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The Use of IS*900* PCR and ELISA for the Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Raw Cow Milk in Egypt

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Abstract: *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease in cattle and it has been suggested that this organism may be associated with Crohn's disease in humans. Diseased cows shed this organism into both milk and feces. The objective of this study was to use IS*900* PCR and ELISA kit to detect MAP in raw cow milk in Egypt. IS*900* PCR revealed that 9 milk samples out of 13(69.2%) raw cow milk samples collected from a dairy farm at El Monefeia governorate,Egypt, were positive to IS*900* PCR. Results indicated that animals have shed the *Map* in their milk or the milk had been contaminated with the feaces of the diseased animal, which can play as a source of infection to other animals and humans if this milk was drunk. ELISA test was also applied on these collected milk samples and it was found that all samples were negative to ELISA. These results reflected that these clinically diseased animals were shedding the MAP in milk without developing antibodies in their sera which mainly appear in the late stage of the disease. Egyptian national programme is needed to decrease the risk of exposure to MAP in both humans and animals.

Key words:*Mycobacterium avium* subsp p*aratuberculosis* · MAP · IS*900* PCR · ELISA · Raw cow milk · Crohn's disease · Johne's disease

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP), the recognized pathogen of Johne's disease (JD), causes chronic granulomatous enteritis in cattle, sheep and other ruminants [1, 2] and results in significant economical loss to the dairy industry [3]. It is also a suspected pathogen of Crohn's disease in humans [4]. This pathogen has been cultured from cows with clinical or subclinical JD in both their milk and feces [5, 6]. Confirmed MAP isolates were cultured from 1.8% of the commercially pasteurized milk samples in the U.K [7]. Similar data were published from the U.S.A. [8]. In the U.K. study, 11.8% of samples of retail milk were MAP-positive by PCR. In Switzerland, 19.7% of bulk-tank milk samples were IS*900* PCR positive [9]. Goat and sheep bulk tank milk samples were also PCR-positive for IS*900* (23.0 and 23.8%, respectively), providing presumptive evidence for the presence of MAP in Switzerland [10]. MAP has been cultivated from cheese [11, 12] as well. Unfortunately, the culture methods require 6 to 16 wk confirming that a sample is negative for MAP [13]; therefore, a rapid and sensitive protocol for detection of MAP is important for development of a JD control program. Efforts have been made in the last few decades to develop protocols for the detection of MAP in feces, milk, tissue, food and environmental samples using various methods. Serology and fecal culture, however, are the most commonly used tests in the field [13, 14]. Polymerase chain reaction (PCR) is an ideal method for rapid and accurate detection [15]. Progress has been made recently to improve the sensitivity of PCR-based tests for MAP in milk [16 - 18].The aim of this study was to detect MAP in Egypt using IS*900* PCR and ELISA.

MATERIALS AND METHODS

Samples: Only 13 raw cow milk samples from clinically diseased animals were collected from a dairy farm from El –Monefia, Egypt and were tested for the presence of MAP using PARACHEK®2 kit ELISA product no.633271 and IS*900* PCR.

ELISA Testing: (PARACHEK®2 kit product no.633271). All reagents were equilibrated except the conjugate. Milk samples were diluted 1:2 in green diluents and mixed thoroughly 3-5 times. Samples were covered and incubated at room temperature (22°C±3) for 30 minutes.

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Only 100 µl of the test and control samples were added to appropriate wells of the coated plate and the plate was shaken. The plate was covered with a parafilm and incubated at room temperature for 30 minutes. Conjugate 100X concentrate have been diluted to be ready for use at the end of this incubation. The plate was washed 6 times with wash buffer by using a plate washer with a suitable washing program. Only 100 µl of freshly prepared conjugate solution were added to each well. Each plate was covered with a parafilm and incubated at room temperature for 30 minutes. The plate was then washed. Only 100 µl of enzyme substrate was added to each well. The plate was covered with a parafilm and incubated for 30 minutes. Only 50 µl of stop solution was added and the results were read at 450 nm filter after 2-20 minutes from the incubation time (End Point Method).

Calculation of cut off value =Mean Negative Control + 0.150 (for Bovine Samples).

