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Original article

EVALUATION OF CLASSICAL AND RAPID METHODS FOR ISOLATION AND IDENTIFICATION OF *MYCOBACTERIUM BOVIS* IN CATTLE IN BULGARIA

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Summary

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Bovine tuberculosis is still a serious problem with major economic impact in many countries. The aim of study was to evaluate the diagnostic capabilities of the classical and some modern, rapid methods for isolation and identification of *Mycobacterium bovis*. In the period 2015–2018 from 29 outbreaks in 10 different regions of Bulgaria, 1193 lymph nodes from slaughtered cattle were examined by pathoanatomical, bacteriological, PCR and immunochromatographic methods. Of the 283 bacterial isolates, 263 were identified as *M. bovis* – member of the *M. tuberculosis* complex.

Key words: bacteriology, bovine tuberculosis, conventional PCR, immunochromatographic test, lymph nodes, pathoanatomy

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic disease characterised by the progressive development of specific granulomatous lesions or tubercles in the lymph nodes, lung tissues or other viscera. The causative agent of bovine tuberculosis is *Mycobacterium bovis*, a member of *Mycobacterium tuberculosis* complex (MTC). All MTC members are phylogenetically related and can cause tuberculosis in several animal species and men (Brosch *et al.*, 2002). At the genome level *M. bovis* shows 99.95% identity with *M. tuberculosis,* the agent of human tuberculosis (Garnier *et al.*, 2003).

Tuberculosis remains a significant health, social and economic problem due to the increasing number of sick people and animals, not only in developing but also in industrialised countries. In the countries where bTB is still common and pasteurisation of milk is not practiced, 10 to 15 per cent of human TB cases are caused by *M. bovis* (Good & Duignan, 2011). Humans are rarely affected by bTB, but people of some professions such

as veterinarians, farmers and workers in slaughterhouses may be at higher risk (Robinson *et al.*, 1988; Rodriguez *et al.*, 1995; Taylor *et al*., 2007).

The development of reliable and rapid screening tests is of great importance and strongly recommended for disease control and for faster confirmation of bTB infection in slaughtered cattle. The different polymerase chain reaction (PCR) methods, introduced into the laboratory diagnostic practice offer significant potential in this respect. PCR-based methods can be used both for diagnosis and for scientific and epidemiological studies.

The present study evaluated the diagnostic capabilities of classical pathoanatomical and microbiological methods for the diagnosis of bTB used in our country and some modern, rapid methods for isolation and identification of *M. bovis* introduced in the world laboratory diagnostic practice: different microbiological methods, the MGIT TBc ID immunochromatographic test based on the presence of MPT64 antigen specific for MTC, respectively for *M. bovis* and the conventional IS6110 PCR technique.

MATERIALS AND METHODS

Diagnostic materials

In the period 2015–2018, a total of 1193 diagnostic materials from 29 tuberculosis outbreaks in 10 regions of the country were examined for tuberculosis lesions at the National Reference Laboratory (NRL) for tuberculosis in the animals, Sofia, Bulgaria.

Mandibular, retropharyngeal, bronchial, mediastinal, and mesenteric lymph nodes from slaughtered bovine animals that responded positively or doubtful to tuberculin, were subjected to a detailed pathoanatomical examination (PA). The tissue suspensions from all diagnostic materials were examined microbiologically on liquid and solid media as described below. Samples positive in the liquid media were tested by immunochromatographic analysis and by conventional PCR.

Isolation of mycobacteria from solid media

Lymph nodes both with and without visible lesions were further processed for isolation of mycobacteria in accordance with World Organisation for Animal Health (OIE) protocols (OIE, 2018). Samples were homogenised and decontaminated with the BBL MycoPrep ™ Specimen Digestion/Decontamination Kit (BD, USA), containing N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH). The sediment obtained after processing the samples was resuspended in 1 mL of phosphate buffer and 100 µL of each sample were cultured on a Löwenstein-Jensen medium with pyruvate and a Stonebrink medium with pyruvate and PACT at 37 ^{o}C. Primary culture was observed after 3–4 to 6–8 weeks. Smears were prepared from the sediment of the treated samples and bacterial isolates and stained by Ziehl-Neelsen (BD TB Stain Kit ZN for warm staining of mycobacteria; Liofilchem, Italy).

