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Paratuberculosis in cattle in Turkey detected by PCR*)

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Summary

In this study 96 randomly selected fecal samples of two-year old or older cattle from Trakya district of Turkey were investigated for the presence of Mycobacterium avium subsp. Paratuberculosis by PCR. A pair of genus-specific primers, derived from the IS 900 gene of the agent, was used as a target sequence for the PCR. The specific DNA was not detected in any of the 96 fecal samples, and all of them were considered to be PCR-negative. In conclusion, although the absence of specific DNA in the samples of cattle raised in the Trakya district is an optimistic sign, further studies on John's disease are needed.

Keywords: paratuberculosis, cattle, Turkey

Paratuberculosis (Johne's disease) is a chronic, contagious disease of cattle, sheep, goat and other ruminants, characterized by weight loss and granulomatous enteropathy, and caused by *Mycobacterium avium subsp. paratuberculosis* (3, 15, 20, 25). Race, climatic differences and farm managements have been reported as important in epidemiology of the disease (6, 7). The infection is not only world-wide and causes significant economical losses in cattle industry, but also a possible association between *M. avium subsp. paratuberculosis* infection and Crohn's disease in humans has been described (1, 18, 26). It has been considered that the agent can be transmitted to humans via milk since the organism has been isolated from milk of clinically (25) and sub-clinically (23) infected cattle, and might survive pasteurisation (2). In addition, paratuberculosis has been reported in monogastric animals such as nonhuman primates (monkey) (17) and rabbits (14).

Traditional diagnostic methods currently include culture of feces and tissues, serological tests such as ELISA and Complement Fixation. Long incubation time and high cost are disadvantages of the isolation techniques. Serological tests are not sensitive since specific antibodies may not be detectable in early course of the infection (4). The developments in molecular biology have promised more accurate and rapid diagnostic methods. At present, PCR techniques based on specific IS 900 gene are being commonly used (12, 28).

Although there have been some studies on bovine paratuberculosis in the central Anatolia (27) and Elazig province (the eastern Anatolia) (10), the prevalence of the disease in the western Anatolia and Trakya district, where the dairy cattle population is concentrated, is unknown.

In this study, determination of the prevalence of the disease in cattle in Trakya district of Turkey by PCR from fecal samples was aimed.

Material and methods

Fecal samples. Accepting the estimated prevalence of the infection in Trakya district as 50%, level of confidence as 95% and desired absolute precision as 10%, fecal samples were randomly collected from two year old or older, 96 dairy cattle raised in different farms. The samples were collected by scraping the rectum mucosas, and transferred to the department laboratory under cold chain in sterile petri dishes. The samples were immediately used for DNA extractions.

Positive control. Positive DNA samples for PCR were kindly obtained from Dr. Tony Rouillard (AES Laboratories-France) and University of Firat, Veterinary Faculty, Dept. of Microbiology.

DNA extraction. DNA extraction was performed according to the manufacturer's (Macherey-Nagel NucleoSpin cat. no. 740952.50) protocol. The extracted DNA samples were kept at –20°C until the time of use.

PCR. PCR amplification was performed according to the manufacturer's (Adiagen Ref: 042-1) protocol. Briefly, 2 µl of template DNA was added into the PCR mix (48 µl of A1 + A2 reagent). This mixture was overlaid with 50 µl of mineral oil and placed in PCR Thermocycler (Biometra Uno-themoblock). DNA amplification was obtained with one cycle of 37°C for 30 sec., 94°C for 5 min., followed by 45 cycles of denaturations at 94°C for 15 sec., annealing at 62°C for 30 sec., and synthesis at 72°C for 40 sec. A final extension step at 72°C for 10 min. was included at the end of the cycles. The PCR mixtures were cooled at +4°C until the time of use.

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In the samples in which internal control bands have not been seen; 1 ml of extracted DNA was diluted tenfold in sterile water to eliminate the probable inhibitory factors in the feces and reamplified again.

