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Detection of bovine tuberculosis by tuberculin test and polymerase chain reaction in Van, Turkey*

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Abstract: The aim of this study was to identify the presence and prevalence of bovine tuberculosis in the eastern Anatolian border province of Van and its villages and to collect the preliminary data required for programs for the prevention and control of this zoonotic disease. The tuberculin test was performed on 210 cattle. Nasal swab and milk samples were obtained from the animals tested. Three cattle that had a positive tuberculin test also tested positive for the DNA target, a 580-bp fragment of IS6110 specific for members of the *Mycobacterium tuberculosis* complex. This fragment was also recovered from milk taken from the third animal tested positive for tuberculin. All the other cattle were negative for bovine tuberculosis on both tuberculin test and PCR assay. The results of the tuberculin test and the PCR were in close correlation with each other. Van is a border province in eastern Anatolia. In the city center and its villages, the prevalence of bovine tuberculosis was estimated as 1.42%.

Key Words: *Mycobacterium bovis*, bovine, tuberculin test, IS6110, PCR

Van'da (Türkiye) polimeraz zincir reaksiyonu ve tüberkülin testi ile sığır tüberkülozunun taranması

Özet: Bu çalışmanın amacı, Doğu Anadolu Bölgesi'nde bir sınır ili olan Van ve yöresinde sığır tüberkülozunun varlığı ve yaygınlığının tespit edilmesi ve bu zoonoz hastalıktan korunma ve kontrolü için ihtiyaç duyulan ön bilginin toplanmasıdır. Tüberkülin testi 210 sığıra uygulandı. Hayvanlardan toplanan nazal svap ve süt örnekleri polimeraz zincir reaksiyonu (PZR) ile test edildi. PZR'de *Mycobacterium tuberculosis* grubu bakterilerin IS6110 bölgesine spesifik olan (580-bp'lik) primerler kullanıldı. Tüberkülin testi pozitif olan 3. hayvandan alınan süt örneklerinde PZR ile pozitiflik saptandı. Tüberkülin testi ve PZR sonuçları birbirleri ile uyumlu bulundu. Van şehir merkezi ve köylerinde sığır tüberkülozunun yaygınlığı % 1,42 bulundu.

Anahtar Sözcükler: *Mycobacterium bovis*, sığır, tüberkülin testi, IS6110, PZR

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Introduction

Bovine tuberculosis has a high incidence throughout the world, especially in developing countries. As an infectious disease, it can create important public health problems as a zoonosis (1,2) and causes significant economical losses (3,4). In addition to the economical losses it causes, because of the zoonotic property of the disease and its chronic progressive nature, eradication efforts have been undertaken in several countries. In those countries that operate bovine tuberculosis eradication programs, human infections caused by *Mycobacterium bovis* have been reduced significantly (5).

The tuberculin test in its various formats has been widely used as a diagnostic test because of its ease of use at herd level. The isolation of the causative agent, *M. bovis,* as the definitive diagnostic finding from clinical samples using classical microbiological methods is very time consuming. Developments in rapid molecular diagnostic techniques have presented other means of diagnosis that have significantly reduced the time to diagnosis, when compared to the classical methods currently used (6-9).

Van is a province in eastern Anatolia that shares borders with Iran and Iraq. From time to time, there is illegal transfer of animals from these neighboring countries. This creates the opportunity for the entry of bovine tuberculosis, as well as other diseases, into Turkey. Therefore it is crucial to identify the presence of bovine tuberculosis and monitor its prevalence in such border provinces at regular intervals. This approach is necessary to protect the health of the national herd and also applies to the control of entry of zoonotic and other infectious animal diseases. This study is aimed at identifying the presence and prevalence of bovine tuberculosis in the province of Van and its villages, thereby providing preliminary data for programs for the prevention and control of this zoonotic disease.

Materials and Methods

According to 2004 official figures the number of cattle in Van is around 80,000 (approximately 45,000 culture breed and 35,000 domestic breed). Sampling was carried out using the random sampling method based upon the predicted animal prevalence of 1.5% with the confidence interval of 95%.