Isolation of mycobacteria from liquid medium

The BD MGIT TBc ID Testing Kit (B&D & Company, USA) was used to speed up the isolation of mycobacteria. The kit includes a barcoded tube containing a modified Middlebrook 7H9 broth medium with fluorescent indicator for the growth of mycobacteria, embedded in silicon of the bottom of the tube (MGIT, Mycobacteria

Growth Indicator Tube) and a fluorescence reader (BD MicroMGIT Reader). Fifty hundred μL of the processed samples were cultured in this MGIT media supplemented with OADC enrichment and PANTA antibiotic mixture. The tubes with inoculated samples were incubated at 37 °C and read daily after the second day with the fluorescence reader for increase of fluorescence, to detect the presence of viable bacteria. In the presence of growth after 4–5 days of culturing, the medium should contain about 10^4–10^7 CFU/mL of mycobacteria. Samples showing middle to intensive fluorescence on reading with MicroMGIT Reader were examined microscopically to detect acid-fast bacteria in Ziehl-Neelsen stained smears. After day 14, samples that did not show a positive result were considered negative.

Immunochromatographic assay

The presence of bacteria from MTC, including *M. bovis* was confirmed by the rapid immunochromatographic test (BD MGIT TBc ID Test) (B & D & Co, USA). The test detects the mycobacterial protein MPT64 secreted by mycobacteria in liquid culture during cultivation in modified Middlebrook 7H9 medium. The total test duration is up to 15 minutes and the reactivity is determined by colour development. The protein MPT64 fraction forms a conjugate with anti-MPT64 mouse monoclonal antibodies fixed on the membrane of the apparatus. The antigen-antibody complex flows laterally across the membrane to the reaction zone where the complex is captured by a second antibody specific for MPT64. If the MPT64 protein is present in the tested samples, a clearly defined pink-violet band (T) identical to test control (C) is observed, proving the presence of *M. bovis*.

DNA extraction

For the purpose of the study, DNA was isolated directly from tissue samples and bacterial isolates from liquid and solid media. After decontamination of the tissue samples, extraction of DNA was performed with NucleoSpin® Tissue kit (Macherey-Nagel GmbH & Co. KG). For isolation of DNA from mycobacterial strains the Seeplex MTB /NTM ACE isolation kit (Seegene, USA) was used. In both cases, the supernatant containing genomic DNA was used for PCR amplification.

PCR amplification

For DNA amplification, the *Mycobacterium bovis* amplification kit (Genekam Biotechnology AG, Germany) was used. The amplification process was performed in a Thermal Cycler (Techne TC-412, UK) at the following temperatures: 1 cycle denaturation, 5 min at 94 °C followed by 30 cycles melting 30 s at 94 °C, annealing 30 s at 58 °C, extension 30 s at 72 °C and 1 cycle elongation 5 min at 72 °C. The PCR products were electrophoresed in 2 % GelRed stained agarose gel in 1× TAE running buffer. The samples in the gel were visualised on a UV Transilluminator (Upland, CA, USA) and the size of resulting PCR products was reported.

RESULTS

Clearly visible tuberculosis-specific lesions were detected in 283 (23.7%) samples out of all pathoanatomically examined 1,193 diagnostic materials (Table 1). Changes were observed mainly in mesenteric and mediastinal lymph nodes that harboured a large number of miliary and larger gray-whitish tubercles with pronounced calcification or a predominant

Table 1. Results of the tests performed by different methods

Pathoanatomical examination (n=1193)	Negative		910 (76.3%)*	
	Positive		283 (23.7%)*	
Microbiological testing (n=1193)	*A) MGIT liquid medium*			
	Negative		910 (76.3%)*	
	Positive		283 (23.7%)*	
	B) Solid medium			
	Löwenstein-Jensen + pyruvate		*Stonebrink + pyruvate and PACT*	
	Negative	930 (77.96%)*	Negative	930 (77.96%)*
	Positive	263 (92.9%)**	Positive	263 (92.9%)**
MGIT TBc ID test (n=283)	Negative		not tested	
	Positive		263 (92.9%)**	
PCR (n=1193)	*Tissue*		*Culture*	
	Negative	930 (77.96%)*	Negative	not tested
	Positive	263 (92.9%)**	Positive	263 (92.9%)**

* percentage of the total number of analysed samples (n=1193); ** percentage of positive isolates in the MGIT liquid medium (n=283).

caseous centre. On Ziehl-Neelsen stained smears, single microscopically pink, short, rod-shaped bacteria were observed.

