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Comparison of the Dot-immunobinding Assay with the Serum Agglutination Test, the Rose Bengal Plate Test and the Milk Ring Test for the Detection of *Brucella* Antibodies in Bovine Sera and Milk

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With 2 figures and 3 tables

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Summary

In this study, *Brucella* antibodies in bovine sera and milk were detected using the dot-immunobinding assay (DIA), the serum agglutination test (SAT), the Rose Bengal plate test (RBPT) and the milk ring test (MRT). For this purpose, a total of 116 paired blood and milk samples collected at the same time from 56 aborted and from 60 healthy dairy cows was examined. In DIA, a nitrocellulose membrane (NCM) was used as the solid phase. Antigen adsorbed on the NCM was extracted from *Brucella abortus S99* by heat treatment. The results obtained by DIA were compared with those of SAT, RBPT and MRT. Of the 116 paired blood and milk samples, 24 were positive and 72 were negative by all tests used. Serum samples of six aborted cows were positive by DIA, SAT and RBPT but the milk samples were negative by DIA and MRT. Serum and milk samples of four aborted cows gave positive reaction only by DIA tests. The remaining six aborted cows were negative only by MRT and two of them were negative by both RBPT and MRT. Four sera of healthy cows were found to be positive only by SAT.

Introduction

Brucellosis is an important zoonotic infection causing abortion and infertility in bovine. The isolation and identification of the micro-organisms from the infected animals are necessary for the diagnosis of the disease. But, Brucellae may not always be isolated from infected animals. Therefore, the detection of antibodies against Brucellae using serological tests is important for the diagnosis of brucellosis (BLOBEL and SCHLISSER, 1982). The serum agglutination test (SAT), the Rose Bengal plate test (RBPT), and the complement fixation test (CFT) are routinely used to detect *Brucella* antibodies in bovine serum. The milk ring test (MRT) is commonly used for detecting *Brucella* antibodies in milk (ALTON et al., 1988).

Several studies reported the use of various enzyme-linked immunosorbent assay (ELISA) tests for the detection of antibodies to *Brucella abortus* in both bovine sera (VAN AERT et al., 1984/1985; SUTHERLAND and HOLLANDER, 1986; MACMILLAN and BRADSHAW, 1991) and milk (HECK et al., 1980; KERKOFS et al., 1990). In these solid phase enzyme immunoassays, a polystyrene plate is widely used as the adsorbent matrix. Nitrocellulose and Nylon membranes were also found to be suitable as alternative solid phases (BLAIS et al., 1989). Immunoassay on such membranes was first described as the dot-immunobinding assay (DIA; HAWKES et al., 1982), and recently DIA using enzyme-conjugated antibody was termed dot-ELISA (KUMAR et al., 1985; CHAND et al., 1988). The assay does not require special laboratory equipment such

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as an ELISA reader compared with conventional ELISA and was found to be specific, sensitive, rapid and economical.

Recently, the dot-ELISA has been used for the diagnosis of rabies (HEBERLING et al., 1987), amoebiasis (KUMAR et al., 1985), for the detection of antibodies to bluetongue virus (AFSAR et al., 1992) and to rinderpest (RAMADASS et al., 1993) in cattle. The efficacy of dot-ELISA on nitrocellulose membranes (NCM) using soluble and whole *Brucella* antigens for the detection of *Brucella* antibodies in bovine and ovine sera has also been reported (CHAND et al., 1989a; GÜRTÜRK et al., 1997).

The present study was performed to evaluate the efficacy of DIA compared with SAT, RBPT and MRT for the detection of *Brucella* antibodies in paired sera and milk collected at the same time from aborted and healthy cows.

Materials and Methods

Serum and milk samples

A total of 116 paired serum and milk samples collected from 56 aborted and from 60 healthy dairy cows of different farms was examined. Vaccination against brucellosis was not being performed on these farms. The samples were collected from all aborted cows after 4 or 8 weeks of abortions. Paired blood and milk samples were taken at the same time from cows. After the testing of milk samples with MRT, the milk and serum samples were stored at −35°C until use.

DIA

Extraction of antigen. Antigen was extracted from *Brucella abortus S99*. The bacteria were cultivated at 37°C on tryptic soy agar supplemented with 2 % glycerol. After 72 h, the micro-organisms were harvested with 0.15 mol/l sodium chloride containing 0.5 % phenol and washed with the same buffer. The extraction was performed using the method as described previously (JIMENEZ DE BAGÜES et al., 1992). Briefly, 1 ml of *Brucella abortus S99* suspension at 0.2 g wet weight/ml of 0.15 mol/l sodium chloride was autoclaved and after centrifugation (4000 r.p.m., 45 min) the supernatant was precipitated with 3 ml of cold ethyl alcohol (5°C overnight). The resulting precipitate was recovered by centrifugation (4000 r.p.m., 15 min) and dissolved in 0.5 ml distilled water. The antigen was stored at −35°C until used.

