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Research Article

Investigation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in Fecal and Bulk Milk Samples from Dairy Farms in Thrace Region of Turkey

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Abstract

The Thrace, which is Turkey's European part as well as the adjoining parts of Southern Bulgaria and north-east Greece has a strategic importance for being a vaccination buffer zone for Europe as declared by FAO in 1999. The objective of the study was to better understand the occurence of MAP in this animal disease free area of Turkey by applying *F57* Real time PCR assay and *IS900* nested-PCR. In this study, 270 feces samples from the dairy cattles over 2 years old in 30 randomly selected dairy farms, 45 raw milk samples from each of the bulk tanks belonging to these dairy farms and the villages located in Thrace were collected. Nine fecal samples were used to create the pooled fecal sample for a dairy farm before performing DNA extraction. All the samples were initially subjected to a *F57*-Real time PCR analysis, and subsequently an insertion sequence *IS900* nested-PCR was performed to verify the results. However, the results revealed that MAP genom could not be detected in any pooled fecal and milk samples. In conclusion, the occurrence of MAP in this part of Turkey may likely be very lower than the capability of the detection limit of the used Real time PCR assay. Furthermore, the results once more confirmed the difficulty of MAP detection in asymptomatic animals and milk samples by performing PCR technique only.

Keywords: *Mycobacterium avium subsp. paratuberculosis, Real time PCR, Nested-PCR, Feces, Milk*

Türkiye Trakya Bölgesindeki Süt İşletmelerinden Toplanan Fekal ve Çiğ Süt Örneklerinde *Mycobacterium avium* subsp. *paratuberculosis* (MAP) İncelemesi

Özet

Trakya Türkiye'nin Avrupa topraklarında olup, Güney Bulgaristan ve Kuzeydoğu Yunanistan sınırlarının keşiştiği bir bölgedir. FAO 1999 yılında bu bölgeyi zoonozları erken önlemek bakımından stratejik önemi olan Avrupa aşılama tampon bölgesi olarak duyurmuştur. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) varlığı Trakya'da net şekilde anlaşılamamıştır. Bu çalışmada zoonozlardan ari olarak kabul edilen Trakya'da MAP varlığını *F57* Real time PCR assay ve *IS900* nested-PCR yöntemlerini uygulayarak incelemek amaçlandı. Araştırmada, rastgele seçilen 30 adet süt işletmesinde 2 yaş ve üzeri sığırlardan 270 adet fekal örnek ile bu işletmelerin ve bulundukları köylerin süt toplama tanklarından 45 adet çiğ süt örnekleri toplandı. Bir süt işletmesini temsilen 9 adet fekal örnek tek havuz örnek olacak şekilde karıştırıldı. Örneklerden DNA izolatları elde edildi. İzolatlara *F57*-Real time PCR assay uygulandı. Elde edilen sonuçların *IS900* nested-PCR yöntemi kullanılarak doğrulaması yapıldı. Sonuç olarak, havuz fekal örnekleri ve çiğ süt örneklerinde MAP genomu tespit edilmedi. Trakya bölgesinde MAP varlığının *F57*-Real time PCR yönteminin tespit sınırının altında kaldığı sonucuna varıldı ve asemptomatik hayvanlar ve süt örneklerinde yalnızca PCR yöntemi ile MAP tespitinin zorluğu doğrulanmış oldu.

Anahtar sözcükler: *Mycobacterium avium subsp. paratuberculosis, Real time PCR, Nested-PCR, Fekal, Süt*

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis called Johne´s Disease (JD), which is an infectious, chronic, and granulomatous enteropathy of the ruminants providing milk and/or meat as food source for human consumption [1]. JD is characterized by severe symptoms such as diarrhoea, reducing milk production and weight loss [2,3]. During a long pre-clinical period more than 2 years it persists and multiplies in subepithelial macrophages to lead to a chronic transmural inflammatory reaction. This pathogen is periodically shed in feces, milk and semen of MAP infected dairy cattles [4,5].

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Although a causal link between MAP and Crohn's disease (CD) has not been proved, MAP may be involved in CD in humans, and unfortunately no cure for CD is currently known [6,7]. The incidence rates for CD in some European Countries (EU) were given as $5.2/10^5$ in Germany, $6.4/10^5$ in France, $2.3/10^5$ in Italy, and $5.9\text{-}11.7/10^5$ in England, respectively [8]. In an epidemiological study conducted in Turkey the incidence of CD was found to be $1.4/10^5$ while its prevalence was estimated as 7.7%, which was higher than in Asia but lower than in Europe [9].