The bacteriological examination of the 1,193 diagnostic materials from lymph nodes suspect for *M. bovis* resulted in isolation of 283 (23.7%) bacterial cultures. In 263 (92.9%) of them bacteriological growth with characteristic for *M. bovis* morphology of colonies was observed (Table 1). On the Stonebrink medium, the first M. bovis colonies appeared after 18–20 days, while on the Löwenstein-Jensen medium the colonies developed more slowly. The specific growth pattern of mycobacteria appeared at the end of the 4th week (in some isolates after 6–8 weeks). In both media wet, gray-white to yellow-coloured *M. bovis* colonies were observed.

Upon inoculation of all tested samples in the Middlebrook 7H9 fluorescence medium and reading with MicroMGIT Reader after 4–5 days, the appearance of specific fluorescence, characteristic for MTC bacteria, respectively for *M. bovis* were observed for 283 (23.7%) of the tested samples. The remaining 910 (76.3%) samples without fluorescence or with

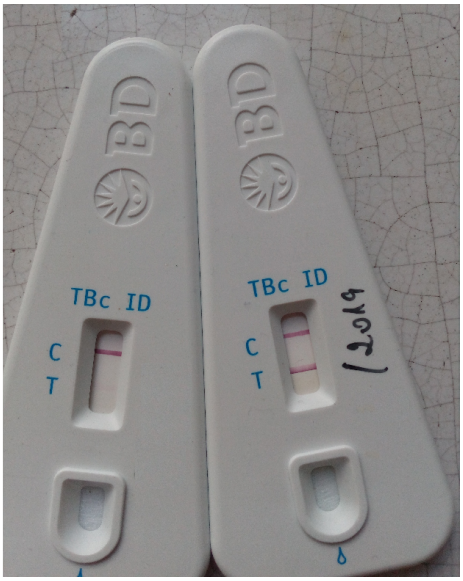

Fig. 1. Positive sample containing MPT64 antigen detected by immunochromatographic BD MGIT TBc test.

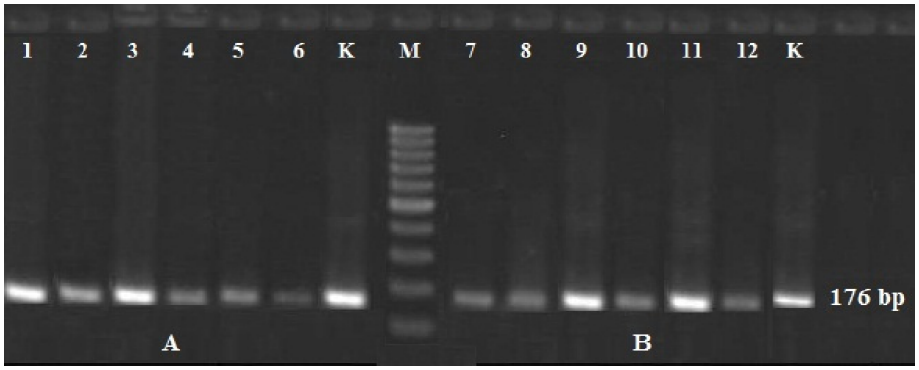

Fig. 2. 176 bp PCR products from lymph node tissue samples (lanes 1–6) and bacterial isolates (lanes 7–12); lane K: reference *M. bovis* 6186 strain; lane M: 100 bp marker.

weak fluorescence after 14 days were considered negative.

Only 263 (92.9%) of the Middlebrook positive samples subjected to immunochromatographic analysis showed two clearly defined pink-violet bands of the control (C) and the tested samples (T) (Fig. 1; Table 1).

