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Research Article

Assessment of the Use of PCR as an Early Diagnostic Indicator of Bovine Tuberculosis in Dairy Farms

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ABSTRACT

Background: Zoonotic tuberculosis has been mainly associated with the consumption of bovine dairy products and its control has been prioritized by the Food and Agriculture Organisation.

Objective: As such, the aim of this study is to assess whether the polymerase chain reaction (PCR) can be used as an early diagnostic indicator of Mycobacterium bovis infection in bovine dairy farms.

Methods: Milk samples (n=78) were collected from all the animals older than 8 years of age, in all (n=4) bovine dairy farms located in a specific agricultural region of Greece, with high prevalence in bovine tuberculosis. Detection of DNA belonging to slow growing members of the genus Mycobacterium was conducted in pooled samples using two PCR assays targeting 16S-rRNA and 65-kDa heat shock protein (*hsp65*). Randomly selected PCR products were submitted to sequence analysis for confirmation of the specificity of the amplification process. DNA isolation and PCR testing were conducted in compliance with ISO17025 accreditation requirements.

Results: The overall percentage of positivity was 47.7%, and ranged among farms from 0% to 76.9%. PCR-positive animals were detected in both farms that were at the time of investigation positive with the tuberculin skin test (TST), whereas the only farm with a negative TST record tested also negatively with PCR. Interestingly, one farm that was negative with TST since 2012 but had a long prior record of high level TST-positivity, tested positively with PCR.

Conclusion: In conclusion it can be stated that PCR can be used for the detection of mycobacteria in pooled samples of milk collected from the older animals of a dairy farm, as an early and sensitive diagnostic indicator. This can support TST monitoring for the control of bovine tuberculosis, and improve detection of farms, in which routine monitoring should be intensified. The specific approach offers significant practical benefits that compensate for the additional cost of PCR.

Keywords: Bovine tuberculosis; Zoonotic tuberculosis; Control of tuberculosis; Test-and-removal; Tuberculin skin test; Polymerase chain reaction; Early diagnostic indicator.

INTRODUCTION

Bovine tuberculosis is caused by Mycobacterium bovis, a member of Mycobacterium tuberculosis complex, which affects many animal species and the human [1,2]. In most cases, human infection is associated with the consumption of dairy products prepared with milk from infected animals, but can also result from direct contact with them [2-4]. In developed countries, the measures applied to control bovine tuberculosis have resulted in considerable decrease in the prevalence of Mycobacterium bovis infections in humans, which are currently reported to correspond to only 0.5-7.2% of all culture-positive cases of tuberculosis [2]. In developing countries however, the control of zoonotic tuberculosis is not equally effective, hence it was prioritized by the Food and Agriculture Organisation with recommendations for holistic measures covering humans, animals, and the environment [5].

In most developed countries the control of bovine tuberculosis relies mainly on test-and-removal of animals that react positively

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to the tuberculin skin test (TST). In Greece, the specific strategy has been applied since 1977. Today, all bovines older than 6 weeks are tested annually with the TST, and reactors are culled. Farms are categorized in levels of health status, and stock movement is restricted so that contact of potentially affected animals with healthy is avoided. The records of disease monitoring document significant improvement in the control of bovine tuberculosis at national level but indicate the existence of high-prevalence areas within the country, one of which is the West Attica prefecture. For the specific region, the prevalence of bovine tuberculosis in year 2016 at farm level was 17.39%, almost six times higher than that which corresponds to the entire country (3.57%); It is noteworthy that in year 2013, the relevant percentages were 30.43% and 4.24%, respectively (data from the national database of the Ministry of Rural Development and Food).

The significantly higher prevalence of bovine tuberculosis in specific geographic areas could be associated with the Mycobacterium bovis infection being enzootic in the local wild animal population, which makes disease control challenging [6,7]. In addressing this challenge at farm level, one approach would be to assess measures that could be applied to improve the effectiveness of test-and-removal. Within this context, this investigation was conducted in order to assess whether the polymerase chain reaction (PCR) could be used as an early diagnostic indicator of Mycobacterium bovis infection in bovine dairy farms that are routinely monitored with the TST.

