MANAGEMENT OF THE BOVINE TUBERCULOSIS DIAGNOSTIC IN ROMANIAN FARMS, IN THE LIGHT OF THE MULTIDRUG-RESISTANT TUBERCULOSIS EMERGENCE

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Abstract

Tuberculosis (TB) is still a public health concern in numerous countries. Livestock can play an important role in the spreading of TB and, consequently, the prevention of tuberculosis in farm animals is an important goal. This study shows the relevance of the diagnostic of the infections produced by Mycobacterium spp. in Romanian slaughtered cattle and consider the opportunity of antimycobacterial susceptibility testing in a veterinary laboratory. Phenotyping and genotyping of the Mycobacterium isolates between 2015 and 2016, revealed 567 strains of M. tuberculosis var. caprae and 22 strains of M. tuberculosis var. bovis. These data support the risk of TB emergence in cattle herds and the human population with M. tuberculosis var. caprae.

Rezumat

Tuberculoza (TB) continuă să fie o problemă de sănătate publică în numeroase țări. Șeptelul poate juca un rol important în controlul global al tuberculozei, iar prevenirea tuberculozei în fermele de animale este un obiectiv major. Acest studiu prezintă diagnosticul de laborator al infecțiilor cu Mycobacterium spp. la bovinele din România și analizează oportunitatea introducerii testului de sensibilitate la antimicobacteriene în laboratorul veterinar. Tipizarea izolatelor de Mycobacterium din perioada 2015 - 2016 a stabilit că 567 tulpini erau M. tuberculosis var. caprae și 22 M. tuberculosis var. bovis. Aceste date susțin riscul emergenței tuberculozei bovine și umane cu M. tuberculosis var. caprae.

Keywords: Mycobacterium, bovine tuberculosis, veterinary laboratory diagnostic

Introduction

Despite the important progress achieved by the European Union (EU) in the battle with tuberculosis (TB), this disease is still a public health threat for several countries, with 55,337 humane cases reported in 2017 by the 31 EU and European Economic Area (EEA) countries, of which 72.10% were newly diagnosed [32]. In the same year, Romania reported 14,000 cases and one of the highest EU/EEA notification rates of tuberculosis in the 0 - 4 year-olds groups [32]. Moreover, Romania has the highest EU/EEA incidence of tuberculosis in humans, representing 25% of the tuberculosis load [33] and it is one of the WHO European Region’s 18 high-priority countries for tuberculosis control [34].

In light of all this data, which covers only tuberculosis in the human population, any action designed to prevent, diagnose and manage infections with tuberculous and non-tuberculous mycobacteria in domestic animals; it is a valuable achievement for the global plan to eradicate TB.