From 283 bacterial cultures obtained from the liquid media, DNA was isolated to confirm the presence of *M. bovis* by conventional PCR. Only 263 samples were PCR positive and the remaining 20 samples were PCR negative.

All samples (263) showed growth of mycobacteria on solid media and a positive result from the immunochromatographic test were also PCR positive. The PCR products obtained showed a specific size of 176 bp (Fig. 2). Tissue suspensions of PA negative materials (930) in the PCR assay showed negative results (Table 1).

DISCUSSION

Mycobacterium bovis has the widest range of hosts of all known pathogens and has been diagnosed worldwide (Taylor *et al*., 2007; Good & Duignan, 2011). Monitoring of bovine tuberculosis is carried out using the tuberculin skin test. The diagnostic materials (lymph nodes and other organs) of the positive or doubtful reacting to tuberculin animals are sent for confirmation in the NRL (Sofia, Bulgaria) where diagnostic methods recommended by the OIE are used (OIE, 2018). Conventional methods for the identification of mycobacteria include the detection of acid-resistant bacilli in Ziehl-Neelsen stained microscopic preparations, followed by culturing on specific culture media and biochemical testing.

The microscopic examination is a widely used and easily applicable method. In tissue smears, however, mycobacteria can't be always detected or because of their small number, or because their lysis (Saggese & Phalen, 2005). Therefore the method is not specific and insufficient for diagnosis and has only indicative character (Pollock, 2006). Bacteriological examination is still a major diagnostic test accepted as the "gold standard" for the detection of mycobacteria. Although time-consuming, it is the only 100% sure method that does not produce false-positive results (De la Rua-Domenech *et al.,* 2006; OIE, 2018).

Isolation and typing of suspected cultures using the standard method can take up to 2–3 months due to the slow growth of mycobacteria (Koneman *et al.,* 2001). This requires the introduction of new faster and more accurate methods in the laboratory diagnostic practice for detection of *M. bovis*. The solid media – of Stonebrink and Löwenstein-Jensen used in our study, in which the glycerol used for cultivation of *M. tuberculosis* was replaced with Na-pyruvate, provided more favourable conditions for the development of *M. bovis*. Corner & Nicolacopoulos (1988) recommend both nutrient media as suitable for primary isolation of mycobacteria. However, our results showed faster growth of mycobacteria in Stonebrink egg-based medium containing pyruvate and PACT, which shortened the isolation time of *M. bovis* by more than one week. O'Reilly & Daborn (1995) and Grange *et al.* (2010) reported similar observations. According to other authors, cultivation on Stonebrink media takes 42 to 91 days (Do Rosário *et al.*, 2014). Our observations show that usually the initial growth of mycobacteria begins after the 21–28th day. In some cases however, the beginning of the primary growth of mycobacteria was observed after 45–50 days. Therefore such samples should be monitored at least for 12 weeks (OIE, 2018). The growth of bacteria can also be affected by possible differences in the composition of the media, produced by different companies, as we have observed in our practice.

The World Health Organization (WHO) recommends the use of liquid medium for accelerating the growth of mycobacterial isolates (WHO, 2007; 2018).

In an attempt to improve traditional microbiological methods, modified liquid media that allow the visualisation of the micro colonies in the initial stages of growth and the preliminary identification of the mycobacteria by the morphological characteristics of the colonies have been developed (Dib *et al.,* 2006; Silva *et al.,* 2007). According to Do Rosário *et al.* (2014) when liquid media are used for culturing, growth of mycobacteria occurred much earlier (between 12–25 days) than in the Stonebrink medium. In our studies for the isolation and typing of *M. bovis*, the modified fluorescence Middlebrook 7H-9 liquid medium was used, in which usually slow-growing mycobacteria from MTC developed significantly faster. Positive samples were identified by reading the fluorescence intensity with a BD MicroMGIT Reader. The Middlebrook 7H9 enriched with OADC and PANTA is one of the most commonly used liquid culture media for mycobacteria in recent years in clinical laboratories worldwide (Machado *et al*., 2014; WHO, 2018). Battaglioli *et al.* (2014) compared the simple Middlebrook 7H9 with modified liquid MGIT medium and demonstrated a higher sensitivity of the fluorescent MGIT medium. The fluorescence Middlebrook 7H-9 liquid medium is favourable for the growth and development of tuberculous and non-tuberculous mycobacteria. For their differentiation, the immunochromatographic test (MGIT TBc ID Test) was successfully used. Thus, out of the 283 samples tested in the liquid fluorescence Middlebrook 7H9 medium, only 263 (92.9%) were positive by immunochromatographic analysis (Table 1).

