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Monitoring of Brucella Reactor does Following Milk Examination Using Different Techniques

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Abstract: Milk samples from 129 does were collected and monitored for *Brucella* antibodies using immunological tests such as Milk Ring Test (MRT), Whey Agglutination Test (WAT), Whey Antiglobulin Coombs Test (WCT) and milk ELISA (m ELISA) using *Brucella* Periplasmic protein antigen. Results obtained from these tests were compared to PCR and bacterial isolation. The highest incidence of positive reactors was given by Whey Antiglobulin and Whey Agglutination Test (9.3%) while the lowest incidence was given by bacterial isolation (*Br. melitensis* biovars 3, 3.8%). PCR showed the highest agreement with the bacterial isolation, while WAT and WCT showed the lowest one. PCR showed a high sensitivity of 1×10^4 *B. melitensis* CFU mL⁻¹ of milk. The results of mELISA here suggests its efficiency to be used as a screening test and/ or confirmatory test, while the modified MRT still need more investigations to diagnosis caprine brucellosis.

Key words: Caprine, *Br. melitensis*, milk, PCR, diagnosis

INTRODUCTION

Brucellosis is an important widespread infectious zoonotic disease, caused by bacteria of the genus *Brucella*. Human infection occurred mainly due to direct contact with blood, placenta, fetuses, or uterine secretions of infected animals. Also, consumption of infected and raw animal products (especially milk and milk products) represents an important source of health hazard to consumers. These organisms localize in the supra-mammary lymph nodes and mammary glands in 80% of infected animals which continue to excrete these pathogens in their milk throughout their lives and have a significant role in the epidemiology of the disease (Hamdy and Amin, 2002; Chand *et al.*, 2005; Gupta *et al.*, 2006).

There is no effective or approved *Brucella* vaccine to be used in humans, and therefore the control of the disease in animal reservoirs is paramount for suppression of human disease (Godfroid *et al.*, 2005; Gupta *et al.*, 2006).

Detection of *Br. melitensis*-antibodies in individuals of a sheep or goat flock is considered to be adequate for control measures to be initiated. Therefore, a particularly sensitive method of detecting *Br. melitensis*-antibodies can be more useful than a specific method, such as isolation of the causative agent, which should be used when an eradication program reaches its final stages (Lithg-Pereira *et al.*, 2001; Burriel *et al.*, 2004).

The diagnosis of brucellosis in dairy goats involves either the isolation of *Brucella* from milk samples or the detection of antibody in serum or milk. However, these methods are not wholly satisfactory. Bacteriological isolation is time consuming procedure and handling the micro-organism is hazardous. Serological methods are not conclusive, because not all infected animals produce significant levels of antibodies and because cross-reaction with other bacteria can give false negative results (Alton *et al.*, 1988).

The milk ELISA applied for detecting *Brucella* antibodies in lactating ewes was comparatively more sensitive and superior than MRT (Biancifiore *et al.*, 1996; Chand *et al.*, 2004). Therefore, it would be important to examine milk of does by milk-ELISA as has been used in bovine brucellosis.

Recently, the milk based PCR assay has been used for detection of *Brucella* sp. It is a promising alternative for conventional bacteriological techniques due to its speedy, safety, high sensitivity and specificity (Gupta *et al.*, 2006).

This research was carried out to evaluate the efficiency of different immunological tests, bacteriological examination and PCR in the diagnosis of caprine brucellosis from milk samples. Special interest was given for selecting the more useful test for the early detection of *Br. melitensis* in goats milk.

MATERIALS AND METHODS

This research was done in corporation with the Egyptian Veterinary Service Organization (Brucella Test and Slaughter Program) during the period from November 2004 to July 2005.

A total number of 129 milk samples collected from does reared in forms of small flocks at different localities in Kafr El- Sheikh Governorate (lies on the coast of the Mediterranean Sea) at different stages of lactation. These samples were examined immunologically, bacteriologically and by using PCR) for detection of Brucella infection.

For each test, equal quantities of milk from the right and left teats were pooled and positive and negative controls were used with each test run.

Immunological tests:

- Milk-Enzyme Linked Immunosorbent Assay (mELISA) was carried out according to Narayanan *et al.* (1983) using the Periplasmic Protein Antigen (SBP₅₀) prepared from *Br. abortus* S-19 (Zhan *et al.*, 1993). Optical densities equal to or higher than double of those of the negative controls were considered positive (Bassiri *et al.*, 1993).
- Modified Milk Ring Test (MRT) was performed according to Mikolon *et al.* (1998) using individual milk samples (1 mL) containing 300 µL of Brucella-negative cow cream. Thirty microliter of test antigen were added to each sample, mixed and incubated at 37°C and tests were read after 1, 3, 4 and 8 h. A positive sample was defined as one in which precipitation of the dyed antigen complex allowed whole or partial clearing of color from the milk or in which any clumping of dyed antigen occurred in the milk column. Weak positive reactions on the MRT were considered positive.
- Coombs Test (WCT) and Whey Agglutination Test (WAT): were carried out as outlined by Alton *et al.* (1988) using *Br. abortus* strain 99 antigen.

