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The Efficiency of ELISA and PCR in Detecting Subclinical Paratuberculosis in the Saudi Dairy Herds

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Abstract: This study was aimed to reveal the presence of John's disease in the Saudi dairy herds using the newly developed diagnostic tests, ELISA and PCR. A total of 687 serum and fecal samples were collected from dairy cattle of four different ages, one, two, four and six years age cattle of three geographically different dairy farms. IDEXX ELISA revealed 15 (2%) positive samples and 17 (2.5%) samples were inconclusive. PCR test were used only to test 62 ELISA-negative samples that their OD readings were the highest and all the inconclusive samples. The PCR disclosed more positive samples ($22/62 = 32\%$), interestingly among the samples of the two-year old cattle. The study has conclusively confirmed the presence of the disease in the Saudi dairy herds. It has also approved the effectiveness of ELISA and PCR tests in revealing the MAP infection at the subclinical stage.

Key words: Saudi Arabia, John's disease, paratuberculosis, MAP, ELISA, PCR

INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes John's disease in domestic and wild ruminant like, cattle, sheep, goats, deer, antelope and bison (Stabel, 1997) worldwide. In Saudi Arabia, John's disease was reported in sheep, goat and camel (Alluwaimi *et al.*, 1999; Gameel *et al.*, 1994). However, the extent and scale of MAP infection in the dairy herds was not established.

The disease inflicts wide range of economical losses on dairy industry (Stabel, 1997). In the United States the losses caused by John's disease exceeded \$1.5 billion/year (Jones, 1989).

Long incubation period is the main characteristic feature of MAP infection. Ingestion of fecal material, milk or colostrum is the main route of infection. Infected cattle shed low amount of bacteria during the subclinical stage. However, during the clinical stage the shed organisms in feces increase dramatically. At the clinical stage, infected animals manifest chronic diarrhea, emaciation, decrease milk production and infertility (Stabel, 1997).

Early diagnosis of MAP infection represents one of the major obstacles in successful control of the disease (Valentin-Weigand, 2002). During the subclinical phase, the immune responses are dominated by cell-mediated immunity whereas; humoral immune responses prevail

at the clinical phase (Coussens, 2001, Valentin-Weigand, 2002). Different versions of Enzyme Linked Immunosorbent Assay (ELISA) and molecular based techniques like Polymerase Chain Reaction (PCR) were introduced in the last decade to assure the early diagnosis of MAP infection. However, these techniques are sensitive if they are applied in the suitable combination to achieve clear cut diagnosis (Collins, 1996; Paolicchi *et al.*, 2003).

ELISA remains one of the most applied tests for the early detection of MAP infection. Overwhelming studies examined the sensitivity and specificity of ELISA in the detection of subclinical MAP infection (Nielsen and Toft, 2006; Collins, 2002; Collins *et al.*, 2005; Stabel, 1997; Whitlock *et al.*, 2000). The specificity of ELISA pretreated or pre-absorbed with *Mycobacterium pheli* was shown to range from 96-98% (Collins, 2002). The sensitivity of ELISA is influenced by age, level of shedding in feces and the mathematical method for calculating the sensitivity (Collins, 2002). The sensitivity of ELISA at the early stage of the infection is about 15 and 47-48% in the moderately shedding animals. However, it reaches 88% in animals with clinical signs (Whitlock *et al.*, 2000). Whitlock *et al.* (2000) considered animals shedding less than 10 colonies per tube are more likely to be seronegative, while animals shedding more than 70 colonies per tube are considered strong seropositive.

Discovery of the distinct IS900 in the MAP genome in the late 1980s has opened the way for development of PCR techniques to enhance the early diagnosis of MAP infection. The IS900 is 1451 pb and is repeated 15-20 times in the MAP genome (Vansnick *et al.*, 2004). The sensitivity of commercially available kit that is based on detecting IS900 could reach 60% (Stabel, 1997). The specificity and sensitivity of direct PCR system was enhanced by developing new primers to detect as low as 1 CFU of MAP (Vansnick *et al.*, 2004). The sensitivity of PCR is superior to ELISA in detection of subclinical MAP infection (Gasteiner *et al.*, 2000) culture 30 days earlier than the appearance of the colonies (Paolicchi *et al.*, 2003).

This study was intended to detect the presence of MAP infection in the Saudi dairy herds and to examine the versatility of the newly developed diagnostic tests, ELISA and PCR, to reveal the subclinical infection in different ages without the need to retest with the fecal culture. Establishing the presence of MAP infection in the Saudi dairy herds is vital in view of compelling evidence that incriminate the MAP in the initiation of Crohn's disease (Naser and Naser, 2007).

