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SHORT REPORT
Five-year surveillance of human tuberculosis caused by *Mycobacterium bovis* in Bologna, Italy: an underestimated problem

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SUMMARY

Human tuberculosis (TB) caused by *Mycobacterium bovis* surveillance is affected by a lack of data. The aims of the present study were: (i) to estimate the proportion of human TB caused by *M. bovis* over a period of 5 years in Bologna, Northern Italy, which, like most Western European countries, has been declared bovine TB-free; (ii) to compare the genetic profiles of *M. bovis* strains identified in humans with those circulating in cattle in the last 15 years in Italy. Among 511 TB patients, the proportion of human TB caused by *M. bovis* was 1·76%, significantly associated to extra-pulmonary localization ($P = 0{\cdot}004$) and to being elderly ($P < 0{\cdot}001$) and Italy-born ($P = 0{\cdot}036$). The molecular epidemiology analysis by spoligotyping and Multilocus Variable Tandem Repeat Analysis confirmed that most *M. bovis* strains from Italy-born patients matched those circulating in cattle herds in Italy between 2001 and 2016. Two cases of *Mycobacterium bovis* BCG infection were also characterized. In conclusion, the rate of human TB caused by *M. bovis* was not negligible, highlighting the relevance of molecular typing in evaluating the effectiveness of programmes designed to eradicate TB in cattle in Italy.

Key words: Bovine tuberculosis (bTB), human tuberculosis (TB), Multilocus Variable Tandem Repeat Analysis (MLVA), *Mycobacterium bovis* (*M. bovis*), spoligotyping.

INTRODUCTION

Mycobacterium bovis (*M. bovis*), a component of the *Mycobacterium tuberculosis complex* (MTBC), can infect a wide range of mammals including humans. The pathogenesis, lesions and clinical findings of tuberculosis (TB) caused by *M. bovis* are indistinguishable from those caused by *M. tuberculosis* (MTB). The main mechanism of transmission to humans is the consumption of contaminated dairy products and, less frequently, contact with diseased animals and human-to-human contact [1]. Consequently, the zoonotic potential of *M. bovis* has raised some public health concerns [2].

According to the last European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) report, in Europe bovine TB (bTB) infection, detection and control is heterogeneous; prevalence ranges from absence of infection to 17·7% of herds test-positive in England and Wales [3]. In some Western European countries,

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the incidence of *M. bovis* in cattle herds has dramatically decreased; in Italy for instance, it fell from 10% in the 1960s to 0·78% in 2015 following a national bTB eradication programme. Official bTB-free status has been achieved in some Italian regions; however, TB in cattle persists in others, particularly in Southern Italy (Sicily 3·71%) [4].

The real incidence of human TB caused by *M. bovis* is underestimated because routine identification techniques often do not differentiate different MTBC species and there is no national surveillance system.

The aim of the present study was to estimate the proportion of cases of human TB caused by *M. bovis* over a period of 5 years in Bologna, Emilia-Romagna (ER) region, Northern Italy. This is a rural setting, representative of most other Western European countries, with bTB-free status, low TB incidence, but a high rate of immigration from countries with high prevalence.

We provide an update of the demographic and clinical characteristics of human TB caused by *M. bovis* compared with that due to MTB. In addition, we report the comparison of *M. bovis* genotypes isolated from humans with those circulating in cattle, by geographic region, using spacer oligonucleotide typing (spoligotyping) combined with Multilocus Variable Tandem Repeat Analysis (MLVA), to obtain the highest degree of epidemiological discrimination for a standard approach [5].

METHODS

A retrospective study (2011–2015), approved by the Ethics Committee of St. Orsola-Malpighi University Hospital of Bologna (Italy) (Approval number 1105/2016), was conducted on 511 MTBC strains collected at the Microbiology Unit. MTBC was identified by Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) directly on specimens. When Xpert was not required by clinicians or the direct specimen was Xpert-negative, Xpert or MGIT TBc Identification Test (Becton Dickinson, Franklin Lakes, NJ, USA) was performed on positive cultures. Drug susceptibility test to first-line anti-tubercular drugs was performed by the automatic MGIT 960 system (Becton Dickinson).

Considering *M. bovis*'s intrinsic resistance to Pyrazinamide (PZA), strains with at least PZA resistance were characterized at species level by Genotype MTBC (Hain Lifescience, Germany).

Spoligotyping was performed as described by Kamerbeek *et al.* [6] and patterns were identified by comparing profiles with the international *M. bovis* Spoligotype Database [7]. MLVA typing was performed with 12 markers including five ETRs and seven QUBs/MIRU markers selected for their high *M. bovis* genotypic discriminatory power as described by Boniotti *et al.* [8]. The spoligotyping and MLVA patterns obtained were run against the national animal database (ITAN-TB), which contains more than 5000 genotypes isolated from cattle in Northern Italy since 2000 and from the rest of Italy since 2007.