DNA extraction, IS*900* PCR and electrophoresis [19]: DNA pellets were extracted [20]. The extracted DNA samples were applied to IS*900* PCR according the following program: 1 cycle at 94 C, 10 min; 50 cycles at 94°C, 59 sec, 60°C, 30 sec and 72°C, 59 sec; followed by a final extension cycle at 72°C, 10 min.The following highly sensitive IS*900* primers were used.

Forward: 5'-CCGCTAATTGAGAGATGCGATTGG-3'.
Reverse: 5'-AATCAACTCCAGCAGCGCGGCCTCG-3'.

The electrophoresis grade Agarose was prepared in 1x electrophoresis buffer to reach the required 1.5% concentration. The Agarose was cooked in a microwave with agitation till being clear. The Agarose was allowed to cool, then 0.5 µg/ml ethidium bromide was added. The Agarose was poured in the electrophoresis mould to make 4 mm depth. The comb was inserted and left to solidify. The comb was removed gently. The TAE buffer was poured until covering the gel. The sample was injected with loading dye and sunk in the well. The cathode and anode were matched with power supply at 100 volt. The current was stopped when the loading dye reached 2:3 to the gel. The transilluminator was used to detect the desired 229 bp band.

RESULTS

IS*900* PCR Results: Nine milk samples out of 13 were positive to IS*900* PCR(69.2%) and gave a 229 bp molecular size band confirming the presence of IS*900* sequence specific for *Mycobacterium avium subsp. paratuberculosis* as shown in Fig. 1.

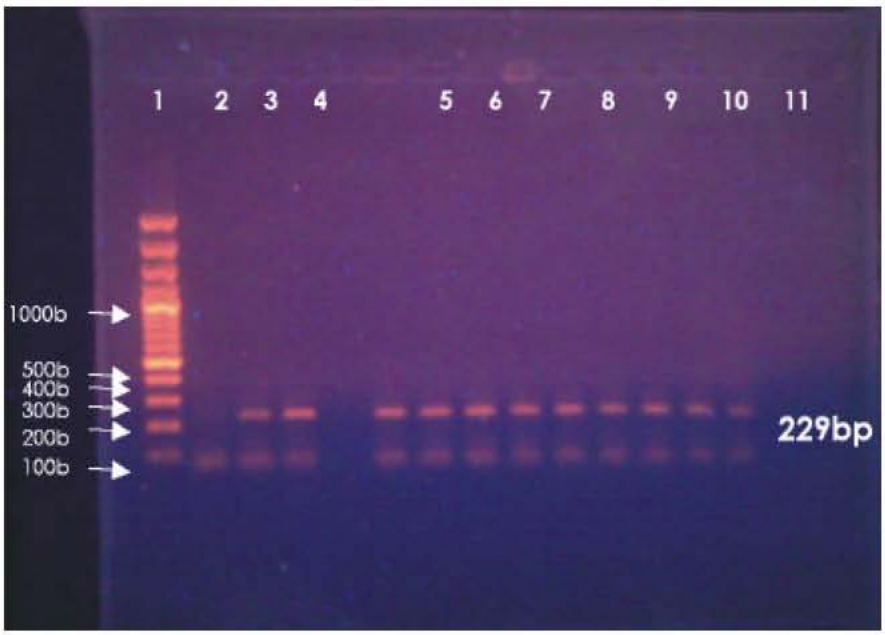

Fig. 1: IS*900* PCR application on 13 raw cow milk samples targeting 229 bp amplicon
Lane 1 corresponds to 100 bp DNA ladder.
Lane 2 corresponds to spiked milk with ***BCG*** strain as negative control.
Lanes 3 and 4 correspond to spiked milk cream and whey with standard strain of ***Map*** as positive control.
Lanes 5-13 correspond to positive IS*900* PCR milk samples.