DIA procedure. DIA was carried out as described previously (KUMAR et al., 1985). A NCM of 0.45 μm average pore size (Schleicher and Schüll, Dassel, Germany) was cut into pieces (0.5 × 0.5 cm) which were employed as the solid phase in the DIA. For the binding of the antigen on the NCM, NCM pieces were washed in distilled water and dried at room temperature (RT). Approximately 0.5 μl of antigen at a dilution of 1:1000 in phosphate-buffered saline (PBS; 0.01 mol/l pH 7.2) was dropped by means of a microcapillary into the centre of each NCM piece. They were kept at RT until dried and then incubated in PBS-T (PBS plus 0.05 % Tween 20) containing 5 % milk powder for 1 h at 37°C to block the nonspecific binding.

To test the serum and milk samples, the prepared NCM pieces were washed three times with PBS-T and incubated with dilutions of serum and milk samples in PBS-T for 30 min at 37°C. After washing five times with PBS-T, they were incubated with conjugate at a dilution of 1:500 in PBS for 30 min at 37°C. Horseradish peroxidase-conjugated rabbit antibovine immunoglobulin G (whole molecule) was used as a conjugate (Sigma, St Louis, USA). Finally, NCM pieces were washed again five times with PBS-T and the binding was revealed with freshly prepared substrate. In order to prepare the substrate, 3 mg of 4-chloro-1-naphthol (Merck, Darmstadt, Germany) was dissolved in 1 ml of methanol and then 5 ml of PBS and 0.02 ml 30 % hydrogen peroxide were added per ml of solution.

A positive reaction occurred as the development of a blue colour against a white background in a few minutes. Nitrocellulose pieces were washed in distilled water and dried at RT and the colour development on the site of the antigen was assessed. Colour development appearing as a circular dot did not change for a long time at RT. Colour development at 1:100 dilution of test sera and milk was considered as positive, and positive and negative controls were used for each test. A pool of paired sera and milk of three aborted cows with brucellosis was used as the positive control and a pool of 12 paired serum and milk samples taken from cows of a *Brucella*-free herd was the negative control.

The test was performed in a plastic plate with 24 wells 1.6 × 1.6 cm square and the reagents were taken in a volume of 1 ml. The optimal dilutions of antigen and conjugate were determined after preliminary experiments employing different dilutions of pooled negative and pooled positive sera in a chequer board analysis.

Other serological tests

SAT was performed as described previously (ALTON et al., 1988). The antigen for SAT was procured by Linear Chemicals (Badalona, Spain). The results of the tests were calculated in IU/ml using standard serum containing 1000 IU/ml. The standard serum was kindly supplied by the Animal Disease Research Centre Institute in Pendik, Istanbul and showed 50 % agglutination at the dilution of 1:640. The serum samples showing a titre of 100 IU/ml and above were considered positive in SAT.

The antigens for RBPT and MRT were kindly supplied by the Animal Disease Research Centre Institute in Pendik, Istanbul and the tests were performed according to their recommendations.

Results

Colour development with positive serum and milk was decreased with high dilutions of antigen. No colour reaction at the site of the antigen with negative serum and milk was observed even if low dilutions of serum and milk (1:25 or 1:50) were used (Figs 1, 2).

In total, 116 paired milk and serum samples from 56 aborted and 60 healthy cows were examined by DIA, SAT, RBPT and MRT for the detection of *Brucella* antibodies. The number of positive results with DIA, SAT and RBPT from sera of aborted cows were, respectively, 40, 36 and 34 and from milk samples, 34 were positive with DIA and 24 with MRT. Only four sera from healthy cows were found to be positive with SAT, but not with DIA and RBPT. All the milk samples from healthy cows were negative with DIA and MRT (Table 1).

The agreement of the results obtained by DIA with SAT, RBPT and MRT is given in Table 2.

When the results were compared, paired serum and milk samples of 24 cows were found positive with all the tests and 72 were negative with all the tests used. Sera of six aborted cows were positive by DIA, SAT and RBPT but the milk samples were negative by DIA and MRT. Serum and milk samples of four aborted cows gave a positive reaction only by DIA. The remaining six aborted cows were negative only by MRT and two of them were negative by both RBPT and MRT. Four sera of the healthy cows were found to be positive only by SAT (Table 3).