MATERIALS AND METHODS

Sample collection

This investigation was focused at the dairy farms of an agricultural region of the West Attika prefecture, Greece, with a long record of bovine tuberculosis. The total animal stock of all bovine dairy farms (n-4, Farms A-D) located in the specific region was 536 animals. Information regarding the year of farm establishment, the number and the age of the animals, and the TST farm record was collected on site through personal interview. Based on this information, samples of milk (n=78, 1 sample/animal, sample volume: 20 ml) were collected aseptically between July and August 2018 from all animals of the targeted farms, older than 8 years of age (Table 1).

After collection, samples were stored in isothermal containers on ice, and were transported to the laboratory within less than 2 h. Upon arrival, the samples were divided in two portions, one of which was stored at -80 °C, and the other was mixed with the respective portion of another sample of the same farm. Thus each pooled sample consisted of two portions, or of three, when the number of samples collected in a farm was odd (Table 1). The pooled samples were stored at 4-6°C until they were processed for DNA isolation and PCR for the detection of Mycobacterium sp.

DNA isolation

Milk samples (n=78) were processed for DNA isolation using a commercially available kit, according to the instructions provided by the manufacturer (Nucleospin® Tissue, Macheray-Nagel GmbH & Co. KG, Germany).

The quality of the DNA product was assessed for purity and integrity by submerged gel electrophoresis followed by image analysis using a Bio-Rad ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories Inc., U.S.A.), and by optical density count at 260/280 nm, using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc., U.S.A.). PCR inhibition was assessed using an assay targeting the β-actin gene [8]. Fragmented DNA products were discarded, and DNA isolation was repeated from the original sample. For those samples that PCR targeting β-actin did not produce a positive result, the assessment for the detection of PCR inhibitors was repeated after dilution of the DNA solution in ddH2O (1:5 v/v).

Processing of milk samples for DNA isolation was conducted in parallel with positive and negative controls (DNA controls), consisting of 200 μl autoclaved Mycobacterium sp. liquid culture (DNA C+), and 200 μl ddH2O (DNA C-), respectively. The number of DNA controls corresponded to 10% of the samples processed per batch.

DNA isolation and PCR testing were conducted in compliance with ISO17025 accreditation requirements.

Polymerase chain reaction

Detection of DNA belonging to slow growing members of the genus Mycobacterium was conducted using two PCR assays targeting the genomic regions 16S-rRNA [9], and 65-kDa heat shock protein (*hsp65*) [10] (Table 2).

Farm	Farm and TST-record	Sample number collected per farm	Sample number tested per farm	PCR		
				16Sr-RNA	hsp65	Result
A	Established in year 1975, Total animal stock: 100, TST-record: Negative since 2012	23	11	5 of 11	3 of 11	5 of 11
				45.50%	27.30%	45.50%
B	Established in year 2006, Total animal stock: 210, TST-record: Positive since 2016	27	13	9 of 13	7 of 13	10 of 13
				69.20%	53.80%	76.90%
C	Established in year 2006, Total animal stock: 26, TST-record: Negative (always)	10	5	0 of 5	0 of 5	0 of 5
				0%	0%	0%
D	Established in year 2004, Total animal stock: 200, TST-record: Positive since 2014	18	9	3 of 9	2 of 9	3 of 9
				33.30%	22.20%	33.30%
Total						
4		78	38	17	12	18
				44.7 ± 5.8%	31.6 ± 4.8%	47.4 ± 5.9%

Table 1: The information and the samples that were collected from Farms A-D and the outcome of their analysis using PCR for the detection of Mycobacterium sp.

Primer code name	Oligonucleotide sequence (5'-3')	Target region	Size*	Reference
MycGenF	AGAGTTTGATCCTGGCTCAG	16S rRNA	1500	Rogall et al., [9]
MycGenR	TGCACACAGGCCACAAGGGA			
HspF	ACCAACGATGGTGTGTCCAT	hsp65	439	Telenti et al., [10]
HspR	CTTGTCGAACCGCATACCCT			

*Size of the of amplification product in base pairs

Table 2: The oligonucleotide primers, the target regions, and the size of the amplification product of the PCR assays incorporated to the detection of DNA belonging to Mycobacterium sp., in samples of milk.

