IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX SPECIES IN BULGARIA

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ABSTRACT

Tuberculosis is caused by closely related mycobacterial species, designated as *M.tuberculosis* complex (MTBC), which includes : *M.tuberculosis* sensu stricto, *M.africanum*, *M.canettii*, *M.bovis*, *M.caprae*, *M. microti*. There is an increase in human TB cases caused by *M.bovis* or *M.caprae* in the EU. Although Bulgaria is not a bovine TB-free country, the species identification inside the MTBC is not routinely performed for human isolates and the presumably animal related pathogens could not be distinguished. This study aimed to reveal the presence of *M.bovis*/*M. caprae* as an aetiological agent on the territory of Bulgaria.

For the period from 2022 to 2025, a total of 175 MTBC strains were further examined to differentiate the species. GenoType MTBC VER 1.X was used as a reliable identification tool.

Almost all cases were found to be *M.tuberculosis*/*M. canettii* (n=173; 98.85%). The prevalence of *Mycobacterium bovis* was 0.57%. *M. bovis* BCG also was represented by a single isolate (0.57%). *M.africanum*, *M.microti*, or *M.caprae* have not been detected so far.

Species identification in the MTBC is an essential step in order to limit the transmission from animal to human and to refine the treatment of the affected individuals.

Key words: *M. tuberculosis, M.bovis, M.caprae*

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INTRODUCTION

Tuberculosis (TB) is an infectious disease of public importance , and a leading cause of death among the infectious diseases of all time. According to WHO data, in 2022 10.6 million people were infected with tuberculosis worldwide, and 1.3 million died [1]. Besides the human population, tuberculosis affects domestic and wild animals, forming endemic zoonotic reservoirs

The disease is caused by a group of mycobacteria designated as *M.tuberculosis* complex (MTBC), which includes different mycobacterial species and subspecies sharing 99.9% of their DNA sequence but differing in their host preferrence [2]. The most common species infecting humans are: *M.tuberculosis* sensu stricto (Lineage 1-4, Lineage7), *M.africanum*, *M.canettii* [2,3,4]. The species adapted primarily to animal hosts are *M.bovis*, *M.caprae*, *M. pinnipedii*, *M. microti*, *M. mungi*, *M. orygis*, *M. suricattae*, chimpanzee bacillus, dassie bacillus [4]. Although prone to invasion, establishment and spread in a particular animal species, most of the mentioned mycobacteria are capable of infecting other mammalian species, i.e. - human [5, 6, 7, 8, 9]. *M.tuberculosis* sensu stricto is the leading aetiological agent of human tuberculosis, but has been also reported in sick animals [8]. *M.africanum* has significance as a pathogen in West Africa, where it causes up to half of the tuberculosis in the human population. [10]. It could be found also in imported cases with migrants from endemic countries [11, 12]

Out of the animal-adapted species, *M.bovis* and *M.caprae* are the most common causes of TB in humans. Transmission of bovine TB from animal to human occurs primarily through close contact and consumption of unpasteurized milk from infected animal [13]. However, routine surveillance of human and animal populations is insufficient , particularly in the countries where bovine tuberculosis is endemic and laboratory capacity is limited. Cases of zoonotic tuberculosis in humans are uncommon in countries with controlled bovine tuberculosis and high food

safety standards [14].

The clinical manifestation of *M.bovis* infection is identical to *M. tuberculosis* sensu stricto, but the extrapulmonary localization is more frequent. The treatment of *M.bovis* infection in men includes rifampicin, isoniazid, and ethambutol with extended duration 9-12 months. The pyrazinamide is excluded from the treatment regimen due to the innate resistance of *M.bovis*. Even when properly assigned, *M. bovis* infection has often unsatisfactory treatment outcome and high mortality rates [15].

Meta-analysis of the published cases between 2009 and 2019 showed that *M.bovis* causes 9.7% of TB cases in humans worldwide, the rates varying between 0.4% and 76.7% in different countries [16]. The reported cases of human TB caused by *M.bovis* or *M.caprae* in the EU for 2022 were 130, 16 of whom died. Compared with the reported cases from the previous year, there was a 13.2% increase, keeping the trend from the recent years [17].

