Macedonian Journal of Animal Science, Vol. **2**, No. 1, pp. 63–66 (2012)
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In print ISSN 1857 – 6907
Received: July 20, 2009
On line ISSN 1857 – 7709
Accepted: November 15. 2009
UDC: 637.1.055 : 579.873.2
Original scientific paper

MYCOBACTERIUM AVIUM SUBSPECIAE *PARATUBERCULOSIS* IDENTIFICATION IN MILK BY MEANS OF IS900 PCR

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Between the numerous emerging pathogens, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) reports a primary interest in its detection for its possible correlation with Crohn's Disease in man (5). Milk and its subproducts may be very important in transmitting *Mycobacterium avium* subsp. *paratuberculosis*. The potential sanitary risk is related to the germ capacity in surviving to routine pasteurization treatments (1). These study objectives were the determination and the optimization of DNA extraction and purification assays to the direct *Mycobacterium avium* subsp. *paratuberculosis* (MAP) detection in milk, by means of PCR. The obtained results with the applied method are based in the milk and its subproducts chain inspective and control actions, relating to the increasing paratuberculosis etiological agent presence and the potential zoonotic transmitting.

Key words: *Mycobacterium avium* subsp. *paratuberculosis* (MAP); milk; PCR; SPF (Specific Pathogen Free)

ИДЕНТИФИКАЦИЈА НА *MYCOBACTERIUM AVIUM* SUBSPECIAE *PARATUBERCULOSIS* ВО МЛЕКОТО СО ПОМОШ НА IS900 PCR

Меѓу бројните откриени патогени, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) има примарно значење за неговата детекција поради неговата можна поврзаност со Кроновата болест кај луѓето. Млекото и млечните производи можат да бидат многу важни во пренесување на *Mycobacterium avium* subsp. *paratuberculosis.* Потенцијалниот здравствен ризик е поврзан со способноста на оваа бактерија за преживување на рутинските третмани на пастеризација. Предмет на оваа студија е преку екстракцијата и прочистувањето на DNA со помош на PCR директно да се открие *Mycobacterium avium* subsp. *paratuberculosis* (MAP) во млекото. Резултатите добиени со применетиот метод се базирани на акциите на инспекција и контрола на млекото и млечните производи, поврзано со зголеменото присуство на паратуберкулозниот етиолошки агенс и потенцијалната зоонотска трансмисија.

Клучни зборови: *Mycobacterium avium* subsp. *paratuberculsis* (MAP); млеко; PCR, SPF (Specific Pathogen Free)

INTRODUCTION

Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic granulomatosis infection of the intestinal tract of wild and domestic ruminants. The symptoms of this disease include diarrhea, reduced milk production, emaciation, and ultimately death in infected animals, and infections result in significant economic losses for individual farms and the dairy industry, worldwide.

A traditional "gold standard" testing method for Johne's disease (MAP infection) is the fecal culture. But this method is time-consuming, requires a long incubation period of 8 to more than 16 weeks for bacterial recovery. Because of these difficulties, several PCR tests have been developed to detect MAP [4, 8]. Although these assays offer the benefits of sensitivity and speed, they cannot distinguish between viable and nonviable MAP cells. Clinical similarities have been observed between Johne's disease in cows and Crohn's disease in humans, and both viable MAP and MAP genetic material have been found in some patients diagnosed with Crohn's disease [4, 5, 10]. However, no cause and effect relationship between MAP and Crohn's disease has been defined and no documentation proving zoonotic transmission from a cow to a human has been recorded [15]. If there is a causal relationship between MAP and Crohn's disease, investigations into possible vectors through which MAP is spread should provide useful information. Cattle infected with MAP can shed live organisms in their milk [13]. Some researchers have demonstrated that MAP is not able to survive commercial pasteurization [13] or have been critical of the differing pasteurization methodologies used [10], whereas other researchers have found that this organism can survive pasteurization under conditions simulating those used in commercial facilities [2, 5].

MATERIALS AND METHODS

Sample preparation. – This method optimizing consists in *SPF* (*Specific Pathogen Free*) milk used originating from Paratuberculosis free farms, which previously have been surveyed by periodic ELISA tests and cultural examinations. Logarithmic dilutions of the *Mycobacterium avium* subsp. *paratuberculosis* strain were performed starting on a solution containing 10^5 mycobacterium/ml of each diluted sample an equal amount (500 µl) was used for the 4.5 ml *SPF* full milk experimental contamination.

DNA extraction and purification. – DNA extraction and purification was realized switching up to the Dneasy Tissue Kit (QIAGEN, Hilden, Germany). The indicated protocol from the producer company was modified at the amount of the starting sample, buffers time and the rinses number. The milk amount (5 ml) experimentally contaminated was mixed with 2.5 ml Lysis Buffer ATL and with 250 µl K Proteinase (20 mg/ml). After the overnight incubation at 56°C in shaking conditions, were proceeded adding 2.5 ml Buffer AL and then passing to another thermal treatment at 71°C for 1 h. The mixed product, with 2.5 ml absolute ethanol, was passed to QIAamp spin column in various centrifugation cycles at 13000 rounds per 1 min. The absorbed DNA in silica gel QIAamp membrane, was submitted to Buffer AW_1 and Buffer AW_2 washes. At last, the DNA was diluted with 80 µl Buffer AE.