All batches of samples were tested in parallel with positive and negative controls (PCR controls), consisting of DNA solutions that were previously confirmed as PCR-negative (PCR C-), and positive (PCR C+) for Mycobacterium sp., respectively. Positive controls were in all cases bovine isolates of Mycobacterium bovis. As for DNA isolation, the number of PCR controls corresponded to approximately 10% of the samples tested per batch.

Randomly selected PCR products, which corresponded approximately to 50% of the PCR-positive samples, were submitted to sequence analysis for confirmation of the specificity of the amplification process. Sequence analysis was performed on both strands using the BigDye® Terminator Cycle Sequencing Kit and PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, California, USA). Results were analyzed and compared to deposited sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

The outcome of the PCR analysis that was conducted in the milk samples collected from dairy cows for the detection of DNA belonging to Mycobacterium sp., is recorded in Table 1. The overall percentage of positivity was 47.4% (18 of 38); among farms, positivity ranged from 0% (Farm C) to 76.9% (Farm B). The outcome of the sequence analysis of the PCR amplification products was confirmatory in connection with all the positive results submitted to this analysis that these corresponded to members of the genus Mycobacterium (100% alignment query cover and percentage of identity), (BLAST). In more detail, the proportion of mycobacterial species and strains detected at the specific levels of alignment query cover and percentage of identity were the following: Mycobacterium bovis (strain SP38, 30, 1595, BCG-1 Russia), (21.1%); Mycobacterium tuberculosis variant bovis strain 2002/0476 chromosome (5.3%), and Mycobacterium tuberculosis variant bovis BCG strain [strain 26, 3281, ATCC 35743, Korea 1168P, Russia 368, Mexico, Moreau RDJ, Tokyo 172 DNA, Pasteur 1173P2, Tokyo 172 substrain TRCS, (52.6%), and BCG-S48, BCG_S49 chromosome (10.5%)].

Comparing the level of positivity with regards to each of the two PCR assays that were incorporated in this study, indicates higher proportions (44.7 ± 15.8%, 17 of the 38) for that which is targeted to 16S-rRNA [9] compared to the one targeting *hsp65* (31.6 ± 14.8%, 12 of the 38) [10] in connection with the three farms for which positive results were recorded (Farms A, B, and D), though the difference is not statistically significant (p=0.12).

The TST has been used for decades for the control of tuberculosis and is recognized as an efficient means of routine monitoring that is easily applicable even in resource-poor settings [11,12]. In many countries, the positive results recorded with the TST have to be confirmed with specific laboratory tests. These are selected based on the requirements of the diagnostic approach, and may include, serology, Ziehl-Neelsen staining of tissue sections or smears of aspirates, in vitro isolation, and PCR. The latter was used in this study because the interpretation of PCR results is not influenced by acquired immunity and the analysis can be conducted within only a few hours, as opposed to cultivation that requires incubation of selective media for several weeks [11,13]. The issue of acquired immunity, and more specifically, the diagnostic problem associated with the seroconversion of animals previously exposed to mycobacterial pathogens, should be considered expected in high-prevalence areas. This problem could complicate the interpretation of serology tests even due to mycobacterial species that are widely spread in the environment, especially if the relevant antibody titres are not monitored regularly at herd level [11,14,15]. In this regard it is worth noting that Greece is enzootic for paratuberculosis of ruminants, which is caused by Mycobacterium avium subspecies paratuberculosis, a pathogen recognized as a source of false-positive TST results [16,17].