The estimated incidence of *M.bovis/M.caprae* infection in humans in the EU is 0.03 per 100,000 population for bovine TB free countries and 0. 04/100,000 for the member states with outbreaks of bovine TB in herds. There was a rise in the prevalence of bovine TB among herds, affecting 500 more herds than the previous year [17].

Countries in the EU that have reported outbreaks of bovine TB in herds were Bulgaria, Croatia, Cyprus, Greece, Malta, Romania, Ireland, some parts of Spain, Portugal and Italy. There were 29 reported epidemic outbreaks of bovine TB in herds in 10 different regions in Bulgaria for 2015-2018 [18]. The existence of natural reservoirs of the infection in Bulgaria has also been proven [19, 20, 21]. Although our country is not bovine TB free, the species identification inside the *M.tuberculosis* complex is not routinely performed for human isolates in the TB laboratories and the *M.bovis* or *M.caprae* infections could not be distinguished.

The study aimed to reveal the presence of *M.bovis/M. caprae* as an aetiological agent on the territory of Bulgaria.

MATERIALS AND METHODS

Patients, clinical isolates and study period

The study included only clinical isolates, previously confirmed as *M.tuberculosis* complex either at the peripheral TB laboratories or at the National Reference Laboratory of Tuberculosis (NRL TB). A total of 175 MTBC strains were examined: n=34 isolates from 2022, n=57 - from 2023, n=74 from 2024, and n=10 from 2025 respectively. Each patient was represented by a single strain. No sensitive personal information was gathered or disposed of in this paper.

Identification

Positive cultures on solid (Löwenstein-Jensen) or liquid media (MGIT) were tested for their affiliation to *M.tuberculosis* complex via rapid immunochromatographic test detecting the MPT 64 antigen [22].

DNA was extracted with GenoLyse® VER 1.0 [23] according to manufacturer's instructions.

The species identification inside the *M.tuberculosis* complex was performed by GenoType MTBC VER 1.X (Hain Lifescience GmbH – A Bruker Company): a PCR and subsequent reverse hybridization which provides a reliable discrimination of *M.africanum, Bacillus Calmette-Guérin, M.bovis, M.caprae, M.microti*. whereas *M.tuberculosis* and *M.canettii* cannot be differentiated from one another with the test [24]. GenoType MTBC relies on detection of specific for MTBC 23S ribosomal DNA fragment, the polymorphisms *of gyrB* DNA, and the RD1 deletion of *M. bovis* BCG [25, 26, 27].

RESULTS

The 175 MTBC strains enrolled in this study were retrieved mainly from pulmonary specimens (n=170, 97.1% : sputa, bronchoalveolar lavage, or aspirate). The non-pulmonary specimens (n=5, 2.9%) were presented by pleural fluids (n=2), bone biopsy (n=1), and lymph node biopsies (n=2). The male/female ratio was: 2.4:1, the mean age of the patients was 48.6.

Most of the patients were urban residents (71% vs. 29% living in a rural area (). The number of isolates varied between the districts and is displayed in the table below.

Almost all of the cases were identified as *M.tuberculosis/M.canettii* by GenoType MTBC (n=173; 98.85%). A single isolate was found to be