Bacteriological examination: Bacteriological examination of milk samples was performed according to Alton *et al.* (1988).

Polymerase chain reaction (PCR): Extraction of DNA from milk samples was done according to Gupta *et al.* (2006). Amplification and detection of *Br. melitensis* DNA was carried out as outlined by Bricker and Halling (1994). Amplification products (731 bp) were separated by electrophoresis through 1.5% agarose gel. To

determine the sensitivity of the PCR assay, 10-fold dilution series of *Br. melitensis* 16M were added to Brucella- free caprine milk. The final concentrations of *Br. melitensis* in milk were 1×10⁵, 1×10⁴, 1×10³, 1×10² and 1×10¹ CFU mL⁻¹. Brucella- DNA were extracted from aliquots of 0.5 mL from each dilution and processed by PCR as described above.

RESULTS

Table 1 shows that immunological tests and PCR were superior to bacteriological isolation for detecting brucella reactors in goat' milk. WAT and WCT give the highest incidence of positive reactors (9.3%) while the lowest incidence was given by bacterial isolation (3.8%). *Br. melitensis* biovars 3 was the only isolated strain.

Table 2 indicates that PCR showed the highest agreement (100%) with the bacterial isolation while WAT and WCT showed the lowest figures (60%).

Table 3 reveals that PCR and mELISA showed the highest specificity while, the WAT and WCT showed the lowest figures.

PCR was indicative of brucellosis in 6.2% of the does milk samples as shown in Fig. 1 by the typical PCR product specific for *Br. melitensis* (731 base pair).

Detection limits of PCR (Fig. 2) using decreasing numbers of *Br. melitensis* per milliliter of milk showed a high sensitivity limit of PCR of 1×10 CFU mL⁻¹ of does milk.

Table 1: Incidence of positive brucella reactors following examination of goat milk using different immunological tests, bacteriological examination and nested PCR

mELISA		MRT		WAT		WCT		Bact.Isol		PCR	
No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
9	6.9	11	8.5	12	9.3	12	9.3	5	3.8	8	6.2

No. of samples = 129

Table 2: Correlation between PCR and positive reactors of different immunological tests in bacteriologically positive does milk samples

Bact.Isol	mELISA		MRT		WAT		WCT		PCR	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
5 samples	4	80	4	80	3	60	3	60	5	100

Table 3: Correlation between PCR and negative reactors of different immunological tests in bacteriologically negative doe's milk samples

Negative bact.	mELISA		MRT		WAT		WCT		PCR	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
121 samples	120	99.1	118	97.5	117	96.6	117	96.6	121	100

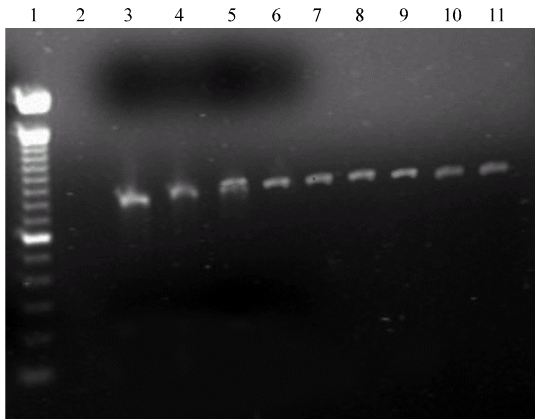


Fig. 1: PCR amplification of *Br. melitensis* DNA from milk samples. Lane 1: Molecular weight marker; Lane 2: Negative control; Lane 3: positive control with *Brucella* DNA; Lane 4-11: Milk sample DNA PCR

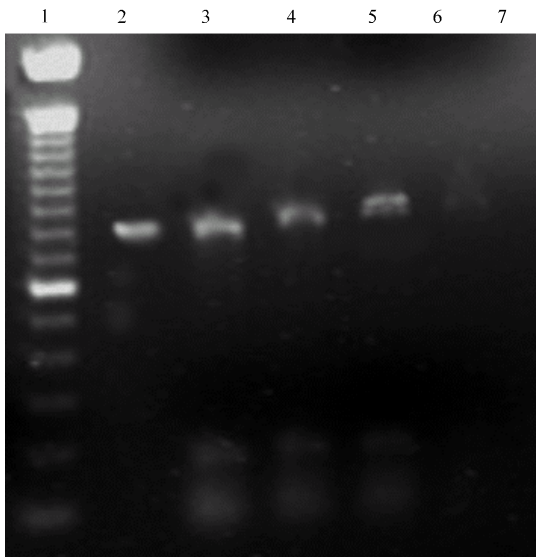


Fig. 2: Detection limits of *Br. melitensis* in milk samples by PCR. Lane 1: Molecular weight marker; Lanes 2-6: Containing decreasing numbers of *Br. melitensis* per milliliter of milk; Lane 2: 1×10^5 CFU mL⁻¹; Lane 3: 1×10^4 CFU mL⁻¹; Lane 4: 1×10^3 CFU mL⁻¹; Lane 5: 1×10^2 CFU mL⁻¹; Lane 6: 1×10^1 CFU mL⁻¹ and Lane 7: Negative sample