RESULTS

PZA resistance was detected in 40 of the 511 MTBC strains: nine *M. bovis* (1·76%), two *M. bovis* BCG (0·39%) and 29 MTB.

The age, sex, country of birth and sample source of the nine patients with TB caused by *M. bovis*, and two patients infected by *M. bovis* BCG, are listed in Table 1. Seven of the nine *M. bovis* strains were isolated from extra-pulmonary samples (77·8%). Compared with the remaining 500 TB cases due to MTB (71·8% respiratory, 28·2% non-respiratory), *M. bovis* was strongly associated with extra-pulmonary localization ($P = 0·004$). Six of the nine (66·7%) *M. bovis* strains were isolated from native Italians with a mean age of 70·6 years, while most TB cases due to MTB occurred mainly in younger patients (mean age 34·4 years, $P < 0·001$) who were foreign-born (71·3%, $P = 0·036$).

Two *M. bovis BCG* strains were isolated from extra-pulmonary samples. According to clinical records, patient GU developed an osseous infection after immunotherapy with BCG (OncoTICE®) for bladder cancer 5 years previously, while patient GS had a localized infection after being vaccinated with a BCG strain at birth in Croatia.

Spoligotyping and MLVA results are shown in Figure 1. The most common spoligotype of *M. bovis* strains was SB0120 (five out of nine, 55·5%), which was also detected in cattle. All SB0120 strains were from Italian patients and exhibited different MLVA types. Three of the five SB0120-MLVA profiles were already present in the ITAN-TB database, confirming that *M. bovis* strains circulating in animals matched those infecting humans. In particular, the profiles from patient BA (84 years old, from ER region) and CF (36 years old, from Calabria region, Southern Italy) were found in 11 TB-positive herds from 2008 to 2017 in Southern Italy, while the genotype from patient MF (89 years old, from ER region) was found in more than 200 outbreaks from 2001 to 2017 including two positive herds in ER region in

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Table 1. *Epidemiological characteristics of patients with Mycobacterium bovis and M. bovis BCG infection, St. Orsola-Malpighi University Hospital, Bologna, Italy, 2011–2015.* (Italian regions were specified in brackets.)

Patient	Age (year)	Sex	Country of birth	Sample source	Strain
FV	62·8	M	Italy (Emilia-Romagna)	Bronchoalveolar lavage	*M. bovis*
BA	84·5	F	Italy (Emilia-Romagna)	Bronchoalveolar lavage	*M. bovis*
GW	16·5	F	Tunisia	Lymphnodes	*M. bovis*
MF	89·6	M	Italy (Emilia-Romagna)	Lymphnodes	*M. bovis*
EWH	39·0	F	Morocco	Lymphnodes	*M. bovis*
HC	56·7	M	Romania	Cerebrospinal fluid	*M. bovis*
RM	80·3	M	Italy (Emilia-Romagna)	Intestinal biopsy	*M. bovis*
PAM	75·0	F	Italy (Emilia-Romagna)	Osseus biopsy	*M. bovis*
CF	36·0	M	Italy (Calabria)	Osseus biopsy	*M. bovis*
GU	80·9	M	Italy (Marche)	Osseus biopsy	*BCG*
GS	0·4	F	Croatia	Pus	*BCG*