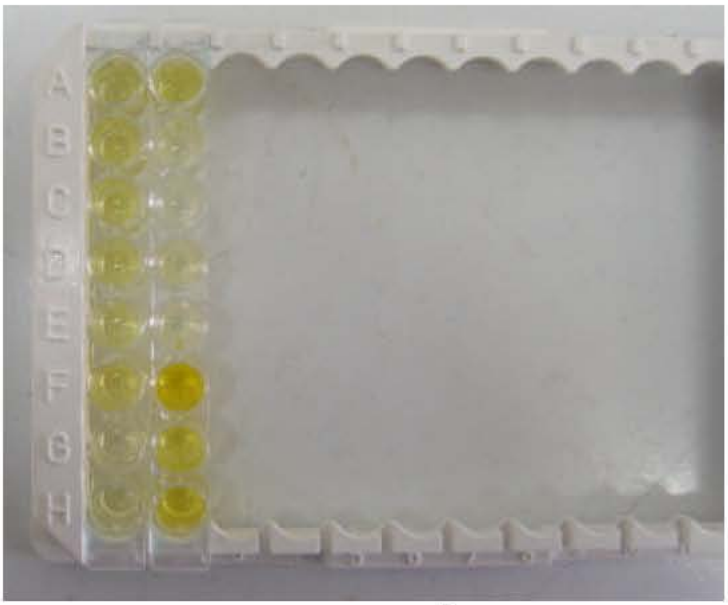

Fig. 2: ELISA test on raw cow milk samples using PARACHEK®2 kit for 13 cow milk samples respectively
Strip 1. Raw cow milk samples from 1-8.
Strip 2. From well A to well E negative raw cow milk samples from 9-13.
Strip 2. Well F positive control.
Strip 2. Wells G and H negative controls.

ELISA Results: All raw cow milk samples were negative to the presence of *Map* antibodies as shown in Fig. 2.

DISCUSSION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a recognized pathogen that affects many species of ruminant and non-ruminant animals [19]. It is the etiological agent of Johne's disease (JD) in cattle. Symptoms are progressive weight loss and chronic diarrhea associated with granulomatous enteritis. Subclinical infection of cows results in reduced milk production and fertility and signifies a considerable economic loss for the global cattle industry [21]. Infected animals with clinical disease and subclinical infections shed MAP bacteria in both milk and feces. Detectable levels of this organism have been found in milk from both clinically infected cattle and asymptomatic carriers [5, 22]. Live MAP organisms were recovered from some samples of retail pasteurized milk and several studies reported survival after exposure to high temperature short time pasteurization [7, 23].

Crohn's disease is an inflammatory gastrointestinal tract disease in humans, presenting with similar symptoms and pathological changes in the gut as Johne's disease in cattle. Therefore, it was suggested that MAP could be one of the etiologic factors of the disease [24, 25]. MAP is possibly passed on to humans through contaminated milk and dairy products although shedding levels appear to be low, especially in subclinical cases (2-8 cfu/50 ml), [26]. *Mycobacterium avium* subsp. *paratuberculosis* has been cultured from the breast milk of patients with active CD [27]. Although the role of this organism in CD is unclear, cow milk can be a source of human exposure to the organism [28]. Due to the possible link between M. avium subsp. paratuberculosis and the development of Crohn's disease in humans [29, 30] M. avium subsp. paratuberculosis has become of interest as a public health issue.

Both efforts to manage Johne's disease and studies of heat resistance of *M. avium subsp. paratuberculosis* have been hampered by the lack of rapid, specific detection tests for viable *M. avium subsp. paratuberculosis* cells. An ideal *M. avium subsp. paratuberculosis* detection test would be low cost, rapid and specific and provide live/dead differentiation. Culture is currently regarded as the definitive method for the detection of viable *M. avium subsp. paratuberculosis* bacteria; however, *M. avium subsp. paratuberculosis* is an extremely fastidious organism and requires the longest incubation periods of all the mycobacteria cultured to date (6 to 16 weeks). Additionally, harsh

chemical decontamination of samples is required to suppress growth of competitive microorganisms, which can reduce the sensitivity of culture to detect *M. avium subsp. paratuberculosis* [31].

Various mycobacterial species contain a number of highly specific insertion sequences (IS) integrated in the genome [32]. Some of those genetic elements can be used to distinguish species of mycobacteria [4, 33].