Regarding the structure of the experimental plan it should be noted that this investigation relied on two well-documented PCR assays that were designed for the detection of slow growing mycobacteria [9,10]. The diagnostic overlap of the selected assays was used as a means of increasing the reliability and the sensitivity of the detection methodology, given that the genomic regions targeted by them are different. The latter was of course recognized also as a potential source of discrepancy in the result recorded with each of the two PCR assays, which was confirmed by the outcome of this investigation. In more detail, both assays incorporated in this study produced a positive result in 61.1 ± 22.5% (11 of the 18) of the samples that reacted positively with either of them. The relevant percentages recorded for Farms A, B, and D were 60% (3 of 5), 60% (6 of 10), and 66.7% (2 of 3), respectively. The percentage of samples that reacted negatively with both PCR assays was 73.1 ± 17.0% (19 of 26) of those for which a negative result was recorded with either of the tests employed, whereas the relevant percentages for Farms A, B, C, and D were 75% (6 of 8), 33.3% (2 of 6), 100% (5 of 5), and 85.7% (6 of 7), respectively (Table 1).

Among the farms included in this investigation, the comparatively highest level of positivity was recorded in Farm B (76.9%), whereas the lowest in Farm C (0%). It must be noted that the high level of positivity that was recorded in all positive farms is associated with the diagnostic approach that was followed, and more specifically with the age of the animals tested and the strategy of sample analysis. In connection with the former, the fact that sample collection was conducted specifically from all animals of the most advanced

age in each farm increases for obvious reasons the likelihood of positivity. The analysis of pooled samples instead of individual is a factor also contributing to overestimation of true positivity, for two reasons. The first is that a pooled sample of two portions only one of which is negative would react positively, whereas for a pooled sample to react negatively, both its constituting portions would have to be negative. The second reason is the minimum detection limit (MDL) of PCR relative to the amount of mycobacterial DNA present in the tested samples, since those containing the targeted analyte at levels below the method's MDL would react negatively if tested individually but possibly positively, if pooled [18-20].

The analysis of pooled milk samples collected from the older animals is of course inappropriate for the assessment of true positivity at farm level. However this approach was selected as an early diagnostic indicator aiming to improving detection of PCR reactors, hence of those farms, in which routine monitoring for tuberculosis should be intensified. The result of this investigation assessed in connection with the TST-record of the tested farms indicates the suitability of the proposed approach. In more detail, PCR-positive animals were detected in both farms that were at the time of investigation TST-positive, i.e., Farms B and D, whereas the only farm with a negative TST record, i.e., Farm C, was also negative with PCR. Interestingly, Farm A that was negative with TST since 2012 but had a long prior record of high level TST-positivity, tested positively with PCR, which indicates that the latter constitutes a diagnostic indicator that, at least for the specific farm, proved more sensitive compared to TST. In this respect the proposed approach is consistent with a control program that would be more efficient in detecting TST-negative shedders of mycobacteria, which is a critical element of effective disease control, since the specific animals contribute significantly in maintaining the infection within the farm.

Though admittedly this study was not structured in order to determine risk factors associated with PCR-positivity at herd level, the outcome of the investigation that was conducted indicates that PCR-positivity is associated with TST-record but not with the farm's current TST-status. In terms of the applicability of the proposed approach it is perhaps worth noting that routine collection of milk samples can be conducted more easily compared to TST, since it is not invasive and it minimizes exposure of the operator to bio-safety hazards, such as affected animals or PPD. Testing pooled samples, instead of individual, decreases the overall cost of monitoring substantially, which is consistent with the financial constrains that constitute a common problem in the application of programs of routine testing. In connection with this it is noteworthy that the overall cost of the proposed PCR analysis is less than 6 euro/sample, whereas the time required for testing 40 samples with one set of devices (thermo cycler and electrophoresis tray) is approximately 2h. Furthermore, collection of milk samples could be conducted at the local milk industries, which simplifies further the procedure of sample collection and decreases the cost of personnel and sample transportation, since, as opposed to farms, milk industries are usually located in easily accessible areas.

CONCLUSION

In conclusion it can be stated that PCR can be used for the detection of mycobacteria in pooled samples of milk collected from the older animals of a farm, as an early and sensitive diagnostic indicator. This can support TST monitoring for the control of bovine tuberculosis, and improve detection of farms, in which routine monitoring should be intensified. The specific approach offers significant practical benefits that compensate for the additional cost of PCR.

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