MATERIALS AND METHODS

Serum and fecal samples: A total of 687 serum and fecal samples were collected from apparently healthy cows from three major herds, one in the central region (herd A) and two from the Eastern province (herd B and C) and 16 samples were collected from a small herd (only 18 cattle) at the Eastern province. The total number of samples that were collected from herd A (25000 cattle) were 238 samples, whereas 221 samples were collected from herd B (14000 cattle) and 218 samples from herd C (7500 cattle). Serum and fecal samples were collected from four different ages, 173 samples from 1 year, 158 samples from two year, 155 samples from 4 year and 201 samples from 6 year old cattle. The serum and fecal samples were kept at -30°C until the analysis.

ELISA (IDEXX): Enzyme linked immunosorbent assay (IDEXX Scandinavian AB, Osterbybruk, Sweden) was used to detect anti-MAP antibodies in the serum samples of different ages. The test was performed according to the manufacturer's direction and as reported elsewhere (Collins, 1996; 2002).

ELISA (ID Vet): The second ELISA Kit (ID VET, Montpellier, France) was also used. After preabsorption step. 100 µL of the previously neutralized samples and controls were transferred to the coated ELISA microplate and incubated for 45 min at room temperature. After

thorough washing, 100 µL of 1X conjugate was then added to each well and incubated for 30 min at room temperature. One hundred microlitre of substrate solution was then added to each well after rewashing, the plate was incubated for 15 min at room temperature. Then 100 µL of stop solution was added to each well. The plate was read by ELISA reader (Thermo Labsystem, Finland) at dual wavelength 450 and 620 nm.

Detection of MAP-DNA by PCR and immunorevelation in microplate: The MAP IS900 probe in fecal samples was detected using commercial kit of PCR and immunorevelation in microplates (Instiut Pourquier, Montpellier, France). The test is comprised of four major steps, Sample preparation, DNA purification, Amplification of IS900 and revelation. The method was applied according to the manufacturer's directions, briefly,

Sample preparation: Approximately 1.5 g of each faecal sample was placed in 5 mL sterile distilled water and after vigorous shaking the samples left to stand for 5 min. A 50 µL of the supernatant was transferred to 1.5 mL tube.

DNA purification: Initially the sample lysates was performed by adding 200 µL of binding buffer and 40 µL of proteinase K and after vigorous shaking the preparation was incubated at 70°C for 30 min then at 95°C for 30 min. The purification of DNA was then carried out using Roche DNA purification kit (Basel, Switzerland). To the 50 µL of the supernatant 100 µL of isopropanol was added and mixed vigorously and the whole amount was transferred to the combined column-collection tube assembly. The column was centrifuged for 1 min. The flow through was removed and 500 µL of inhibition removal buffer was added and the column was centrifuged again for 1 min. The column then washed twice with wash buffer, the DNA then eluted in 1.5 mL sterile tube by adding 60 µL prewarmed (70°C) elution buffer by centrifugation for 1 min.

Amplification of IS900: The reaction mixture 1 and reaction mixture 2 was added in 48 and 2 µL, respectively. Then 2 µL of, negative, positive or purified DNA sample was added in the appropriate tubes. The mixture was amplified in the 2400 thermocycler (GeneAmp® PCR system, Applied BioSystems, USA). The mixture was heated at 37°C for 5 min and at 95°C for 5 min and finally amplified in 45 repeated cycles, First DNA probe was melted at 95°C for 30 sec, annealed at 62°C for 30 sec and extended at 72°C for 40 sec and the final step at 72°C for 8 min. The reaction was kept at 8°C to be cooled.

Revelation of amplified product: The amplified product (10 µL) was added to 50 µL in biotinylated amplified product on streptavidin coated plates. The plate then incubated at room temperature for 30 min. The sample was then denatured by adding 50 µL of the denaturation reagent and incubated for 10 min at room temperature. The plate then washed with 1X washing solution (three washings). The Mycobacterium hybridization solution (50 µL per each well) and 50 µL of the internal control hybridization solution were added to the appropriate wells. After one hour incubation at 37°C, the wells were rewashed and 50 µL of the conjugate was dispensed. The revelation of the reaction was followed by adding 50 µL of TMB substrate and incubation at room temperature for 10 min. The reaction was stopped by 50 µL stop solution per well and the plate was read by ELISA reader (Thermo Labsystem, Finland) at 450 nm.

RESULTS

IDEXX ELISA: Analysis of serum samples with the IDEXX ELISA, revealed only 15 out of 687 (2%) positive samples, whereas 17 (2.5%) samples were inconclusive. No positive samples were found among one and two year cattle. However, 8 positive (5.7%) were detected among 4 year and only 7 positive (3.7%) samples were among the 6 year cattle (Table 1). The higher number of positive samples was in herd A, 8/25000 (0.032%). Herd B indicated the lowest positive number, 1/14000 (0.0071%). Whereas, the positive samples in herd C were 6/7500 (0.08%).