IS900-PCR. – Extracted DNA amplification was evaluated by means of PCR, performed on a final volume of 25 µl, using 12,5 µl HotStarTaq Master Mix 2X (QIAGEN, Hilden, Germany) and the content of 0.125 µM Primer oligonucleotids, as described by [11]. The amplification program predicted an initial denaturizing at 95°C for 15 min, followed by 35 denaturizing cycles at 94°C for 30 sec, annealing at 54°C for 30 sec and by extension at 72°C for 45 sec. The reaction was realized on a Mastercycler personal (Eppendorf, Milano, Italia). Amplifications specificity was insured by the enzymatic dissolution using *Mse*I [9]. Amplification products were analyzed by means of electrophoresis on agarose gel and were visible by ethid bromurium coloring.

RESULTS AND CONCLUSIONS

The individualized nucleic acids extraction and the purification method, based on the use of K proteinase and silicium filter tubes, randomly applied for full fat milk analyzing has insured the complete sample dissolution and has demonstrated an easy execution. This system also brought out amplified DNA, ensuring so the vantage of an appropriate target molecules number and of the inhibitors removal. In fact, PCR-IS900 used for the verification of the extracted and purified DNA amplification based on experimentally contaminated milk portions with logarithmic dilutions of *M. avium* subsp. *paratuberculosis,* evidenced a limit of the revelation (LOD) equal to 10 organisms for ml. The analysis revealed the expected results as previously described by Khare et al. [9] (2004). The described extracting procedure is demonstrated as widely applicable for the *Mycobacterium avium* subsp. *paratuberculosis* identification in the unelaborated milk by means of PCR. Comparing to the immune – magnetic division, widely used for the *Mycobacterium avium* subsp. *paratuberculosis* detection, in various types of food samples [9, 7, 14], the above described procedure results less costly and laborious. This method also consists in short times performances, an aspect not to be unconsidered for its importance on the experimental definition of the analytical control strategies. The obtained results with the proposed method are in concordance with the inspective action and with the milk chain control and its subproducts, as the interests to face the paratuberculosis (PTB) etiological agent are increasing, and as the zoonotic potential still unknown can be detected emergently.

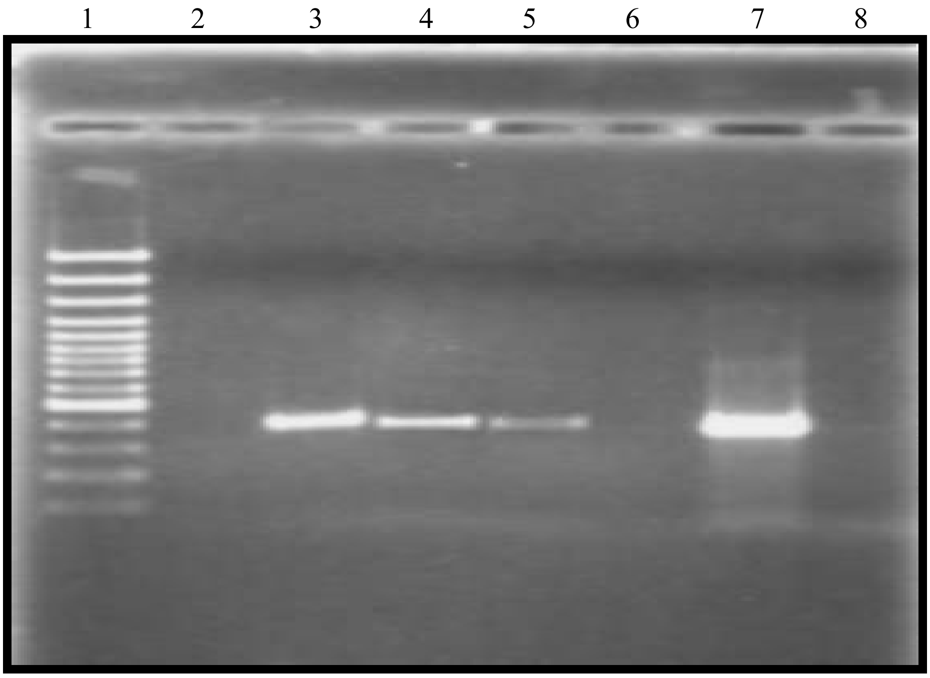

Fig. 1. Electrophoretic profile obtained by experimentally contaminated milk with logarithmic dilutions with *Mycobacterium avium* subsp. *paratuberculosis* (400 bp).

Lane 1: 100 pb DNA Ladder;
Lane 2: negative control sample (DNA *Mycobacterium avium* subsp. *avium*);
Lane 3: 103 mycobacteriums/ml;
Lane 4: 102 mycobacteriums/ml;
Lane 5: 10 mycobacteriums/ml;
Lane 6: <10 mycobacteriums/ml;
Lane 7: positive control sample (*Mycobacterium avium* subsp. *paratuberculosis*);
Lane 8: negative reagent (no ADN)

Acknowledgments. The authors thank Prof. Dr. M. G Tantillo. and Dr. Novello L. (Dipartimento di Sanita e Benessere degli animali, Facolta di Medicina Veterinaria, Universita di Bari, Italy) for their great support on this study.

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