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DETECTION OF *Mycobacterium avium* subsp. *paratuberculosis* IN COW'S MILK

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ABSTRACT

In the present study, *Mycobacterium avium sub sp. paratuberculosis* (MAP) infection was detected by serological and molecular methods in herds of dairy cattle. Both milk and blood samples of 88 suspected affected dairy cattle with paratuberculosis were collected from ten dairy farms from different Governorates in Egypt. California Mastitis Test (CMT) was performed on milk samples. Indirect Enzyme Linked Immuno Sorbent Assay (ELISA) was used for detection of MAP antibodies in milk and serum samples. Polymerase Chain Reaction (PCR) was utilized for molecular identification of MAP from milk samples. 7 (7.95%), 33 (37.5%), 26 (29.55%) and 22 (25%) of 88 milk samples were CMT (++), CMT (+), suspicious and negative, respectively. According to ELISA results of milk samples, 24 (27.27%) and 64 (72.73%) were positive and negative, respectively. On the basis of the ELISA results of serum samples, it was found that 26 (29.55%), 1 (1.14%) and 61 (69.32%) were positive, suspicious and negative, respectively. Concerning PCR of milk samples, MAP DNA was detected in 21 (23.86%) samples.

Keywords: MAP, ELISA, PCR, Milk, Serum

INTRODUCTION

MAP belongs to the family of *Mycobacteriaceae*, it is a subsp. member of the *Mycobacterium avium* complex, which causes paratuberculosis, an infectious enteric disease of ruminants, also called Johne's disease (JD) **(Neuendorf and Ackermann, 2015).** Dairy cattle affected with JD shed MAP in their milk **(Streeter *et al*., 1995)**. MAP can be isolated from milk, supramammary lymph nodes and lymph fluid samples from the udder **(Khol *et al*., 2012)**.

Unfortunately, MAP is able to resist pasteurization conditions; confirmed MAP isolates were cultured from 1.8% of commercially pasteurized milk samples in the UK, and similar data were published from USA **(Dimareli-Malli, 2010)**. Moreover, MAP has high environmental survival time due to the thick, waxy cell wall that renders the organism highly resistant.

Suspected affected dairy cattle suffer from "Water-hose" or "pipe stream" diarrhea, hypoproteinemia and intermandibular edema (bottle jaw), therefore they become increasingly lethargic, weak and emaciated **(Tiwari *et al*., 2006)**. These affections lead to severe economic problems such as reduced milk production, reproductive performance and culling value, premature culling, increased replacement costs, and more recently, potential concerns about the safety of the herd's milk **(Council, 2003)**. Additionally, paratuberculosis may cause subclinical mastitis **(Gümüşsoy *et al*., 2015)**; because there is an increase in somatic cell counts in animals with paratuberculosis **(Jurkovich *et al*., 2016)**.

Several countries – Ireland, N. Ireland, Sweden, Ukraine, Australia, New Zealand, USA and others – have made JD a notifiable disease, it may occur either sporadically in Egypt or a minor epidemic within a herd **(El-Sawalhy, 1999)**. MAP was first isolated in Egypt in 2005, since then, the pathogen has been detected in different Egyptian provinces **(Salem *et al*., 2005).** JD was included by the World Organisation for Animal Health in the list of diseases with particular economic significance **(Office International Des Epizooties, 2004)**.

In human, MAP has been discussed as probable causative agent of Crohn's Disease (CD), which is an inflammatory disease of the human intestinal tract **(Behr and Collins, 2010)**. The consumption of milk and dairy products contaminated with MAP may be a possible source of infection for humans **(Hermon-Taylor and Bull, 2002)**, so introducing MAP -free milk and dairy products would provide the least risk for consumers **(Hruska *et al*., 2005)**, therefore it is of interest to rapidly detect MAP in milk for human consumption.

The ELISA is, at present, the most sensitive and specific test for milk and serum antibodies to MAP in cattle, so it is used for indirect detection of MAP in serum or milk **(Neuendorf and Ackermann, 2015)**. PCR is rapid and specific method for MAP detection and could be used for detection of JD at an early infection **(Ibrahim *et al*., 2004)**.

Owing to the seriousness that arises from the presence of MAP in milk, this present study was designed to screen for the present of subclinical mastitis, MAP antibodies in milk and serum, and MAP DNA in milk samples collected from suspected infected cattle using CMT, indirect ELISA and PCR respectively.

