IDENTIFICATION OF MYCOBACTERIUM STRAINS BY PCR AND PCR-REA

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Abstract

The presented study aimed isolation of Mycobacterium strains from cases of bovine tuberculosis in Turkey, and identification of these isolates by PCR and restriction endonuclease analysis (REA). Mycobacteria were isolated from bronchial, mesenteric, and prescapular lymph nodes and tubercle samples from slaughtered cattle, which were either skin-test reactors or showed tuberculous suspected lesions during meat inspection. The samples were cultured in Lowenstein-Jensen medium. Colonies suspected of being Mycobacterium sp. were analysed by the PCR method using the MTUB_c-gyrBf and MTUB_c-gyrBr primers, which amplify the 1,020-bp region of the gyrB gene, that encodes the B subunit of the DNA gyrase (topoisomerase) enzyme in strains of the Mycobacterium tuberculosis complex (MTBC). The REA of MTBC-PCR positive amplified products was performed using the SacII and RsaI enzymes, in order to identify specific strains. The MTBC-PCR analysis of 117 bacterial strains demonstrated that all isolates belonged to the MTBC. All isolates were identified as M. bovis/M. bovis subsp. caprae by RsaI-REA, whilst 93 (79.5%) of the bacterial strains were identified as M. bovis and 24 (20.4%) as M. bovis subsp. caprae by SacII-REA. M. bovis subsp. caprae was isolated and identified for the first time in Turkey from bovine tuberculosis cases.

Key words: cattle, tuberculosis, M. bovis subsp. caprae, identification, restriction endonuclease analysis.

Tuberculosis, which results in severe health problems and economic loss, is a chronic and zoonotic bacterial disease of animals and humans, caused by Mycobacterium strains. The disease is characterised by the formation of white-yellow calcified granulomas (tubercles) formed as a result of the interaction of host immune cells with tubercle bacilli (18). In 2009, 14 million tuberculosis cases were reported in humans worldwide. The rate of infection was reported as 23% in Turkey (21).

Microorganisms of the Mycobacterium tuberculosis complex (MTBC) are the causative agents of human and animal tuberculosis. Of these microorganisms, M. tuberculosis, M. africanum (subtypes I and II), and M. canetti mainly infect humans and non-human primates (2, 20), M. bovis subsp. bovis and M. bovis subsp. caprae are the causative agents of ruminant tuberculosis, and M. microti is mainly found in small rodents (2). Genetically, all members of this complex are extremely similar, having 99.9% similarity at the nucleotide level and identical 16SrRNA sequences (7).

M. bovis and other MTBC species can be differentiated by biochemical tests, and the most distinctive feature of M. bovis is resistance to pyrazinamide (PZA) (6). However, recently, PZA-susceptible M. bovis strains have been isolated and identified. These strains can be distinguished by species-specific molecular methods, and M. bovis is divided into two sub-species as M. bovis subsp. bovis (PZA-resistant) and M. bovis subsp. caprae (PZA-susceptible) (4). Both sub-species can be differentiated only by molecular methods (6).

Differentiation of the MTBC members is of utmost importance (7). Several molecular techniques, being the best alternative to conventional culture and biochemical tests, were designed to differentiate MTBC. A two-step method for the differentiation of these species has been developed, based on the gyrB gene. In the first step, a 1,020 bp fragment of the gene is amplified with specific primers, which do not generate amplicons from other species of mycobacteria. In the second step, the amplicon is digested with restriction enzymes (7, 13, 16). Therefore, the PCR and restriction endonuclease analysis (REA) methods for the identification of Mycobacterium strains isolated from bovine tuberculosis cases were applied.

Material and Methods

Culture. Bronchial, mesenteric, and prescapular lymph nodes and tubercle samples were taken from 135 slaughtered cattle, which were either skin-test reactors or showed tuberculous suspected lesions during meat inspection at three different farms in Turkey (Table 2). The samples were decontaminated and inoculated into Lowenstein-Jensen medium (Merck VM614000, Germany). Culture media were incubated at 37°C for 4-8 weeks (3, 11).
Extraction of DNA. A loop of Mycobacterium-suspected colony was taken from the Lowenstein-Jensen medium, resuspended in 500 µl of Tris-EDTA (TE, 10 Mm Tris, 1 Mm EDTA, pH 8) and incubated at 80°C for 1 h to inactivate the mycobacteria. DNA was extracted from the inactivated samples using the protocol described by Barouni et al. (5) and Del Portillo et al. (10).