DISCUSSION

In *Brucella* infected goats, persistent infection of the mammary glands and supramammary lymph nodes is common with constant or intermittent excretion of the bacteria in the milk during the subsequent lactation

(Alton, 1985). In the same time, clinical signs of mastitis are seldom detectable in naturally infected goats (Alton, 1990) and this constitutes a serious human health hazard. Therefore, this work was carried on goat milk out to evaluate the efficiency of different immunological tests, PCR and bacteriological examination for detection of *brucella* reactors does.

Most of techniques that have been performed well in diagnosing bovine brucellosis (Gall, 2001; Godfroid *et al.*, 2005; Nielsen, 2002) have also been evaluated to a greater or lesser degree for diagnosis of the disease in sheep and goats (Biancifiori *et al.*, 2000; Durán-Ferrer *et al.*, 2002). However, the information available on performance characteristics of these tests is still limited and at times contradictory, (Garin-Bastuji *et al.*, 1998; Anonymous, 2001).

In normal lactating animals, almost all amounts of IgG in milk are derived from blood by a selective transfer (Norcross, 1982). Therefore, milk antibody levels reflect the serological status of the animal and can be safely considered for deciding an animal serologically positive or negative for brucellosis (El-Loly and Ghazi, 2002). Moreover, local antibody synthesis against mammary gland infection and secretion of the reactive antibodies in the milk cannot be ruled out (Chand *et al.*, 2005).

In this study, WAT and WCT showed the highest reactivity among immunological tests, PCR and bacterial isolation. This may be due to their high sensitivity and specificity and this is clear in the great difference between both tests from one side and the PCR in the other side. However, these tests seems more laborious and time consuming.

Despite, the milk ring test that is widely used to detect brucellosis in dairy cattle, it is not sensitive enough to detect brucellosis in sheep (Shimi and Tabatabai, 1981). However, because the test is simple and easy to perform it might be useful to detect *Brucella* antibodies in milk. MRT, like other immunological tests, detects *Brucella* antibodies in milk but its use in sheep and goats has not been recommended (Alton, 1987; OIE, 2000; FAO, 2003) due to the difference between the physiologic properties of goat and cow milk, the milk ring test does not perform well with goat samples .

Since the MRT do not usually result in ring formation in goat milk, many results may subjectively be considered positive. These include the formation of small dyed clumps under the cream layer, clumps in the cream layer and occasional true rings, but most commonly, different degrees of clearing of the milk as the dye precipitates. The problem is that all milk samples have some precipitate, so a subjective decision is made of how much whitening constitutes clearing. Also, milk samples, including

antibody-negative samples, will accumulate more precipitate of the dyed antigen with increasing incubation times. The MRT, therefore, pose substantial problems in standardization and have inadequate sensitivity and specificity (Mikolon *et al.*, 1998). The modification of MRT test performed here perhaps increased the sensitivity of the test but yet it yielded lower reactors than WAT and WCT. The condition may be due this test may yield false negative results in the early stages of *B. melitensis*-infected herds (FAO, 2003).

This study is the first report of using *Brucella* periplasmic protein antigen in Milk ELISA technique. However, it showed the lowest reactivity among all the milk immunological tests, this may be due to the increase in the specificity of the test is on account to its sensitivity. This was proved by the quite similarity in results of milk ELISA and PCR

Bacterial isolation resulted in identification of 5 *Brucella* isolates only out of 129 goat milk samples (3.8%). This low incidence may be due to false negative bacteriological result due to massive contamination of the milk samples, by the inhibition of some *Br. melitensis* strains in the selective medium or by a viability loss before culturing (Romero *et al.*, 1995). Moreover, it was found that nalidixic acid and bacitracin, at the concentration used in that medium, have inhibitory effects for some *Br. melitensis* strains (Marin *et al.*, 1996).

Finally, the present PCR succeeded to overcome many inhibitory substances that may be found in milk such as protein contamination, SDS and phenols (Romero *et al.*, 1995). Moreover, the used PCR protocol showed a sensitivity of 10 CFU mL⁻¹ of milk which is more than efficient in comparison with the bacterial examination. Also, it showed the highest agreement with the bacterial isolation while WAT and WCT showed the lowest one. This procedure also simultaneously determined the type of *Brucella* present in the milk of infected goats.

In conclusion, mELISA can be used as a screening test, followed by PCR as an ideal confirmatory test for fast and accurate diagnosis of caprine brucellosis in milk and possibly may substitute the bacterial isolation. Modified MRT still need further investigations in the field of caprine brucellosis diagnosis.

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