

*MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS* IN RAW CAPRINE MILK*¹Saad, M.F., ¹ Nagah M. Hafiz & ¹Salwa A. Aly¹Cairo University, Faculty of Veterinary Medicine, Department of Food Hygiene & Control, Giza square, Egypt

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ABSTRACT

One hundred and fifty individual caprine milk samples were analyzed for *Mycobacterium avium subsp. paratuberculosis* (*MAP*). Out of 150 samples tested for *MAP*, 53 (35.33%) samples could be detected by Enzyme- Linked Immunosorbent Assay (ELISA) technique. However, one (0.67%) sample was found positive in Polymerase Chain Reaction (PCR) method and failed to be isolated from all the examined samples.

KEYWORDS: Caprine milk, *Mycobacterium avium subsp. paratuberculosis*, Enzyme- Linked Immunosorbent Assay, Polymerase Chain Reaction.

INTRODUCTION

Johne's disease, caused by *Mycobacterium avium subsp. paratuberculosis*, is a chronic granulomatous 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 as a whole (Ellingson *et al.*, 2005). *Mycobacterium avium subsp. paratuberculosis* has been controversially linked with human Crohn's Disease which characterized by Ulcerative Colitis and Inflammatory Bowel Disease. Goat's milk is a potential source of *MAP* transmission in young kids, therefore, it is important to diagnose and control of the disease (Singh & Vihan, 2004; Rath *et al.*, 2011; Dalton *et al.*, 2014). PCR is a rapid and sensitive method for the detection of *MAP* in milk. Almost all PCR protocols target the IS900 insertion sequence, which has been accepted as a standard marker for *MAP* (Englund *et al.*, 2002).

MATERIALS & METHODS**Collection of samples**

One hundred and fifty individual goat's milk samples were taken after washing the udder and teat dipping. The first milk stream was discarded and the samples were collected (250 ml) into sterile bottles then were transferred to the laboratory in an insulated ice box with a minimum of delay to be immediately examined for:

Detection of *MAP* antibodies by ELISA technique (ID-vet kit, France) according to Verna *et al.* (2007)

In a 96-well pre-dilution microplate, samples were diluted 1/2 and controls to 1/12 in Dilution Buffer. 6.10 µl of the Negative Control and 110 µl of dilution buffer 6 were added to wells A1 and B1. 10 µl of the Positive Control and 110 µl of dilution buffer 6 were added to wells C1 and D1. 80 µl of dilution buffer 6 and 80 µl of each sample to be tested were added to the remaining wells and incubated between 5 min and 45 min at 21°C (± 5°C). 100 µl of the

previously neutralized samples and controls were transferred to the coated ELISA microplates and incubated 45 min ± 4 min at 21°C (± 5°C). The wells were empty and each well was washed 3 times with approximately 300 µl of the Wash Solution. Drying of the wells between washings was avoided.

The Conjugate was prepared by diluting the Concentrated Conjugate 10X to 1/10 in Dilution Buffer 3. 100 µl of the Conjugate 1X was added to each well and incubated 30 min ± 3 min at 21°C (± 5°C). The wells were empty. Each well was washed 3 times with approximately 300 µl of the Wash Solution. 100 µl of the Substrate Solution was added to each well and incubated 15 min ± 2 min at 21°C (± 5°C) in the dark. 100 µl of the Stop Solution was added to each well in order to stop the reaction. The Optical Density (OD) was read and recorded at 450 nm. Positive ratio (S/P) percentage was calculated as follows using the control values: S/P = OD (sample) – OD (negative control) / OD (positive control) - OD (negative control) x 100. Less than or equal to 15 % are considered negative and greater than 15 % are considered positive.