The importance of food as a source of exposure to MAP has been assessed by many research groups. Milk and dairy products might be a likely food source for human exposure to MAP as well as a direct contact to the MAP infected animal [10,11]. The MAP occurrence in milk worldwide was estimated to be 1-3% [11,12]. Thus, exposure of humans to MAP should be minimized as a precautionary measure [13]. Due to this fact, the EU has compulsorily developed effective control programs for monitoring it [14].

The epidemiological situation of MAP in Turkey has not been well-understood. A study in 1968 indicated that sheeps in İzmit-Turkey were infected with MAP. This result was followed by another study performed for goats [15]. Towards 2000, MAP was initially found in goats [16]. Subsequently, mycobacterial DNA in milk samples of 500 dairy cows in Elazığ was detected by a polymerase chain reaction (PCR) based on IS900 [17]. A recent study for the dairy cattles in Uşak-Turkey showed that MAP prevalence was ranged between 9.5-17.0% in feces whereas it was between 5.5-17.5% in milk samples [18]. In Kars-Turkey the sero-prevalence of subclinical paratuberculosis in the cattles was found to be 3.5% while it was 41.6% in farms [19].

Culture-based methods are time-consuming as well as having insufficient effectiveness of decontaminating methods. On the other hand, it still holds the advantage of specificity for MAP detection [20]. Recently, molecular-based methods such as PCR has become important for the evaluation of MAP-infected animals and the products of animal origin instead of using culture-based methods as well as immuno-based diagnosis [21].

The objective of the study was to investigate the occurence of MAP in pooled fecal and bulk milk samples collected from the Thrace, which is considered to be the animal disease free area of Turkey by using *F57* Real time PCR and *IS900* nested-PCR assays.

MATERIAL and METHODS

Sample Collection

In this study, 2 dairy farms per village, a total of 30 dairy herds from 15 villages in Thrace were randomly selected. From October 2011 to December 2011, 270 fecal samples from 9 cattles over 2 years per a farm and 45 bulk milk samples from the dairy farms and the villages were collected *(Table 1)*. All samples were placed into sampling bags, and immediately transported to the laboratory in a container at 4°C for sample preparation.

Table1. *Sampling data*
Tablo 1. *Numune bilgileri*

Name of Village	No of Herds	Herd's Cattle Population	No of Animals > 2 years in herd	Distribution of Collected Samples		
				No of Fecal Samples	No of Bulk Milk Samples	No of Bulk Milk Samples from Bulk Milk
Bahçeköy	2	35	20 (57.1%)	18	2	1
Çamlıca	2	44	22 (50%)	18	2	1
Çobançeşme	2	70	34 (48.6%)	18	2	1
İzzetiye	2	34	18 (52.9%)	18	2	1
Karahisar	2	54	32 (59.3%)	18	2	1
Karasatı	3	102	53 (51.9%)	27	3	1
Karlıköy	2	51	28 (54.9%)	18	2	1
Kılıçköy	1	200	60 (30%)	9	1	1
Küçükdoğanca	1	24	11 (45.8%)	9	1	1
Lalacık	2	50	25 (50%)	18	2	1
Orhaniye	3	92	53 (57.6%)	27	3	1
Paşayiğit	2	38	22 (57.9%)	18	2	1
Pırnar	3	94	44 (46.8%)	27	3	1
Siğilli	1	22	10 (45.4%)	9	1	1
Türkmen	2	130	85 (65.4%)	18	2	1
Total	30	1040	517 (49.7%)	270	30	15

Number of Dairy Farms

Minimum number of the herds to be sampled was calculated as 30 [22]. The criteria for decision were selected to be an expected herd level-prevalence of 0.80 (EU prevalance of MAP in herds), a maximum acceptable error rate of 0.10, a probability of Type I error of 0.05 and Z statistic for a level of confidence of 1.96 were choosen, respectively.

Number of Animals

Minimum number of the animals to be samples was taken as 270 [22,23]. The criteria was decided to be an expected animal level prevalence proportion of 0.05, Z statistic for a level of confidence of 1.96, precision of 0.025 [23]. The average number of animals per dairy farm was directly obtained as 9.