The BD MGIT TBc ID test is an express immunochromatographic assay for qualitative identification of MPT64 antigen in positive MGIT cultures and positively stained for mycobacteria by Ziehl-Neelsen (Machado *et al.,* 2014). Globally, currently three rapid MPT64-based immunochromatographic tests (MPT64 ICT)

have been developed for MTC identification: Capilia TB, SD Bioline and BD MGIT TBc Identification Kit. Kumar *et al*. (2011), Agora *et al.* (2015) and Babu *et al.* (2018) evaluated the SD Bioline kit; Brent *et al.,* (2011) and Machado *et al.* (2014) examined the diagnostic capabilities of the BD MGIT TBc kit; and Kandhakumari & Stephen (2017) worked in parallel with all three kits validating them using standard microscopic, bacteriological and biochemical analyses. The authors demonstrated very similar high sensitivity and specificity of these assays, which did not differ significantly from the identification of MTC isolates by conventional methods and believed that the immunochromatographic tests can be successfully used in the national tuberculosis monitoring programs because of their simplicity, cost-effectiveness and speed.

This corresponded with our observations on the BD MGIT TBC identification kit. All samples with positive growth of the solid media (92.9%) and strong fluorescence on the Middlebrook 7H-9 liquid medium were also positive for the immunochromatographic test, which agreed with the results obtained in the subsequent PCR examination of isolates. The first introduction of the test several years ago at the NRL for diagnosis of bTB in Bulgaria enabled the rapid identification of *M. bovis* before the results of the classical bacteriological cultivation on solid media. The test confirms the MTC cultures within 5–10 to 15 min as opposed to few weeks required by conventional techniques. The immunochromatographic MPT64 test is highly appropriate for use in diagnosis of tuberculosis. It can substitute molecular identification methods when their application is not possible, mainly in countries with limited financial resources (Kandhakumari & Stephen, 2017).

The presence of *M. bovis* in the examined tissue samples of lymph nodes with proven lesions and the obtained bacterial isolates was also confirmed by conventional PCR. The resulting PCR products had a size of 176 bp. The development and marketing of PCR kits significantly facilitates the work of laboratory staff and reduce the time for diagnosis (Taylor *et al.*, 2007; Ameni *et al*., 2010). PCR identifies and differentiates quickly the members of *Mycobacterium* genus (Bonovska *et al*., 1999; 2004; Rocha *et al.,* 2017). The test is suitable for the diagnosis of bTB in both live and slaughtered animals, which is of great practical importance for the monitoring of the disease and recovery of the herds (Ameni *et al.,* 2010; Good & Duignan, 2011).

The present investigation is the first comprehensive study in Bulgaria providing evaluation of diagnostic capabilities of few different laboratory methods for detection of bovine tuberculosis. The obtained results showed the advantage of the modified Middlebrook 7H-9 fluorescent liquid medium for primary isolation of mycobacteria as positive results were observed on the 7–10th day. Demonstration of *M. bovis* in the examined samples by immunochromatography and PCR has a high diagnostic value for preliminary diagnosis, as results can be obtained within 1–2 weeks. The Stonebrink solid medium was more suitable for cultivation of *M. bovis* than the Löwenstein-Jensen medium, since the first colonies appeared after 18–20 days.

The use of the combination of the presented methods as a routine approach in the laboratory diagnostic practice can guarantee greater accuracy of the performed studies and provide an earlier preliminary diagnosis. This will allow adequate measures to be taken to limit the

disease impact, improve the control of bovine tuberculosis and significantly reduce economic losses.

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