Isolation of *MAP* according to Singh and Vihan (2004)

Equal amount of freshly milk samples and BBL MycoPrep (Becton, Dickinson and Company, USA) were put in 50 ml tube. The samples were vortexed for 30 second and were left for 30 min. Phosphate buffer up to 50 ml was added. The tubes were centrifuged at 3000g for 20 minutes. The tubes were stood for few minutes and the supernatant were decanted. Finally few drops of sediment were transferred to the Herrold's Egg Yolk Medium tubes slants with Mycobactin J. and VAN, BD Becton, Dickson and Company, USA. The inoculated tubes were incubated at 37°C in CO₂ incubator in slant position with the cap loosened. After one week the tubes were tighten caps and placed in vertical position in the incubator up to 16 weeks. The tubes were read and evaluated for growth weekly. Ziehl-Neelsen smears stain was done to ensure the presence of *MAP* in the isolated colonies according to (Slana *et al.*, 2008).

Detection of MAP in milk by PCR

Extraction of MAP genomic DNA from milk samples according to Paolicchi *et al.* (2003)

At first, milk sample (50 ml) was centrifuged at 4000 rpm for one hour. The whey was discarded, and the pooled fat and pellet was washed four times with Phosphate Buffer Saline at 5000 rpm for 5 min, the mixture was resuspended in 450 µl TE buffer followed by addition of 50 µl lysozyme (10 mg/ml) and incubated overnight at 37°C. Next, 100µl of 10% Sodium Dodecyl Sulfate (SDS) and 50µl of proteinase K (10 mg/ml) were added and incubated at 55°C for 2h. Also, 200µl of 5M NaCl and 160µl Cetyl Trimethyl Ammonium Bromide (CTAB) were added, the suspension was subjected to incubation at 65°C for 30 min. this was followed by chloroform-isoamyl alcohol extraction and isopropanol precipitation. The pellet was washed with 70 % ethanol, resuspended in 30 µl TE buffer, and stored at -20°C until needed for PCR.

DNA extraction from MAP cultures according to Stabel *et al.* (2004)

The colonies were harvested into 1X TE buffer and washed three times. The supernatant was removed; pellet was taken and suspended in 1X TE buffer. Heat block was used at 100°C for 10 minutes, and then the supernatant was transferred in new eppendorf and kept at -20°C for PCR.

IS 900 specific primers used in detection of MAP by PCR:

Forward primer:

5` -CCGCTAATTGAGAGATGCGATTGG-3`

Reverse primer:

5` -AATCAACTCCAGCAGCGGGCCTCG-3`

DNA amplification by conventional PCR:

PCR was performed in 25 µl reaction volume in 200 µl PCR tubes using 5 µl of DNA extracted from milk samples. The previous procedure was repeated on the isolates. Amplification cycles were carried out in programmable thermocycler (Brimus MWG, biotech, Germany).

A number of experiments were performed to optimize the PCR protocol. The optimized PCR assay was finally established using a total volume of 25 µl reaction mixtures contained 5µl of DNA as template, 20 pmol of each primer and 1X of PCR master mix (Dream Taq™ Green PCR Master Mix).

Reaction conditions for IS900 primer were optimized to be:

95°C for 5 min. as initial denaturation followed by 50 cycles of 95°C for 1 min, 60°C for 1 min and 72°C, 59 sec. A final extension step at 72°C for 10 min was followed.

Visualization of the PCR products:

After amplification, 10µl of the PCR product were electrophoresed on 1.5% agarose gel electrophoresis containing 0.5X Tris- Borate- EDTA (TBE) buffer and 0.5 µg/ml ethidium bromide for 60 min. at 70 volts in an electrophoresis unit (BioRad) containing 0.5X TBE as electrophoresis buffer. Amplification products were visualized using ultraviolet transilluminator and photographed using digital camera (FinPix S9600S).

The size of the amplification products were compared with the GeneRuler™ 100 bp Plus DNA Ladder, Fermentas Life Science. All these chemicals were molecular biology grade produced by Sigma Aldrich (USA). Samples showing positive amplification of 229 bp for IS900 primer and were considered positive.