Mycobacterium tuberculosis complex has several variants and a wide host range, each of these causing tuberculosis mainly in the host species: M. tuberculosis var. bovis in cattle, other bovid species, or humans, M. tuberculosis var. caprae in goats, M. tuberculosis var. microti in voles and other rodents, M. tuberculosis var. pinnipedii in marine mammals, M. tuberculosis var. mungi in mongooses, and M. tuberculosis var. oryxis in oryxes (antelope species). However, most M. tuberculosis variants are generally capable of causing human disease, mainly among immunocompromised individuals [23]. The TB clinical form depends on the various factors (e.g., strain virulence, the host immune status, and the route of entry of the pathogen) [3, 13]. Livestock can play an important role in the spreading of several M. tuberculosis strains and, consequently, the prevention of tuberculosis in animals (e.g., bovine) become one of the most important actions in tuberculosis control. Bovine tuberculosis (BTB) is a zoonotic disease which causes significant economic losses due to a high cost of the eradication programs and trade restriction.
on live animals, meat, milk, and other products of animal origin [17, 24]. All of these are completed by public health threats: BTB is one of the seven neglected zoonotic diseases [11, 30]. Studies on the best BTB preventive actions recommend the covering of at least following five actions: (1) Restricting the contact between cattle and wild reservoir (e.g., badger in the UK; bison, elk, and deer in the USA); (2) Managing of the cattle feed and water; (3) Banning of the infected animals’ movement between herds; (4) Reducing of the risk from neighbouring herds; (5) Minimising of the infection risk from cattle manure [1, 2, 8, 12]. Vaccinating cows is banned in most countries in the world, enabling vets to continue to use the TST in BTB control programs. However, a novel vaccine and complementary TST to protect cattle against BTB have been recently proposed by Chander et al. which could allow better protection of animals with the new BCG vaccine, while maintaining a novel DIVA skin test that will detect BTB [9]. In TB’s diagnostic, laboratories play the central role by using a wide spectrum of techniques, most of them able to detect directly the etiological agent. The direct diagnostic can be done by the following methods: (a) Microscopy and culture; (b) Mycobacterial speciation by biochemical assays; (c) Mycobacterial antigen detection by monoclonal sera; (d) Analysis of lipid composition by chromatography; (e) Detection of DNA or RNA of mycobacterial origin. Indirect methods are based on the detection of IgG or IgM antibodies against mycobacteria or cellular immunity via skin tests [31]. In BTB’s diagnostic, the World Organisation for Animal Health (OIE) recommends similar methods as those described in TB. The demonstration of acid-fast bacilli by microscopic examination, as a presumptive confirmation method, is followed by the mycobacteria isolation on selective culture media and their subsequent identification by cultural and biochemical tests or nucleic acid recognition methods. Indirect methods include the TST (the prescribed test for international trade), the gamma-interferon assay (approved for use in several national programmes including in the European Union), the lymphocyte proliferation assay (not used for routine diagnosis) and the ELISA for measurement of humoral immunity [19]. Also, accurate and early diagnosis of TB should use conventional diagnostic methods in combination with modern molecular and immunological techniques [28]. The TB affects numerous domestic and wild mammalian and bird species, having major economic and public health implications [6, 15]. In developed countries, BTB control is carried out by test and slaughter of infected animals, based primarily on the tuberculin skin test (TST) [28]. TST has some limitations and only one round of testing using standard interpretation may fail to detect 20 - 25% of infected cattle [10]. However, TST sensitivity can be increased by using the interferon-gamma blood test. Also, false negatives TST can occur as a consequence of the Johne's disease, immune-suppression of the animal, delayed hypersensitivity not yet developed, the terminal stages of TB infection and errors when carrying out the skin test [10, 19]. Many developed countries pay compensation to the farmers in the test-and-slaughter programs, perform also post-mortem examinations of the carcass, and implement control strategies in herds proved to be contaminated by using laboratory methods of diagnostic [19]. TB treatment is done only in the human population, while in animals it is not performed. By not treating BTB, resistance emergence of Mycobacterium tuberculosis variants against anti-TB drugs will be very low. However, the occurrence of drug-resistant tuberculosis (DR-TB) became a real human threat, including a large spectrum of resistance, representing a red flag of burden TB even for developed low burden tuberculosis European countries [3]. In Romania, the surveillance, monitoring, and eradication of several cattle diseases are part of the national action plans and are carried out following the World Animal Health Organization (OIE) requirements [5, 7, 19, 26, 27]. In this study, we aimed to show the relevance of the diagnostic of the infections produced by Mycobacterium spp. in Romanian slaughtered cattle in the context of multi drug-resistant tuberculosis emergence.

Materials and Methods

Biological samples
The biological samples from this study were composed of lymph nodes from 590 bovines with the TST positive or inconclusive results, slaughtered for the confirmation of tuberculosis through laboratory examinations. The principles and recommendations of the European Union Reference Laboratory for Bovine Tuberculosis (Madrid, Spain) are applied throughout the diagnostic conduct. The more important rules applied to tuberculosis are Council Directive 64/432/EEC who defined the status officially free for tuberculosis in bovine herds; Regulation 852/2004 and Regulation 853/2004 who establishes the procedures for the post-mortem inspection at slaughterhouse [35-37]. Morphopathological examination
The lymph nodes were sliced at about 1 - 2 mm, and the section surfaces examined very carefully for typical or suspicious lesions. The macroscopic appearance of the tuberculous lesions will be gray-white or yellowish nodules, not encapsulated, firm or hard, with a yellowish yellow centre, usually with calcifications. Microscopic examination and culture
Bacteriological exams were performed at the National Reference Laboratory for Bovine Tuberculosis (Institute
of Diagnosis and Animal Health, Bucharest, Romania) in accord with Chapter 3.4.6. - Bovine tuberculosis of the OIE Terrestrial Manual 2018. Briefly, samples of lymph nodes collected from a bovine with positive or dubious results in the tuberculin tests were used to prepare direct smears stained with the Conventional Ziehl-Neelsen (ZN) method. The smears were performed from the caseous lymphadenitis lesions or the concentrated pathological material destined for culture and biological tests. Tissue specimens for culture were firstly homogenised using a mortar and pestle, decontaminated with an equal volume of 2 - 4% sodium hydroxide solution for 30 minutes at 37°C and then neutralized with a few drops of 10% HCl or 5% oxalic acid in the presence of a colour indicator. The sediment was used for culture and microscopic examination.

The sediment was inoculated on solid egg-based media Lowenstein–Jensen with sodium pyruvate, Malachite green and glycerol free, Middlebrook 7H10 or 7H11 with egg, Malachite green, bovine serum, oleic acid and albumin, and Herrold's egg yolk agar with sodium pyruvate. Cultures were incubated for a minimum of 8 weeks at 37°C, with a weekly examination to detect growth.