ID VET ELISA: The ID VET company (ID VET, Montpellier, France) has offered us their newly developed ELISA kit to compare its efficiency in detection of the anti-MAP antibodies. Ninety-one serum samples (including positive, inconclusive and high OD reading negative samples) were retested and appeared compatible to data obtained by the IDEXX ELISA.

DNA-PCR of fecal samples: The PCR of the MAP-DNA IS900 probe in fecal cultures was applied only on 62 samples which included the inconclusive samples (16 samples) and the negative samples (46 samples) of all ages that their Ods recorded the highest. The total percentage of positive PCR samples were 21 out of 62 (33%). The PCR analysis revealed 10 (16%) positive samples from the ELISA-inconclusive samples and only 12 (19%) samples of the ELISA-negative samples. The distribution of the PCR positive samples according to the age is summarized in Table 2.

Table 1: The total analysis of IDEXX ELISA according to age of cows

Age	Positive	Negative	Inconclusive	Total	Age (%)
One year	0.0	172.00	1.00	173	25.18
Two year	0.0	155.00	3.00	158	23.00
Four year	8.0	140.00	7.00	155	22.60
Six year	7.0	188.00	6.00	201	29.26
Total	15.0	655.00	17.00	687	
Total (%)	2.1	95.34	2.47		

Table 2: The distribution of the samples retested with PCR-ELISA test according to age

Age	ELISA		
	Negative	Inconclusive	PCR Positive
One year	13	0	1 (N ¹)
Two year	17	3	7 (5N ¹ , 2IN ²)
Three year	6	6	6 (2N ¹ , 4IN ²)
Six year	10	7	8 (4N ¹ , 4IN ²)

¹No. of ELISA-negative samples that were positive by PCR, ²No. of ELISA-inconclusive samples that were positive by PCR

DISCUSSION

In Saudi Arabia, John's disease was reported in sheep, goat and camel (Alluwaimi *et al.*, 1999; Gameel *et al.*, 1994). However, the extent and scale of MAP infection in dairy herds was not established.

In this study two commercial ELISA kits and PCR, were employed to study their efficiency in detecting the subclinical MAP infection in the Saudi dairy herds. The results obtained with IDEXX ELISA correspond to reports elsewhere (Gasteiner *et al.*, 2000). The overall positive and inconclusive samples are [32 (4.6%)] confined to higher ages (4 years and 6 years). However, IDEXX ELISA test failed to reveal any positive results among the young cattle. ELISA sensitivity is greatly reduced with samples of low shedder animals. Whitlock *et al.* (2000) have classified the low shedder animals as the most challenging to any test sensitivity. It is therefore, screening of any herds for MAP infection with ELISA will definitely result in missing a considerable number of subclinical infected young cattle. However, the ELISA results of older animals in this study are in accordance with most of the studies reported elsewhere (Gasteiner *et al.*, 2000). Hence ELISA has confirmed the existence of the disease in the Saudi dairy herds, but the overall prevalence of the disease can not be determined unless a massive longitudinal survey is performed. Nevertheless, despite the limited generated data in this work, the disease has been shown to exist in all tested dairy farms.

PCR-ELISA indicated acceptable versatility in detecting positive samples among the young cows. The test disclosed 50% positive results of the retested samples of two year old animals and older groups (4 years and 6 years animals). Several studies have examined the PCR sensitivity in detection of subclinically infected

animals (Gasteiner *et al.*, 2000; Paolicchi *et al.*, 2003; Zimmer *et al.*, 1999; Anklam *et al.*, 2005). Positive correlation was reported between PCR and ELISA of samples from the subclinical shedders (Gasteiner *et al.*, 2000). However, the PCR sensitivity was shown to be less than the fecal culture in screening of samples of clinically (68%) and subclinically (21%) infected cattle (Zimmer *et al.*, 1999). Low sensitivity of PCR in comparing to the fecal culture could be due to the presence of inhibitors (Paolicchi *et al.*, 2003) or presence of more than one *Mycobacterium* species (Anklam *et al.*, 2005).

The finding of this study is important for the studies that aim to determine the prevalence rate of this disease in the Saudi dairy herds. Although, number of the positive samples detected by ELISA and PCR are small, the presence of the disease in wider scale, especially among young cattle can not be dismissed. The national control plan that addresses the culling and vaccination policy is entirely dependent on drawing the prevalence rate of the disease in the Saudi dairy herds.

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