandatory notification and restrictive measures for *M. bovis* only.

The cases of bTB in OTF Countries represents a remarkable threat to public health and international trade. Accordingly, an elevated level of alertness should be kept also in OTF areas, and risk-based surveillance programmes are needed to promptly detect any bTB introduction and spread to domestic or wild animal populations. This is also corroborated by the detection of *M. caprae* in Italy in 2015, which indicated the effectiveness of the regional surveillance plan.

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