The identity of the suspected cultures was confirmed by microscopic analysis to detect acid-alcohol-resistant bacilli and the phenotypic characteristics for differentiation of infrasubspecific M. tuberculosis variants. M. tuberculosis var. bovis is sensitive to thiophen-2-carboxylic acid (TCH) hydrazide, isonicotinic acid hydrazide (INH), para-aminosalicylic acid and streptomycin; it is negative for niacin accumulation and nitrate reduction, and in the amidase test it is positive for urease and negative for nicotinamidase and pyrazinamidase.

**Histopathological examination**

Routine histological processing steps were used for histopathological examination. Briefly, lymph nodes samples were fixed in 10% Formalin (formaldehyde), the paraffin blocks were sectioned by using precision knives (microtome) at 3 - 5 µm and the microscopic examination of slides were done after ZN staining.

**Guinea pig virulence testing (biotest)**

Guinea pigs (young males of 250 - 300 g) were subcutaneously inoculated with 0.5 mL inoculum obtained by free decanting the suspension (about 10 grams of pathological material in one volume of sterile physiological serum) and monitored daily up to 90 days. Two guinea pigs were used per sample. In the case of guinea pig death in the first days after inoculation, the necropsy, bacterioscopic or cultural examination was performed to isolate and identify the bacterium that caused the death of the animals, and the biotest was repeated using guinea pig pathological material.

The study was conducted in the frame of Strategic Plan approved by Order no. 35/2016 of president of Central National Sanitary Veterinary and Food Safety Authority. Mice were housed at the Animal Facility of Faculty of Veterinary Medicine-Bucharest, authorisation number 277/2016, in groups of 2 to 6 individuals in isolated ventilated cages in a standardized environment (22.5 ± 1.5°C, 55 ± 10% relative humidity, 12 h light/dark cycle, 74 changes of air per hour), fed a standard rodent diet ad libitum and had free access to water.

**Genotyping of Mycobacterium species/strains**

For the identification and differentiation of M. tuberculosis var. bovis and var. caprae was used the GenoType MTBC test (Hain Lifescience, Germany). Assays were performed according to the manufacturer's instructions, previously described by Richter et al. (2003) [21]. Briefly, 10 µL of bacterial culture grown on solid medium were suspended in 300 µL ultrapure water, sonicated 5 min, incubated 20 minutes at 95°C, and 5 µL of the suspension was used in the PCR reaction with the Taq DNA polymerase in a final volume of 50 µL. Hybridization was performed at 45°C and 300 rpm with an automated device (TwinCubator; Hain, Germany), in a volume of 20 µL of the amplification product.

**Results and Discussion**

The macroscopic appearance of the lymph nodes examined for the tuberculosis lesions (gray-white or yellowish nodules, not encapsulated, firm or hard, with a yellowish yellow centre, with or without calcifications) revealed the presumptive macroscopically lesions of tuberculosis in 99.83% (589/590) cases (Figure 1).

In accord with OIE Terrestrial Manual [19], for all slaughtered cattle with the TST positive or dubious results, the presumptive diagnosis of infection with M. tuberculosis var. bovis was for all tissues with characteristic histological lesions, respectively: chronic granulomatous caseous-necrotising inflammatory process, mineralisation, epithelioid cells, multinucleated giant cells of the Langhans type and macrophages. The microscopic examination of smears from clinical samples proved to be a relatively insensitive method of BTB diagnosis. These results are in accord with the general point of view which considers the smear microscopy with the light microscope able to detect only ~60 - 70% of TB [29]. Several methods allow identification of mycobacterial species, ranging from biochemical typing to genotyping, but the gold standard test for the TBT confirmation is the cultivation of the organism on primary isolation medium followed by phenotypic or/and genotyping methods [19].

Phenotypic methods of mycobacteria identification took into consideration morphological characteristics, growth rates, growth temperature, pigmentation, and biochemical characteristics. On pyruvate-based solid medium, smooth and off-white (buff) colonies were classified as M. tuberculosis var. bovis. Also, those isolates were positive for urease and negative for nicotinamidase and pyrazinamidase.
Figure 1.
The pathological aspect of bovine tuberculosis. A: chronic granulomatous caseous-necrotising inflammatory process in lymph node; B: acid-fast bacilli (AFB)-positive smear (ZN, x1000); C: multinucleate giant cells of the Langhans type (H&E, x400)