A total of 200 animals kept in 48 barns in 6 villages located in different parts of Van along with 10 animals kept in 4 barns in the center of Van underwent tuberculin tests from April to July 2005. Of these animals 29 were bullocks and 181 were cows.

Nasal swab samples were taken from 26 of these bullocks and 172 of these cows, and milk samples were also collected from 146 of the cows. Sampling of nasal swabs and milk was carried out according to the bovine tuberculosis regulation (10).

Standardization of DNA extraction and amplification was performed using, as a positive control, a *M. bovis* strain obtained from the Tuberculosis Laboratories of the Veterinary Control and Research Institute of the Ministry of Agriculture and Rural Affairs (Etlik, Ankara).

The Reference *M. bovis* strain was grown in Lowenstein-Jensen media. DNA isolated from direct culture and from milk inoculated with the reference strain was used as positive control.

DNA isolation from direct culture was carried out according to the method described by Vitale et al. (11).

DNA isolation from milk samples used as research material was carried out according to the method described by Çetinkaya et al. (12).

The target DNA for amplification was a 580-bp fragment of IS6110, an insertion sequence-like element currently used to identify members of the *M. tuberculosis* complex. The primers used were the oligonucleotides 295 up (5'-dGGACAACGCCGAATTGCGAAGGGC-3') and 851 down (5'-dTAGGCGTCGGTGACAAAGGCCACG-3'), which correspond to base pairs 295 to 318 and 851 to 874 of the IS6110 insertion element, respectively.

PCR amplification was carried out in 50 µl reaction mixtures containing (final concentrations) 2.0 mM $MgCl_2$, 50 mM KCl, 200 mM (each) deoxynucleoside triphosphate, 200 nM of each primer, 5 µl of template DNA solution, 2.5 U of DNA Taq polymerase (Fermentas), and 32 µl double distilled water. The reactions were performed in an automated thermal cycler (ThermoHybaid, USA). The conditions were set as follows: denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min. A 1-min extension period at 72 °C was added after 35 cycles.

PCR products were analyzed by electrophoresis on 1.5% agarose gels containing 0.1 mg of ethidium bromide/ml in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA). The gels were visualized under UV light with a visualization system (Spectronic, USA).

The mammalian PPD and avian PPD tuberculins used in this study were obtained from the Tuberculosis Laboratories of the Veterinary Control and Research Institute of the Ministry of Agriculture and Rural Affairs (Etlik, Ankara). The tuberculin test was performed in accordance with the bovine tuberculosis regulation (10).

Results

Three of the 210 animals tested showed a positive reaction to the mammalian PPD tuberculin. One, a bullock, was positive to the mammalian PPD tuberculin only, while the other 2 (1 bullock and 1 cow) were positive to both mammalian and avian PPD tuberculins. In accordance with the bovine tuberculosis regulation, animals that show a positive response to mammalian PPD are regarded as positive for tuberculosis. The 2 animals that were positive both for mammalian and avian PPD were evaluated in terms of the increase in the thickness of the skin at the sites of inoculation as compared to the thickness of the skin on both sides of the neck and were finally regarded as positive for tuberculosis. That is, 3 of the 210 animals tested were identified as cases of bovine tuberculosis according to the bovine tuberculosis regulation.

Two other animals, namely one bullock and one cow, each showed a positive reaction to the avian PPD tuberculin alone and were regarded as positive for paratuberculosis, in accordance with the above regulation.

Nasal swab samples were obtained from 26 of 29 bullocks and 172 of 181 cows. In 3 nasal swab samples, using PCR methodology, the 580-bp fragment of the IS6100 insertion element that is specific for the DNA of members of the *M. tuberculosis* complex was detected. Milk samples were obtained from 146 of 181 cows, and in 1 of these samples the 580-bp fragment was identified. Regarding the cow from which this milk sample was collected, the nasal swab sample taken from this animal also contained this fragment (Figure). The 3 animals from which nasal swab and/or milk samples that were positive for the 580-bp fragment were taken were the same 3 animals that showed a positive reaction to the mammalian PPD tuberculin and which, on that basis, had been deemed to be bovine tuberculosis positive.