Extraction Procedure of DNA from Pooled Fecal and Bulk Milk Samples

Approximately 2 g from each of 9 fecal samples per herd was put into a clean tube for pooling, i.e. a total of 18 g pooled mixture (9 randomly selected fecal samples/pool; 1 pooled sample/herd). It was homogenized for 5 min by vortexing (Daihan Scientific, South Korea). Then, 1.5 g of pooled feces was mixed with 5 ml of Roche S.T.A.R Buffer Solution in a 15 ml falcon tube followed by vortexing for 30 s (Daihan Scientific, South Korea). The tubes were allowed to stand at room conditions till a clear supernatant is observed. Following that 200 µL of this supernatant was transferred to a 2 ml Eppendorf tube, in which 200 µl of Roche Lysozyme was. Finally, it was incubated at 37°C for 1 h in an incubator (Binder, Germany). In a similar way, 10 ml of bulk milk sample were pippetted into a 15-ml falcon tube. It was centrifuged at 2.500 ×g for 30 min (Hettich, Germany). The pellets were resuspended in 200 µl of Roche Lysozyme, well-mixed by vortexing, following that incubated at 37°C for 1 h. DNA isolation was made according to GENESpin DNA Isolation Kit protocol (Eurofins GeneScan GmbH, Germany). Then, eluted DNA was kept at 4°C for direct use or at –20°C for further processing.

F57 Real-time PCR Application

The kit procedure of MAPsureEasy® (TransMIT GmbH, Germany) was followed. *F57* Real time PCR analysis was performed in a 96-well plate format on Agilent Stratagene Mx3000P Real-time PCR (Stratagene, USA). A 5 µl aliquot of DNA was mixed with 20 µl of Master Mix (12.5 µl of qPCR Master Mix, 1 µl of MAP Oligonucleotid Mix, 1 µl of IAC Solution and 5.5 µl of A. dest). HEX fluorescence was selected because its emission peaks do not extend over each other due existence of inhibitory impurities in feces matrix. FAM was used for IAC. Thermal processing parameters were adjusted as 1 cycle at 95°C for 15 s and 45 cycles at 60°C for 1 min. Reference strain ATCC 19698 as positive control, DNA of a non-MAP mycobacteria as negative control as well as a master mix blank control were included. Each measurement was performed in duplicate with *IS900* nested-PCR method by using primers and PCR conditions [24]. Threshold cycle (C_T) of the assay $C_t \leq 40$ was accepted to be positive in MAP [25].

Determination of Detection Probability

The detection limit of Real-time PCR assay was initially determined by analyzing serial dilutions including MAP reference strain ATCC 19698. Subsequently, the inoculum for spiking was prepared by inoculating 10 ml Middlebrook 7H9 broth (Difco Laboratories, Germany) containing 10% OADC supplement (Becton-Dickinson, Germany), 2 µg/mL mycobactin J (Allied Monitor, USA), 0.05% Tween 80 (Sigma-Aldrich, Germany) and 2.5% glycerol with a colony of the reference strains of MAP from a slant of Herrold's Egg Yolk Medium (BD HEYM, Germany). MAP reference strains were grown in a shaker incubator for 6-8 weeks at 37°C. Broth cultures were centrifuged at 2500 x *g* for 15 min and the pellets were re-suspended in phosphate buffered saline (PBS) by vortexing with a few sterile glass beads (VWR International, Germany) to yield a suspension containing approximately 10^7 MAP cfu/mL. An initial MAP concentration of about 10^7 cfu/mL was set using a photometer and a counting chamber. Serial dilutions prepared from the MAP stock solution ranged from 10^7 to 10^1 cfu/ml using PBS and 1 ml of each of the dilutions were serially added to 10 ml of raw milk and 10 g of feces homogenate. Positive controls used were "raw milk" and "feces" spiked with 10^7 cfu/ml MAP whereas negative controls as raw milk and feces were spiked with equivalent volume of sterile PBS, respectively. Serial dilutions for spiking were also confirmed by DNA extraction, and subsequently by *F57* Real time PCR as described above. All experiments were repeated in triplicate format.

RESULTS

This study assessed the occurrence of MAP in pooled fecal samples from the cattles over 2 years and the bulk milk samples from each of the collection tanks installed in the randomly selected dairy farms and the villages located in the Thrace Region of Turkey by using a *F57* Real time PCR and *IS900* nested-PCR assays. The results revealed that MAP genom could not be detected in any pooled fecal and milk samples

DISCUSSION

The Thrace, i.e. Turkey's European part and the adjoining parts of Southern Bulgaria and north-east Greece has a strategic importance as a vaccination buffer zone for Europe [26]. In literature, the studies related to understanding MAP prevalance in this region are significantly limited. MAP could not be detected in fecal samples of 2 years-old and/or older cattle from Thrace by PCR based on *IS900* [27].