MATERIAL AND METHODS

Collection of samples

After examination of 8750 dairy cattle from ten dairy farms from different Governorates in Egypt, a total of 88 suspected affected dairy cattle with paratuberculosis were chosen for collection of milk and blood samples.

California Mastitis test (CMT) of milk samples (Middleton *et al*., 2004) approximately 2 ml of the milk sample were poured in the cup of the test paddle. 2 ml of the reagent (very pure clorhidryc acid 35%, bromocresol purple and sodium dodecilbencenosulfonate) were added on milk, after that the paddle was moved softly with light oscillatory movements so that both milk and reagent were mixed enough for the reaction. The milk samples were graded as negative, suspicious, CMT (+), and CMT (++).

Serodiagnosis of MAP by ELISA of milk and serum

Detection of antibodies of MAP in milk and serum of suspected affected dairy cattle by ELISA (ID-Vet kit, France) (ID SCREEN® Paratuberculosis Indirect Confirmation test) **(Hafiz *et al*., 2016),** the used method was described in the Office International Des Epizooties (OIE) recommendations. The MAP antibodies test kit (ID-Vet, France) was utilized according to the manufacturer's direction.

Detection of MAP in milk by PCR

DNA Extraction

DNA extraction was done using Qiagen extraction kit with some modification as follow: **(Gao *et al*., 2007**)

50 ml or what was available of milk sample was heated in water bath at 95°C for 10 min then cooled in ice water for 10 min. MAP lysis was done according to **Corti and Stephan, (2002)** as follow**:**

In a biosafety cabinet (Bioworkstation), 50 ml of the milk sample was thoroughly mixed with 0.5 ml triton® X-100, vortexed and centrifuged at 4500 rpm for 30 min at 10°C. Cream and whey were discarded and the sediment resuspended in 2 ml mycobacterial lysis buffer (86.6 ml deionized water, 8 ml 5 M NaCl, 2 ml 2 M Tris HCl (pH_8), 3 ml 20% SDS, 400 µl 0.5 M EDTA and 220 µl 15.6 mg/ ml proteinase K), thoroughly mixed by vortex and incubated overnight at 37°C. MAP DNA was released by mechanical disruption of the lysate at ULTRA sonik (104 X, York) for 1 min afterwards; the tubes were immediately put on ice for 15 min and vortexed for thoroughly mixing. After then the lysate was exposed to steps of DNA purification.

Purification of DNA The DNA was obtained or purified the QIAmp DNA Mini Kit by Qiagen according to the manufacturer's instruction (spin protocol) **(Szteyn *et al*., 2014).**

Measuring DNA concentration and purity by spectrophotometer (GeneQuant 1300).

One µl of DNA was diluted with 49 µl distilled water and the optical density (OD) was measured in a quarter cuvette at 260 and 280 nm.
DNA concentration was calculated using the following formula:
DNA concentration (µg/ ml) = 50 x A260 x dilution factor (50).
DNA purity was calculated from the following equation:
DNA purity = A280 / A260 for dsDNA an absorbance ratio of 1.7 to 2.0 is concerned acceptable.

PCR amplification of IS900 gene (Gümüşsoy *et al*., 2015)

The optimized PCR was established using a total volume of 15 µl reaction mixtures in 0.2 ml PCR tubes contained **7.5 µl master mix** (Promega Master Mix), 1µl P90, 1 µl P91 primers (Invitrogen) and 5.5 µl DNA template. The mixture was placed in the thermal cycler (Biometra), which was programmed as follow:
Initial denaturation applied at 94°C for 1 min, number of amplification cycles (30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min) and final extension at 72°C for 10 min.

IS900 P90 & P91primers (Invitrogen)

Primers	Sequence (5' - 3')
P90	GAAGGGTGTTCGGGGCCGTC
P91	GAGGTCGATCGCCCACGTGAC

Gel electrophoresis

To assess the amplified (PCR) product, 4 mm thickness of 1% agarose containing 0.5 µg/ ml ethidium bromides was poured in mini-gel and left till solidify then put in the loading buffer. 2 µl of the PCR products were inserted in the wells of the agarose gel. DNA ladder (100 bp) was used as a marker, positive control (DNA extract of MAp, Vet. Med. Uni., Vienna) and negative control (sterile distilled water) were used and DNA electrophoresis was done at 100 V for 1.30 h. The band patterns were analyzed in a gel documentation system (Viberloumat). The 400 – bp was evaluated as positive for PCR **(Gümüşsoy *et al*., 2015).**The procedures of ELISA and PCR were performed in the Molecular Biology Research Unit, Assiut University, Egypt. The statistical analysis was done by Chi-square method (Pearson, 1900) that performed by SPSS

