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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)

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 AW1 and Buffer AW2 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 MseI [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.

**REFERENCES**