RESULTS

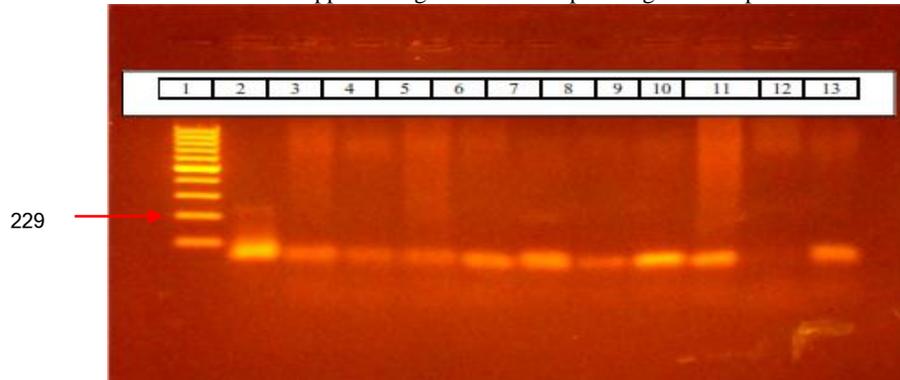
TABLE 1: Incidence of *Mycobacterium avium* subsp. *paratuberculosis* in the examined samples based on ELISA technique, PCR and culture methods (n =150).

Methods	Positive (+)		Doubtful (±)		Negative (-)	
	No. of samples	%	No. of samples	%	No. of samples	%
ELISA	53	35.33	0	0.00	97	64.67
PCR	1	0.67	0	0.00	149	99.33
Culture	0	0.00	0	0.00	150	100.00

n= number of examined samples.

TABLE 2: Comparison of *Mycobacterium avium* subsp. *paratuberculosis* milk ELISA and milk PCR techniques in the examined samples

Tests	Sensitivity % (Sn)	Specificity % (Sp)	Positive Predictive Value (PPV)	Negative Predictive Value (NPV)
Milk ELISA	50.00	73.66	1.82%	99.34%
Milk PCR	50.00	98.69	33.33%	99.34%

FIGURE 1: IS 900 applied on goat's milk samples targets 229 bp molecular size.

Lane 1 corresponds to 100 bp DNA ladder.

Lane 2 corresponds to *MAP* +ve control.

Lane 3 to 12 corresponds to the examined milk samples.

Lane 13 corresponds to *MAP* -ve control.

DISCUSSION

Paratuberculosis (PTBC) is a chronic infectious enteric disease that affects domestic and wild ruminants. It is an economically important disease seen primarily in cattle, sheep, and goats and is caused by *Mycobacterium avium* subsp. *paratuberculosis*. The first case in goats was reported in 1912. The disease was reported in several other European countries, such as Great Britain, Italy, and France, as well as in many Asian and African countries. Caprine paratuberculosis has been reported in the USA, Argentina, and Canada. Milk has been reported as the main transmission source of *MAP* to human and animals (Grant *et al.*, 2001 and Kruze *et al.*, 2006). Infected animals may excrete *MAP* in their feces and milk and spread the infection, which can cause considerable financial losses due to premature culling and death (Ayele *et al.*, 2001). This organism infects animals in the first months of life and elicits a slowly progressive inflammatory response in the gastrointestinal tract that is not clinically evident until months to years later. Clinical signs of this infection are chronic weight loss and unremitting diarrhea. Diarrhea is infrequent in infected goats (Stehman, 1996). In advanced cases, the organism can disseminate beyond the gastrointestinal tract to other organ systems (Whitlock and Buergelt, 1996). Raw goat's milk is consumed as medicine in many parts of the world. Therefore, findings achieved by different studies led the United Kingdom to adopt an increased holding time for commercial milk pasteurization (25 second rather than 15 second at 72°C) in order to increase the lethality of the pasteurization to *MAP* (Grant *et al.*, 2002).