Guinea pigs’ experimental infection revealed 99.83% (589/590) isolates with specific pathological results for *M. tuberculosis* complex: swelling of the regional lymph node, eventual abscess, and persistent ulcer. However, Guinea pig virulence testing did not allow differentiation of *M. tuberculosis* var. *bovis* and *M. tuberculosis* var. *caprae*. Guinea pigs are sensitive to experimental infection with the species of the *Mycobacterium* tuberculosis complex and resistant to *M. avium* infection [26]. Phenotyping and genotyping of the *Mycobacterium* isolates between 2015 and 2016, revealed 567 (96.10%) strains of *M. tuberculosis* var. *caprae*, 22 (3.73%) strains of *M. tuberculosis* var. *bovis* and one (0.17%) strain of *M. avium* subsp. *avium* (Table I).

| Mycobacterium species and infraspecies isolated in Romania (Data recorded between 2015 and 2016 by Institute for Diagnosis and Animal Health, Bucharest, Romania) |
|---------------------------------------------------------------|
| **Year** | **No. of animals** | **Year** | **No. of animals** | **Year** | **No. of animals** | **Total** |
| 2015 | 17 | 2016 | 5 | 2015 | 251 | 2016 | 316 | 2015 | 0 | 2016 | 1 | 590 |
| % | 2.88 | % | 0.85 | % | 42.54 | % | 53.56 | % | 0 | % | 0.17 | 100 |

Considering the infraspecies of *M. tuberculosis* isolated in Romanian cattle with positive or dubious reactions at tuberculin skin tests between 2015 and 2016 (Figure 2), 64.86% (382/589) were Romanian Spotted breed, 33.61% (198/589) Holstein Friesian, and 1.53% (9/589) other breeds. The analysis of infected animals by age categories revealed the highest value of infected cattle at the age group “12 - 50 months” with 50.76% (299/589), followed by group “> 50 months” with 27.00% (159/589) and group “< 12 months” with 22.24% (131/589) (Figure 3).

Figure 2.
Infraspecies of *M. tuberculosis* isolated in Romanian cattle with positive or dubious reactions at tuberculin skin tests, by breed categories (Data recorded between 2015 and 2016 by Institute for Diagnosis and Animal Health, Bucharest, Romania)
In our study, the GenoType MTBC assay proved to be a very useful tool in the rapid identification and differentiation of *M. tuberculosis* infraspecies with an easy-to-perform reverse hybridization assay. Similar results were reported by Richter et al. in their works with *M. tuberculosis* isolates in cultures obtained from clinical specimens [21, 22].

Concerning the lesions collected from the lymph node samples examined in this study, no difference has been found between the pathologic profiles of infections by *M. tuberculosis* var. *caprae* and *M. tuberculosis* var. *bovis*, both as typology and expression. In one laboratory the *M. avium* subsp. *avium* strain was isolated in a bovine, from a non-professional holding.

Increased number of *M. tuberculosis* var. *caprae* (n = 567) in the lymph node samples originated from TST positive animals (n = 590) identified within the national eradication programs, from abattoir surveillance, support the risk of the emergence of this pathogen in cattle herds. This situation should concern the national and international authorities as long as *M. tuberculosis* var. *caprae* is a human pathogen [16] and consumption of unpasteurized dairy products from animals with lesions in the mammary glands is a public health risk [25].

Also, in the light of the recent studies that proved the ability of zoonotic variants of *M. tuberculosis* (*bovis* and *caprae*) to acquire antimicrobial resistance due to treatment of humans with TB [4, 14, 18], the confirmatory diagnosis should include the antimicrobial resistance (AMR) profile of each strain not only in humane tuberculosis, but also in bovine tuberculosis. Considering the DR-TB as a serious threat to global control of TB [20], the detection of multi-drug resistance (MDR-TB), extensively drug resistance (XDR-TB) and totally drug-resistant (TDR) strains in the veterinary laboratory can prevent the emergence of zoonotic DR-TB.

**Conclusions**

Between 2015 and 2016, the *Mycobacterium* isolates from the Romanian cattle with positive or dubious reactions at tuberculin skin tests were 96.10% (567/590) *M. tuberculosis* var. *caprae* and 3.73% (22/590) *M. tuberculosis* var. *bovis*. The most affected breeds were Romanian Spotted (64.86%, 382/589) and Holstein Friesian (33.61%, 198/589), and most frequently reported at the “< 12 months” age group (50.76%, 299/589) and “> 50 months” age group (27.00%, 159/589). Considering the zoonotic risk of *M. tuberculosis* var. *caprae* and *M. tuberculosis* var. *bovis*, a more complete characterization of the isolates identified in the veterinary laboratory by the antimycobacterial susceptibility testing would be recommended. Also, it is necessary to further intensify the active surveillance measures for bovine tuberculosis, mainly in small dairy farms.

**Conflict of interest**

The authors declare no conflict of interest.

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