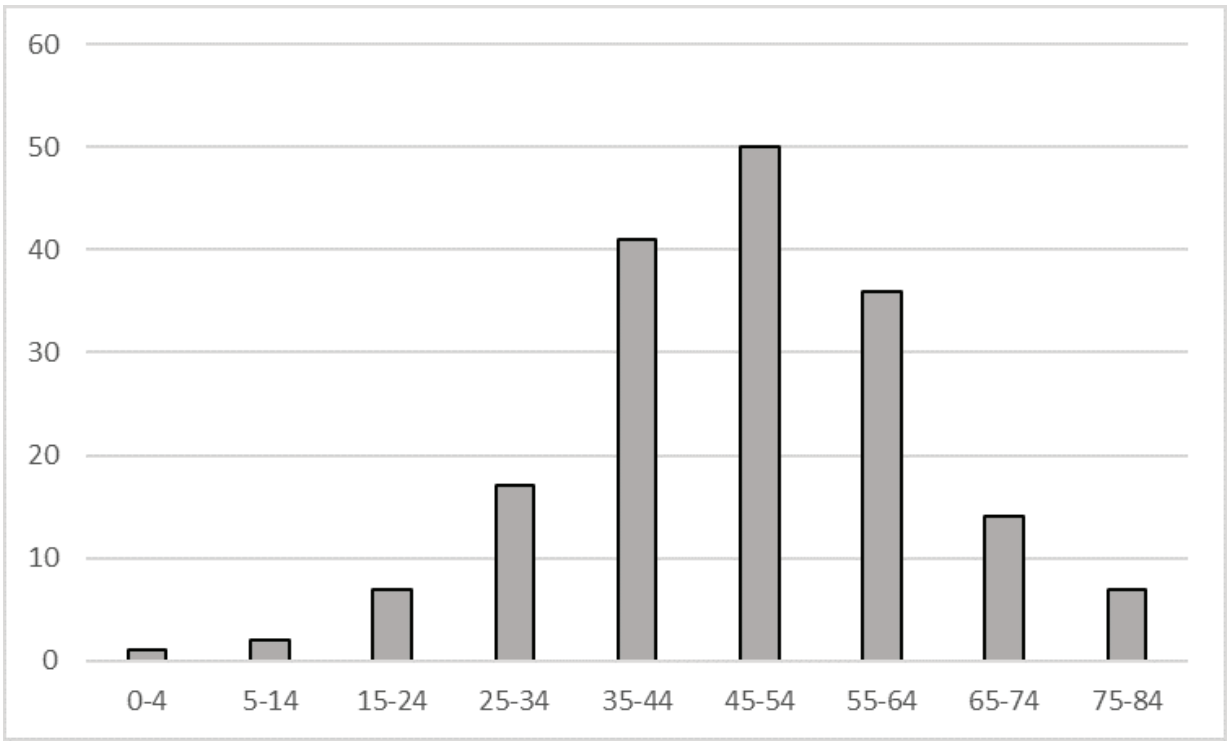

Figure 1. Age structure of TB patients

Table 1. Number of MTBC isolates by region of TB patients' residence

Region	n	Region	n	Region	n
Sofia - city	29	Pazardzhik	7	Pleven	2
Vratsa	18	Montana	5	Razgrad	2
Pernik	14	Ruse	5	Silistra	2
Vidin	12	Burgas	4	Haskovo	2
Targovishte	12	Gabrovo	4	Blagoevgrad	2
Dobrich	10	Sofia - district	4	V.Tarnovo	1
Varna	9	Kardzhali	3	S.Zagora	1
Shumen	9	Lovech	3	unknown	4
Kyustendil	8	Plovdiv	3		

Mycobacterium bovis BCG (n=1; 0.57%) and one was *Mycobacterium bovis* (n=1; 0.57%). *M.africanum*, *M.microti* or *M.caprae* were not detected.

DISCUSSION

The MTBC isolates enrolled in this study were sent in NRL TB from peripheral laboratories for drug susceptibility testing or resistance confirmation. Although the sample size was not representative given the small number of tested strains and the disproportionate district distribution, the results revealed the necessity of routine species identification within the *M.tuberculosis* complex at the national level.

For the study period only two isolates were sent in NRL TB with a requirement for species identification. One of them was found to be *Mycobacterium bovis* BCG (n=1; 0.57%), isolated from a lymph node of 8-month old infant with disseminated infection.

The *Mycobacterium bovis strain* (n=1; 0.57%) was isolated from a foreign 70-year-old woman with pulmonary TB, who had settled down in a rural region of Bulgaria. The strain was sensitive to the first line anti-TB drugs (streptomycin, isoniazid, rifampicin and ethambutol).

The discrepancies in the results and the estimated

rate of over 0.4% for *M.bovis* could be related to the profile of the tested TB patients: most of them were urban residents and the location of the infection was mainly pulmonary. The estimated prevalence of *M.bovis* in Bulgarian TB patients in a previous study was 0.46% [28].

Interestingly, *M.caprae* was not detected in humans, though the same is awell known causative agent of bovine TB in Bulgarian cattle herds [18].

Expectedly, *M.africanum* was not found in this study mainly because the number of imported TB cases in the country is low [29] and the immigrants from the endemic region are heading to the western part of Europe [30].

Species identification within the MTBC is important for the early detection of epidemiological links of disease transmission from animal to human and vice versa, for limiting the outbreaks and for refining the treatment of affected individuals.

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