Mycobacterium avium subsp. *paratuberculosis* is linked with Crohn's Disease, and the possible modes of transmission of the organism from animals to humans should be considered. Although fecal contamination of the udder may account for a portion of subsequent contamination of milk, it has also been demonstrated that animals infected with *M. paratuberculosis* shed the organism directly into their milk (Streeter *et al.*, 1995). Out of 150 milk samples tested, 53 (35.33%) samples could be detected by ELISA technique. However, one (0.67%) sample was found positive in PCR method and failed to be isolated from all the examined samples (Table

1 and Figure 1). Paratuberculosis is characterized by two main stages; the first stage is the tuberculoid stage and is marked by a strong cell mediated immune response. The second stage is the leproid stage, in which humoral immunity is important (Slana *et al.*, 2008). Serological tests are not suitable for newly infected animals, because anti-*MAP* antibodies are not usually produced in the early stage of infection (Gumber *et al.*, 2006). Milk-ELISA is considered an alternative to fecal culture as cost-effective and accurate as goat paratuberculosis screening test (Hendrick *et al.*, 2005 and Salgado *et al.*, 2007).

The goats with a *MAP*-specific antibody response above a defined threshold were considered to be infected. Because the process of infection is said to take place at an early age and antibody detection develops in later stages of disease, only goats of at least 2 years of age, the probability of detecting infection will be increased. In general the probability of antibody detection in an infected goat increases with age (Van Hulzen *et al.*, 2012). Sensitivity and specificity of ELISA technique in comparison to PCR method was determined. This was observed that ELISA technique shown the 50.00 % sensitivity and 73.66 % specificity in comparison to 50.00 % and 98.69 % sensitivity and specificity respectively, in PCR method (Table 2). The lower sensitivity of ELISA technique may be attributed to lower concentration of antibodies in milk than serum. It has been reported that infections by other members of the mycolata such as *Mycobacterium* may cause false-positive serologic tests for paratuberculosis in small ruminants (Salgado *et al.*, 2005). Polymerase Chain Reaction was developed and evaluated for detection of *Mycobacterium avium* subspecies *paratuberculosis*. The rapidity, sensitivity and specificity of the PCR assay would greatly facilitate detection of Johne's disease infection among susceptible ruminants and could be used for detection of Johne's disease at an early stage of the infection (Ibrahim *et al.*, 2004). A disadvantage of the PCR methods currently used for the detection of *MAP* in milk is the fact that they cannot distinguish between viable and nonviable bacteria (Englund *et al.*, 2002). Due to the fact that milk contains a number of other bacteria and milk elements, which can spoil samples if they are stored inadequately, an effort has been made to improve the

culture method of *MAP*, or even to use this method concurrently with other methods of *MAP* detection (Slana *et al.*, 2008). Methods to selectively kill non-mycobacterial flora in milk and decontamination are critical factors to ensure a high sensitivity of *MAP* detection. Raw milk usually contains a high level of contaminants, and the selection of the chemical decontamination step is an important consideration for the successful recovery of *MAP* in culture method (Dundee *et al.*, 2001). The advantage of the culture method of *MAP* is specificity. However, the disadvantages of this method are taken a long time (six weeks or more) and the insufficient effectiveness of decontaminating methods (Harris & Barletta, 2001 and Ayele *et al.*, 2005).

The lower detection rate of *M. paratuberculosis* by milk culture could be attributed to the following causes; the isolation procedure for *M. paratuberculosis* from milk used by most researchers has been adapted from the protocol developed for fecal samples; the number of organisms shed in milk may be few in number (compared with feces) due to intermittent shedding milk or non-processing of fat layer, which may have carried the bacilli along with it during the centrifugation step or due to presence of anti-metabolites in milk causing hindrance in growth of bacilli leading to long incubation period in the milk samples (Van der Giessen *et al.*, 1992 and Whipple *et al.*, 1992).

Due to the lack of awareness about PTBC among goat owners, lack of regulations on animal trade and standard goat husbandry practices, a continuous spread of PTBC among and within goat herds should be expected. The safety of raw milk derived from *MAP* infected dairy goats becomes an important consideration for public health, because *MAP* could be associated with Crohn's disease in humans (Fiorentino *et al.*, 2012).

CONCLUSION

We emphasize the importance of using more than one type of diagnostic technique for the detection of goat's milk positive for paratuberculosis.

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