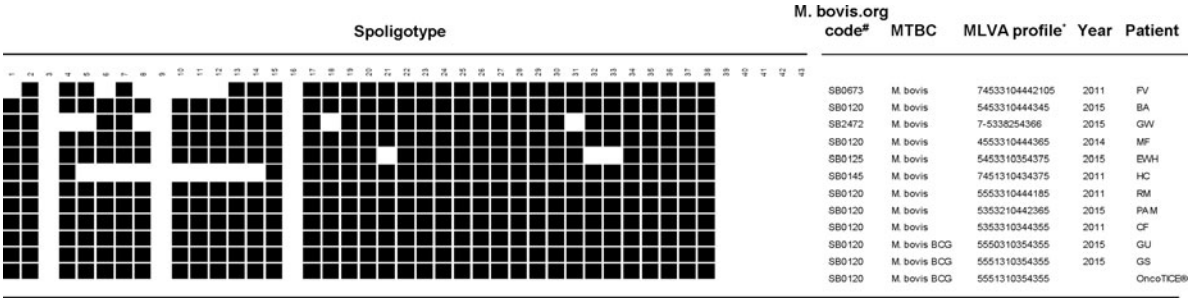

Fig. 1. Spoligotying and MLVA typing results of *Mycobacterium bovis* and *M. bovis* BCG strains isolated from 11 patients with TB. All the isolates showed a different Spoligo-VNTR profile. For the spoligotyping, 43 spacers in the DR locus were hybridized with DNA from *M. bovis* isolates. MLVA was performed by amplification of 12 MIRU-VNTR loci. ■ Presence of the specific spacer at position 1–43 in the DR locus, □ absence of the specific spacer at position 1–43 in the DR locus, #*M. bovis* code available from http://www.Mbovis.org [7], *MLVA markers: ETRA, ETRB, ETRC, ETRD, ETRE, VNTR2163a, VNTR2163b, VNTR4052, VNTR1895, VNTR3155, VNTR3232, VNTR2996 described by Boniotti *et al.* [8].

2003. In contrast, two SB0120-MLVA profiles (from patients RM, 80 years old; PAM, 75 years old, both from ER region) were not present in the database, even though similar profiles with single or double locus variations were found. The other *M. bovis* genotypes identified (SB0125, SB0673 and SB0145) did not correspond to any strains present in the bovine ITAN-TB database and were isolated from two foreign-born patients (EWH, HC) and one Italy-born patient (FV, from ER region), while profile SB2472 from a foreign-born patient (GW) has not been described to date.

Furthermore, both patients infected with *M. bovis* BCG strains exhibited SB0120 spoligotype but MLVA typing differed by one locus. Patient GS, who had a localized infection soon after vaccination, presented a profile identical to that of the vaccine OncoTICE® (5551310354355), while patient GU had a profile similar to OncoTICE® but no tandem repeats were found in the ETD-R locus.

DISCUSSION

This retrospective study was conducted in ER, Italy, a region considered bTB-free since 2007, and therefore representative of most Western European countries. Our results demonstrated that the proportion of human TB caused by *M. bovis* was not negligible (1·76%); comparable results were obtained in a similar Italian setting (Tuscany) during a 4-year survey performed 10 years ago [9]. Despite control efforts in cattle herds, the few published data from Western Europe reported comparable prevalence of TB cases caused by *M. bovis*: 2% in Lyon (France) during the years

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2000–2005 [10], 1·1% in United Kingdom during the period 1999–2015 [11] and 1·5% in Spain between 2006 and 2014 [12].

We compared the demographic and clinical characteristics of human TB caused by *M. bovis* with those of TB due to MTB. Our analysis pointed out that most cases occurred in elderly native Italians and were strongly associated with extra-pulmonary localization, as described by previous authors [9]. This was to be expected since the route of *M. bovis* infection is mainly via contaminated food (especially milk) or direct contact with infected animals.

Furthermore, using a combination of spoligotyping and MLVA, human *M. bovis* strains were typed and compared with those circulating in Italian herds by geographic region. We found that three of the six strains from Italy-born patients matched those found in cattle over the last 15 years. In particular, recent transmission was likely for the only young Italian case, CF from Southern Italy, with known risk of exposure to infected animals (veterinarian), supported by matching the genotype with those isolated in Southern Italian herds during the same period. In contrast, recent transmission was unlikely for two elderly Italian patients both living in ER region; a match was found for patient BA but with herds in a different geographic region, and for patient MF in the same region but 11 years before diagnosis.

In conclusion, in contrast to the results presented in a recent paper by Palacios *et al*. [12], our data suggest that most infections in elderly people were not due to recent transmission, but probably to the reactivation of *M. bovis* infection acquired before the introduction of bTB eradication.

The remaining three profiles from Italy-born patients without matches with animal genotypes could be ascribed to infection with *M. bovis* strains circulating before the creation of the ITAN-TB database, which are no longer in circulation, indicating that control efforts in cattle herds have been effective. On the other hand, the three foreign-born patients with *M. bovis* genotypes not present in the ITAN-TB database could have acquired infection in their countries of origin.

In our study, no drug-resistant *M. bovis* strain was detected (data not shown), in contrast to a recent report from Mexico describing the high rate of primary resistance to Streptomycin among human *M. bovis* isolates as a result of antibiotic use in cattle [13].

In conclusion, our study underlines the need to identify cases of human TB due to *M. bovis*, which is rarely performed. Furthermore, molecular epidemiological analysis can help us to recognize the *M. bovis* genotypes circulating in humans as well as evaluate the effectiveness of TB eradication programmes in cattle.

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DECLARATION OF INTEREST

None.

ETHICAL STANDARDS

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Declaration of Helsinki of 1975, as revised in 2008.

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