In 2007, Turkey firstly reported Food and Mouth Disease in Thrace whereas there was no information of MAP in this reporting [28]. A low prevalance of MAP in animals from Thrace part of Greece was determined by *F57* Real time PCR and *IS900* n-PCR. But, in this study, no MAP could be detected in the collected fecal and bulk milk samples in a defined area of Thrace [29].

In MAP prevalence studies, sampling size is arranged by randomly selecting herds and animals from these herds as wells as considering sensitivity and specificity of the diagnostic test [30]. In this study, sampling size calculations were performed based on prevalences of both MAP-infected herd and MAP-infected cattle as 80% and 5% according to EU Data due to the lack of official data indicating MAP prevalance in Turkey. In this study, minimum numbers of the herds and the animals to be sampled were found as 30 and 270, respectively [22,23].

Estimation of the apparent prevalence of MAP in the dairy herds varies significantly among studies, depending on number of the herd, number of the animals to be tested and the method to be performed [31]. Sensitivity of detection for MAP was greater with a smaller pool size, i.e. 5 versus 10 samples per pool whereas 10 cows per pool was recommended by another study [32,33]. In this study, we performed 9 fecal samples per pool as well as the bulk milk samples from the holding tanks were already pooled naturally. Thus, our MAP negative results might likely be due to the dilutions in the pooled feces and the bulk milks. In this way, probability density for low prevalence herds and infected animals would not be distributed within the reference prevalence of MAP in EU as 80%.

The effective diagnosis of MAP is a challenge due to lack of the clinical signs from sub-clinically infected animals, the difficulties in primary isolation of this hardy bacillus, and possible unknown kinds of the MAP strains [34]. Real time PCR has significant advantages over other methods [20,35]. On the other hand, success of a Real time PCR is dependent on a well-performed DNA extraction from test matrix [25,36]. Thus, commercial kits for DNA extraction would detect MAP from feces with high sensitivity without cultivating bacteria [37]. Bead beating in a lysis solution for cell distruption as well as use of spin column technology can perform more effective DNA extraction especially from fecal samples [38]. In this study, DNA extraction was also conducted by commercial kit supported with lysis solution and spin column technology for the most accurate diagnosis of MAP.

There are the genomes highly homologous to other environmental *Mycobacterium* species. It highly affects the reliability of PCR application [39]. *F57* and *IS900* are MAP-specific genetic elements [40]. Insertion sequence *IS900* is a reference marker for confirmation of MAP whereas it may lead to cross-reactions and possible false positive results [24]. Due to this fact, another alternative genetic element *F57* is used [41]. In contrast to *IS900*, *F57* is not similar to genes on other related organisms [42]. But, *F57* does not provide for as high a sensitivity as *IS900* element with less false positive results [43]. DNA extraction followed by Real-time PCR is a sensitive method making possible a detection limit of MAP like such as log 2.0 cfu/mL or g of raw milk and feces within one day only [44]. In this study, *F57* Real time PCR assay was very reliable because it was tested for specificity by including an internal amplification control (IAC) to exclude false-negative PCRs for smaller *MAP* DNA amounts. In addition to that, *IS900* n-PCR was applied despite of being time-consuming with high risks of cross-contamination [45]. Thus, any other available PCR system was not able to detect any MAP DNA [46].

Diagnostic strategies to detect MAP super-shedder cows in dairy herds have been minimally studied [47]. Similar to our study based on Real time PCR, MAP was determined at a level of 10^4 cfu/g of spiked feces, 1-10 cfu/ml of milk, 2.42×10^1 MAP cells in 1 ml of artificially contaminated raw milk, and 10^2 cfu/g of feces and 10^2 cfu/10 ml of bulk milk, respectively [42,48-50]. Hence, in our study we concluded that number of MAP cells in samples might be lower than detection levels of the assays used.

In this study, MAP negative results might be arised from some limitations. These limitations would be lack of information for MAP prevalance in Turkey, no clinical signs on sampled animals over 2 years, MAP cells in smaller amounts, lower sensitivity of *F57*-Real time PCR assay, dilution effect of pooling, and lack of culture-based method.

In conclusion, screening of MAP should be extended to cover the whole region by increasing sampling size of herds, animals, fecals and bulk milk samples in parallel to including the culture based microbiological method together with molecular-based methods for an effective investigation of MAP throughout this animal disease free area of Turkey in Europe.

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