DNA amplification. The primers MTUB_c-gyrBf-5’-TCGGACGCGTATGCGATATC-3’ and MTUB_c-gyrBr-5’-ACATACAGTTCGGACTTGCG-3’ were used to amplify a 1,020-bp fragment of the gyrB sequence (7, 13). The 50-µl reaction mixture contained: 5 µl of PCR buffer (with KCl), 3 µl of MgCl2 (25 mM), 10 mM of each deoxynucleoside triphosphate, 1 µM of each primer, 1.25 U of Taq DNA polymerase (Fermantas, EP0402), 33.75 µl of sterile distilled water, and 5 µl of DNA sample. PCR amplifications were performed in an Eppendorf thermocycler (Matercycler gradient 5331 000.010, Germany) according to the following protocol; initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The 10 µl of the PCR products were separated by ethidium bromide (5 µg/mL, Sigma) agarose gel (1.5%, w/v) electrophoresis with 100 bp DNA ladder (Fermentas, SM0321). The gels were visualised and photographed by an ultraviolet transilluminator. An amplification product of 1,020 bp was indicative of the MTBC. The DNA from M. tuberculosis H37Rv and M. bovis (AN5) was used as a positive PCR control and sterile distilled water as a negative control (7, 13).

Results

Culture. In total, 117 Mycobacterium strains were isolated from 77 bronchial, four mesenteric, and 18 prescapular lymph nodes, and 18 tubercle samples.

PCR. In MTBC-PZR analysis, all isolates gave positive bands at the 1,020 bp sequence and were defined as the MTBC (Fig. 1).

Table 1

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<tr>
<th>Rsal-REA</th>
<th>SacII-REA</th>
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<tr>
<td>360/560 bp</td>
<td>M. tuberculosis</td>
</tr>
<tr>
<td>360/480 bp</td>
<td>M. bovis, (PZA-resistance)</td>
</tr>
<tr>
<td>360/660 bp</td>
<td>M. microti</td>
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<tr>
<td>No digestion</td>
<td>M. bovis BCG</td>
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<tr>
<td>280/740 bp</td>
<td>M. bovis subsp. caprae (PZA-susceptible)</td>
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Restriction endonuclease analysis (REA). All MTBC PCR-positive isolates, of which the amplified DNA yielded 360/480 base pair bands, were identified as M. bovis/M. bovis subsp. caprae by Rsal-REA (Fig. 2). No DNA digestion was observed in 93 (79.5%) isolates and these were identified as M. bovis, whilst in 24 (20.5%) isolates, the DNA yielded 280/740 base pair bands and accordingly, these were identified as M. bovis subsp. caprae by SacII-REA (Fig. 3, Table 2).

Discussion

Tuberculosis is one of the leading causes of death among humans and animals. Every year, 8 million new active cases occur and 2 million people die due to tuberculosis worldwide (1). Microorganisms of the MTBC are the causative agents of tuberculosis (16). The differentiation of MTBC members is required for the appropriate treatment of individual patients as well as for epidemiological purposes (7). There is a direct correlation between M. bovis infection in cattle and disease in the human population. M. bovis is responsible for about 10% of new cases of human pulmonary and extra-pulmonary tuberculosis. Bovine tuberculosis is an economical and public health threat in developing countries (9).

Table 2

<table>
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<th>Place of origin of Mycobacterium strains</th>
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<td>Origin of Mycobacterium Strains</td>
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<tr>
<td>Farm 1</td>
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<td>Farm 3</td>
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According to novel taxonomy, *M. bovis* is divided into two subspecies, namely, *M. bovis* subsp. *bovis* and *M. bovis* subsp. *caprae* (4). Although *M. bovis* subsp. *caprae* was initially isolated from sheep and goats, further studies confirm its infectivity in humans, cattle, dogs, and pigs (15). Lantos et al. (15) isolated and identified *M. bovis* subsp. *caprae* from a tracheal aspirate obtained from an 8-year-old male Siberian tiger kept at the Budapest Zoological Garden. Cvetcic et al. (8) identified the *M. bovis* strain isolated from tissue samples of six cattle and four pigs raised at the same farm as *M. bovis* subsp. *caprae*. Furthermore, *M. bovis* subsp. *caprae* was detected in farmed deer (12, 17) and a camel (12). In Central Europe, *M. caprae* is the major cause of tuberculosis in cattle, and also the predominant agent of bovine tuberculosis in humans (14, 19). Kubika et al. (14) isolated *M. bovis* subsp. *bovis* (69%) and *M. bovis* subsp. *caprae* (31%) from cases of human tuberculosis in Germany between 1999 and 2001.

In this study, 93 out of the 117 (79.5%) *Mycobacterium* strains, which were isolated from 58 bronchial, three mesenteric, and 16 prescapular lymph nodes, and from 18 tubercle samples obtained from 135 cattle were identified as *M. bovis*, whereas 24 out of the 117 (20.5%) strains, which were isolated from 19 bronchial, one mesenteric, and two prescapular lymph nodes, and from two tubercle samples were identified as *M. bovis* subsp. *caprae*. No data are available on the prevalence of tuberculosis in animals and humans caused by *M. bovis* subsp. *caprae* in Turkey. In our study, *M. bovis* subsp. *caprae* was isolated and identified in cases of bovine tuberculosis in Turkey for the first time. In conclusion, at present, conventional culture remains the gold standard for the detection of *M. bovis* in clinical samples. Nevertheless, the PCR and PCR-REA methods offer the potential advantages of sensitivity, flexibility, and speed. At the moment, *M. bovis* subsp. *caprae* accounts for a high ratio of bovine tuberculosis cases in Turkey.

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References