Figure. Analysis of PCR amplified 580-bp fragment by 1.5% agarose gel elctrophoresis. M: Marker (GeneRuler 100-bp DNA ladder, Fermentas), 1: Milk, 2: Positive control, 3: Nasal swab, 4: Nasal swab, 5: Nasal swab

Discussion

The accurate diagnosis of bovine tuberculosis remains an elusive goal because no method has yet been developed that can precisely detect the presence of the microorganism in live animals in all cases. The tuberculin test currently used around the world renders variable results due to limitations regarding sensitivity and specificity which bring into question its reliability when used to identify individual animals that are infected with *M. bovis*. The accuracy of the tuberculin test depends on several factors, including the use of high-quality tuberculins, the format of the tuberculin test used (e.g. single intradermal comparative tuberculin test, single intradermal tuberculin test, the caudal fold test), the efficiency of the testing procedure, the mode of interpretation of the test result) as well as the immunological responsiveness of the animal at the time of test. Furthermore, a negative result to a tuberculin test does not mean that the animal is not infected with *M. bovis* while, on the other hand, a positive result represents an immunological response, in the form of a delayed hypersensitivity reaction to mammalian PPD tuberculin that is most commonly due to a current infection or a previous exposure to *M. bovis* but may less commonly be due to an infection or exposure to other bacteria that share antigens similar to those of *M. bovis*. In the study, the agreement between PDD tuberculin and PCR was determined as excellent.

In view of these shortcomings, the samples (nasal swab, milk) obtained from animals that had been tuberculin tested for tuberculin was subject to PCR assay in order to determine if such an assay would improve the efficiency of diagnosis.

According to the tuberculin test results, the positivity rate for bovine tuberculosis was identified as 1.42% (3/210) in the animals tested. This rate was very close to the predicted disease prevalence before initiating the study. In our study, of the animals tested with tuberculin, nasal swab samples of 26 bullocks and 172 cows were analyzed with PCR. In 3 of the nasal samples, and in one of the 146 milk samples examined, a 580-bp DNA sequence specific for the *M. tuberculosis* complex was identified (Figure). In one cow both the milk sample and the nasal swab sample contained the target DNA sequence. When the results of the tuberculin test were compared with those of the PCR, the 3 animals evaluated as positive with PCR also showed a positive reaction to mammalian PPD tuberculin in the tuberculin test. No other animals reacted positively to the mammalian PPD tuberculin. Two cattle which had reacted positively solely to the avian PPD tuberculin were negative on the PCR assay; both of these animals were regarded as positive for paratuberculosis in accordance with the bovine tuberculosis regulation.

In a study by Romero et al. (13), of 275 dairy cattle, 184 were found to be positive to the tuberculin test. The researchers obtained blood, nasal swab samples, and milk samples from the animals that were tuberculin test positive. After examining these samples using PCR, 26 animals tested positive. Romero et al. (13) reported PCR as being a more sensitive and specific test than the tuberculin test. Other researchers (9,14-16) have compared classical diagnostic methods with PCR in their studies and reported that PCR was a more sensitive method. In our study, tuberculin test results were in close correlation with the PCR analysis results. Therefore we do not consider that those animals that had tested negative on the tuberculin test are possible sources of infection for the herds of origin.

In summary, this study was carried out in Van which is a border province of Turkey between Iraq and Iran. Three animals that were diagnosed by means of the tuberculin test as cases of bovine tuberculosis. Nasal swab samples taken from all 3 animals (2 bullocks and 1 cow) along with a milk sample taken from the same 3 cows tested positive for the DNA target, fragment 580-bp, on PCR analysis, thus showing a close correlation between the tuberculin test and PCR test results. Samples taken from other animals under study were negative on the PCR assay. The prevalence of bovine tuberculosis in the province of Van was calculated as 1.42%.

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