The specific insertion sequence of MAP is the IS*900* element [34, 35]. Raw milk may be a potential vehicle for the transmission of MAP to human population. Sweeney *et al.* [6] found, that MAP is shed into milk by 12% of subclinically infected cows in a concentration of 2 to 8 CFU per 50 ml milk. Moreover, results of laboratory pasteurization tests of whole milk spiked with MAP showed that MAP was capable of surviving pasteurization of raw milk [36,37]. Nevertheless, literature data are very divergent. Sung *et al.* [38] reported a D-value (decimal reduction time) for MAP in milk of 11 sec at 71°C, so there is a possibility for MAP to survive HTST (high temperature short time) pasteurization if the initial number is >101 cfu ml-1 milk. In a study of whole-pasteurized cows milk obtained from retail outlets in England 7% of these samples tested MAP-positive (IS*900*-positive) by PCR [39]. Grant *et al.* [7] pasteurized raw cows' milk naturally infected with MAP and found, that MAP is capable of surviving commercial scale pasteurization at 73°C for 15 s and 25 s with and without prior homogenization if the bacterial cells are present in sufficient numbers before heat treatment. They also tested commercially pasteurized cows' milk samples from dairy processing establishments for the presence of MAP by culture. Only 1.8% of the tested pasteurized milk samples were MAP-positive [7]. Furthermore, the production process for hard and semi-hard raw milk cheese seems not to completely reduce MAP. An efficient method for detection of MAP bacterium in bulk tank raw milk is important for assessment of this risk factor and the determination of MAP status of dairy farms in a JD control program.

Efforts have been made in the last few decades to develop protocols for the detection of MAP in feces, milk, tissue, food and environmental samples using various methods.

Serology and fecal culture, however, are the most commonly used tests in the field [13, 14].The sensitivity of the ELISA for detection of high shedders was >90% both for individual milk and serum samples [40]. PCR is an ideal method for rapid turn around time [15]. These assays have been successfully used to detect the presence of *M. avium subsp. paratuberculosis* based on the amplification of the sequences IS*900* [41].

The *Map* is contagious infection spreads both horizontally and vertically. The horizontal infection proceeded through the contact with feces, contaminated pastures, litter and equipments used during milking and care instruments. In turn, the vertical infection proceeded through: colostrum, milk as well as interuterinally and through semen as reviewed by Whittington and Windsor [42]. The disease in cattle is spread by ingestion of *Map* from the contaminated environment. The disease persisted after the introduction of infected animals. Infection can be spread vertically to the fetus and semen could be infected with the organism. The primary source of infection in calves was milk from infected cows or milk that was contaminated with the feces of diseased cattle as stated by McFadden *et al* [34]. A possible relationship between Crohn's disease and *Map* have been stimulated by the recent findings by researchers in the United Kingdom which revealed that *Map* DNA could be detected in pasteurized milk samples purchased from retail markets as shown by Millar *et al* [39]. Cattle could be infected early in life by ingestion of Map via colostrum, milk, fecal contaminated teats, water, feeds or surfaces as stated by Stabel [43].

The obtained results confirmed the presence of IS*900* specific to MAP which is either to an infection or contaminated by the feaces of the diseased animal. Milk was considered to be the main source of infection in both cattle and human. Therefore, detection of *Map* in milk was important to diagnose and control the disease in cattle and human by excluding the positive *Map* milk and its animal to prevent the spread of infection. Application of IS*900* PCR was performed to detect the DNA of *Map* in milk and it was found that 9 milk samples out of 13(69.2%) raw cow milk from a dairy farm at El- Monefeia governorate,Egypt, were positive to IS*900* PCR as shown in Fig. 1. In the light of the previous data, the results indicated that animals have shed the *Map* in their milk, which can play a source of infection to other animals and human if this milk was drunk. These results agreed to some extent with those obtained by Millar *et al.* [39] who detected the *Map* in pasteurized milk samples purchased from retail markets. ELISA test was also applied on these collected milk samples and it was found that all were negative to ELISA as shown in Fig. 2, which reflects that these clinicaly diseased animals were shedding the *MAP* in milk without developing antibodies in their sera which mainly appear in late stage of the disease as reported by Stable [43]. IS*900* PCR is considered to be

the best choice to detect MAP in milk in such case despite that IS*900* PCR can not differentiate between living and dead cells. The possible risk of *Mycobacterium avium subsp. paratuberculosis* living cells, dead cells and its structures in milk and food in respect to autoimmune Crohn's disease should be carefully monitored to decrease the risk of exposure for children and people under the highest risk for Crohn's disease and a national programme should be developed for controlling the disease in Egypt in both humans and animals.

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