Detecting of DNA. Ten µl of amplicon and loading solution mix (10 µl amplicon + 2 µl loading solution) were analyzed by electrophoresis in a 2% agarose gel using Tris--Boric acid-EDTA (TBE) buffer. Following electrophoresis which was carried out at 3.4 volts per cm for 1.5 hours, DNA bands were observed by UV lights after ethidium bromide (0.5 µg/ml) staining for 45 min. at room temperature.

In the study, the size marker including 169 bp target DNA, 480 bp internal control DNA and 1028 bp DNA was used to detect the molecular weight of the bands.

Results and discussion

Positive bands of 169 bp were observed in none of the investigated 96 fecal bovine samples. Therefore, all samples were considered as negative.

Numerous researches have been performed on the epidemiology of the infection, and the United States and many other countries have instituted Johne's disease certification programs to eradicate the disease due to economic considerations and the association between paratuberculosis and Crohn's disease in humans (12). In Turkey, although presence of the disease is known, detailed investigations on the prevalance and epidemiology of the disease are needed. In a study of Vural and Atala (27), the seroprevalance of the disease in cattle in Central Anatolia region was determined as 2.7% as a result of Complement Fixation Test. Çetinkaya et al. (10) reported that, 25 (5%) of 500 bovine milk samples taken from Elazıg province and its villages were found to be positive by PCR. In the study, 17 out of 25 PCR positive samples were also determined to be positive by culture. Paratuberculosis was also reported from sheep and goats populations of Turkey (9, 11).

The prevalance of the infection in other countries can vary with geographical differences, control measures, and applied techniques. In Uruguay randomly selected 720 cattle from 36 herds located in every districts of the country were investigated by ELISA, and 16.02% of them have been reported to be positive (19). In a similar study performed in Portugal, spesific antibodies were found 7% of 2120 cattle sera by ELISA (13). As a result of the study carried out intestinal lymph nodes of 1553 cattle slaughtered at abattoirres in South West England, the prevalence of subclinical disease in adult cattle was explained as 3.5% and 2.6% by PCR and culture, respectively (8). On the other hand, it has been reported that there is no isolation from 4000 fecal samples of dairy cattle raised in Sweden, and these results were supported the paratuberculosis-free status of Swedish cattle (21).

It has been reported that, culture of feces that is considered as the referance test needs long incubation time (12 to 16 weeks), is laborious, and has insuficient sensitivity in diagnosis of subclinically infected animals. Also, serological tests and skin tests may give false results. Consequently alternative methods have been investigated and now identification of the organism achieved by PCR amplification of the IS900 gene as a results of developments in molecular biology which promises more accurate and rapid diagnostic tools (5, 22).

Lillini et al. (16) analised 30 fecal samples from paratuberculosis positive cattle, with two different PCR approaches -home-made and the commercial kit ADIVET-. They reported that the results of both methods correlated completely with controls and decided to use PCR as rapid and reliable diagnostic tools for their further studies.

In this study, DNA extracts from fecal samples were amplified by Adiavet Paratub PCR kit which has internal control DNA to check the amplification processes for each sample. For the samples that control bands had not existed after PCR, the extracts were diluted to eliminate possible inhibition factors and then re-amplified. Although this obligatory application increased the reliability of the test, it decreased the amount of template DNA in the extracts. *M. paratuberculosis* specific DNA was not detected in any of the fresh fecal samples obtained from Trakya district where cattle breeding with Holstein genotype is concentrated. This result is hopeful when compared with other limited studies performed in other parts of Turkey. It is considered that this result could be a result of relatively better care and feeding condition relative to other parts of Turkey because of socioeconomic factors. The isolation of *M. paratuberculosis* from semen, testes, bulbourethral glands, prostate and vesicula seminalis of infected cattle suggests that breeding by natural means is a potential source of the infection (3). It has been indicated that the disease has a higher prevalence rate in cattle in which natural mating were being applied (10). In Trakya district, artificial insemination is usually preferred. Therefore, the negative results could also be due to these applications.

In conclusion, the absence of specific DNA in the fecal samples of cattle raised in Trakya district was hopeful. Because of the economic significance and importance in terms of public health of the disease, further studies on John's disease including other farms that have different climate and management conditions in Trakya district and also other districts of Turkey are needed.

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