RESULTS AND DISCUSSION

7 (7.95%), 33 (37.5%), 26 (29.55%) and 22 (25%) of 88 examined milk samples were determined as CMT (++), CMT (+), suspicious and negative, respectively (Tab 1). The obtained results were in harmony with that demonstrated by **Gümüşsoy *et al.* (2015)**. There is an increase in somatic cell counts in animals with paratuberculosis **(Jurkovich *et al*., 2016)**. MAP may be the primary factor in mastitis, or it may be caused by secondary agents **(Gümüşsoy *et al*., 2015)**.

Table 1 Prevalence of mastitis in the examined milk samples based on the result of CMT

No. of examined samples	Degrees of reaction on samples							
	++ve samples		+ve samples		±ve samples		-ve samples	
	No.	%	No.	%	No.	%	No.	%
88	7	7.95%	33	37.5%	26	29.55%	22	25%

Based on the result obtained, ELISA technique revealed a higher number of positive samples in serum samples than in milk samples, whereas when the milk samples were analyzed using PCR, a lesser number of positive samples was revealed (Tab 2). It was found that there is no significant difference between these obtained results by Chi-square method that performed by statistical program (SPSS).

Table 2 Prevalence of MAP infection in the examined samples based on the result of ELISA of milk and serum and PCR of milk

examined samples	Test used	Positive samples		Suspicious samples		Negative samples	
		No.	%	No.	%	No.	%
88	ELISA of milk	24	27.27%	0	0%	64	72.73%
	ELISA of serum	26	29.55%	1	1.14%	61	69.32%
	PCR of milk	21	23.86%	0	0%	67	76.14%

Gel image of PCR products (1% agarose gel) showing

M: 100-bp DNA ladder marker, +ve: positive control (DNA extract of MAP, Vet. Med. Uni., Vienna), Lanes (1-3, 5-8): positive samples for MAP by using IS900 gene (400bp), Lanes (4, 9-13): negative samples for MAP. Results are shown in the figure 1.

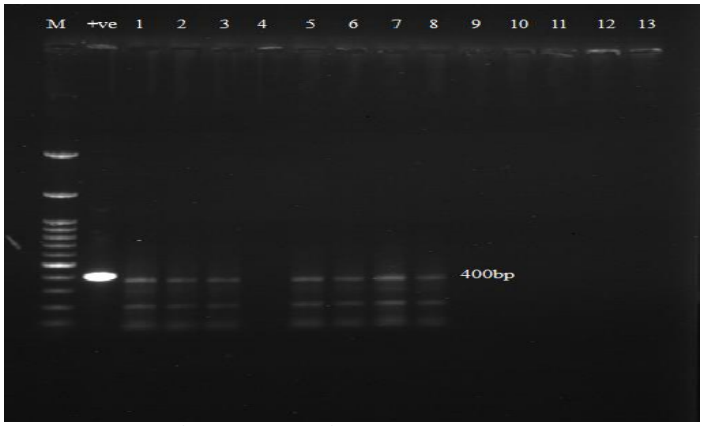

Figure 1 Example of the Gel image of PCR products (1% agarose gel)

It is evident from the data demonstrated in (**Tab 3)** that about 13 (54.17%) of 24 positive cattle by ELISA of milk, 12 (46.13%) of the positive cattle by ELISA of serum and 11 (52.38%) of 21 positive cattle by PCR of milk may be suffered from subclinical mastitis. These results showed that mastitis symptoms in paratuberculosis were subclinical.

Table 3 Correlation between ELISA of milk and serum, PCR of milk and CMT scores

Technique used				CMT scores							
				CMT (++)		CMT (+)		Suspicious		Negative	
	Result	No.	%	No.	%	No.	%	No.	%	No.	%
ELISA of milk	+ve samples	24	27.27%	3	12.5%	10	41.67%	7	29.17%	4	16.67%
ELISA of serum	+ve samples	26	29.55%	2	7.69%	10	38.46%	8	30.77%	6	23.08%
PCR of milk	+ve samples	21	23.86%	0	0%	11	52.38%	4	19.05%	6	28.57%

From the result shown in (**Tab 4)** it is clear that 17 (26.56%) milk samples out of 64 negative milk samples by ELISA were positive by PCR, while 47 (73.44%) were negative. Concerning positive milk samples by ELISA, 4 (16.67%) milk samples out of 24 positive milk samples by ELISA were positive by PCR, while 20 (83.33%) were negative. 17 (27.87%) and 44 (72.13%) milk samples of 61 sera negative cattle by ELISA were positive and negative by PCR, respectively, while 4 (15.38%) and 22 (84.62%) milk samples of 26 sera positive cattle by ELISA were positive and negative by PCR, respectively **(Tab 5)**. The results demonstrated in **Tables 4** and **5** may be attributed to the fact that antibody titers are usually low at the beginning and increase during the later stage of the disease **(Gilardoni *et al*., 2012)**. Moreover, **Gümüşsoy *et al.* (2015)** proved that immune response has been seen at a relatively late stage of infection, the reason for the low sensitivity of ELISA may be due to its inability to identify early positivity. The cause of the negative and doubtful results from the samples of the same positive PCR animals is thought to be due to the humoral response not being active enough in the early stages of infection. Thus negative sample by ELISA and in the same time positive by PCR, The infection may be in the early stage, no enough antibody titer presents for detection. The cause of negative PCR results from the milk samples of the same positive animals by ELISA may be attributed to that cattle affected with paratuberculosis spread MAP intermittently, so the negative milk samples by PCR and in the same time positive by ELISA, it may be collected at the time that the cattle not shed MAP, because shedding of MAP is intermittent **(Neuendorf and Ackermann (2015)**.

Table 4 Correlation between ELISA and PCR of milk

ELISA of milk			PCR of milk			
			Positive samples		Negative samples	
Score	No.	%	No.	%	No.	%
-ve samples	64	72.73%	17	26.56%	47	73.44%
+ve samples	24	27.27%	4	16.67%	20	83.33%

Table 5 Correlation between ELISA of serum and PCR of milk

ELISA of serum			PCR of milk			
			Positive samples		Negative samples	
Score	No.	%	No.	%	No.	%
-v samples	61	69.32%	17	27.87%	44	72.13%
±v samples	1	1.14%	0	0%	1	100%
+v samples	26	29.55%	4	15.38%	22	84.62%

The obtained results in **(Tab 6)** showed that 1 (1.64%) and 60 (98.36%) milk samples of 61 sera negative cattle by ELISA were positive and negative by ELISA of milk, respectively. In contrast 23 (88.46%) and 3 (11.54%) milk samples of sera positive cattle by ELISA were positive and negative by ELISA of milk, respectively. It was noted that some negative cattle by ELISA of milk were positive by ELISA of serum at the same time. These serum samples contain low amounts of MAP antibodies; so they gave positive results in the beginning of positivity. The most plausible explanation for this is dilution of milk antibodies in cows with higher milk production. Milk production is simply one factor that interferes with interpreting results of the ELISA at the individual cow level **(Hendrick et al., 2005).**

Table 6 Correlation between ELISA of serum and milk

ELISA of serum			ELISA of milk			
			Positive samples		Negative samples	
Score	No.	%	No.	%	No.	%
-ve samples	61	69.32%	1	1.64%	60	98.36%
±ve samples	1	1.14%	0	0%	1	100%
+ve samples	26	29.55%	23	88.46%	3	11.54%

Regarding the results in **(Tab7)** it is evident that 4 dairy cattle were positive in all three techniques (ELISA of milk, ELISA of serum and PCR of milk), while 19 dairy cattle were positive by two techniques (ELISA of milk and serum). Also, 22 dairy cattle were positive only by one technique as; 1, 4 and 17 dairy cattle were positive by ELISA of milk, ELISA of serum and finally PCR of milk, respectively.

Table 7 Correlation between the detected positive dairy cattle by using different techniques

+ve dairy cattle		ELISA of milk	ELISA of serum	PCR of milk
4		4 (100%)	4 (100%)	4 (100%)
19		19 (100%)	19 (100%)	0 (0%)
22		1 (4.55%)	4 (3 +ve, 1 ±ve) (18.18%)	17 (77.27%)
Total	45	24	27	21

CONCLUSION

It can be concluded from this study, there is no one reliable technique for the detection of MAP infection and complementary diagnostic techniques must be used for detection. Using a combination of more than one technique to obtain accurate results in the detection of MAP may be useful. MAP constitutes economic and public health significance and maximal efforts should be made to control this microorganism.

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