Discussion

Bacteriological cultures are necessary for the diagnosis of brucellosis but Brucellae cannot always be isolated from the clinical samples. Many serological tests have been developed for the diagnosis of brucellosis, but no single test can detect all infected cows and, therefore, combinations of serological tests are necessary to limit the false-negative results (BLOBEL and SCHLISSER, 1982). RBPT, SAT and CFT are the main serological tests used for the detection of *Brucella* antibodies in bovine sera. MRT used for the detection of *Brucella* antibodies in milk

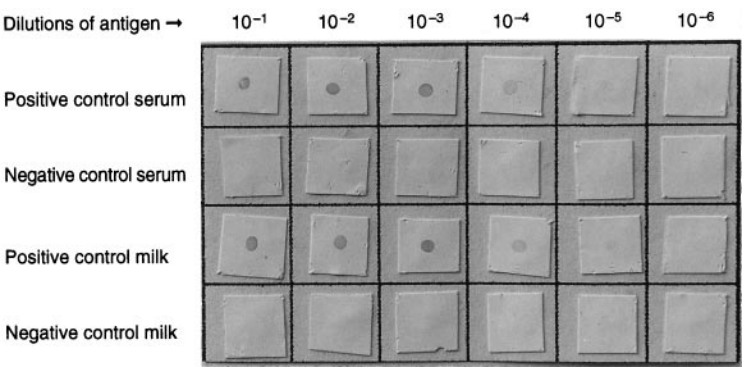

Fig. 1. The colour development in positive and negative control serum and milk with varying dilutions of antigen by dot-immunobinding assay

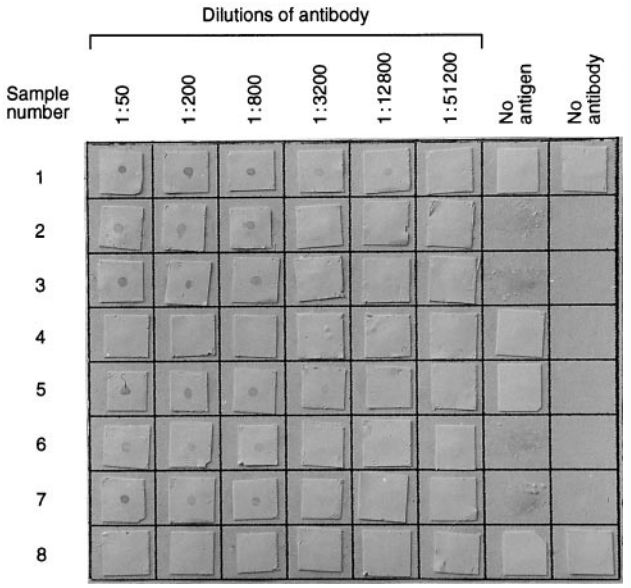

Fig. 2. The colour development in serum samples giving positive (1, 2, 3) and negative (4) reactions and in milk samples giving positive (5, 6, 7) and negative (8) reactions with varying dilutions of antibody by dot-immunobinding assay. No colour development was observed at the sites where no antigen was added or when incubated with PBS-T instead of serum and milk

Table 1. Results from 116 milk and serum samples obtained from 56 aborted and 60 healthy cows

Tests	Positive sera Aborted (n)	Healthy (n)	Positive milk Aborted (n)	Healthy (n)
DIA	40	0	34	0
SAT	36	4	ND	ND
RBPT	34	0	ND	ND
MRT	ND	ND	24	0

ND, not done; DIA, dot-immunobinding assay; SAT, serum agglutination test; RBPT, Rose Bengal plate test; MRT, milk ring test.

is found to be satisfactory to screen brucellosis in dairy herds (ALTON et al., 1988). Recently, ELISAs detecting *Brucella* antibodies in both sera and milk have been developed (HECK et al., 1980; VAN AERT et al., 1984/1985; SUTHERLAND and HOLLANDER, 1986; KERKOFS et al., 1990). Milk samples are easier and more economical to collect than blood. However, the disadvantage in the testing of milk is the lower concentration of immunoglobulin present in the milk as compared with sera (FLORENT et al., 1988).

In the present study, DIA using a NCM was applied for the detection of *Brucella* antibodies in sera and milk from aborted and healthy cows. Results from DIA were compared with those of SAT, RBPT and MRT. The assay was found to be more sensitive than SAT and RBPT for the detection of *Brucella* antibodies in sera from aborted cows. Whereas 40 sera were positive with DIA, 36 were found to be positive with SAT, and 34 with RBPT. CHAND et al. (1989b) also found dot-ELISA on the NCM more sensitive than SAT, RBPT and CFT for the detection of *Brucella* antibodies in bovine sera. The DIA also detected *Brucella* antibodies in milk from

Table 2. Agreement between the results obtained by DIA, SAT, RBPT and MRT

Results	Tests DIA_{serum}	DIA_{milk}	SAT	RBPT	MRT
DIA_{serum}					
Agreement					
Pos/pos		34	36	34	24
Neg/neg		76	72	76	76
Disagreement					
Pos/neg		6	4	6	16
Neg/pos		0	4	0	0
Total		116	116	116	116
% agreement		94.8	93.1	94.8	86.2
DIA_{milk}					
Agreement					
Pos/pos			30	28	24
Neg/neg			72	76	82
Disagreement					
Pos/neg			4	6	10
Neg/pos			10	6	0
Total			116	116	116
% agreement			87.9	89.7	91.4

DIA, dot-immunobinding assay; SAT, serum agglutination test; RBPT, Rose Bengal plate test; MRT, milk ring test.

Table 3. Comparison of the results obtained by DIA and SAT, RBPT and MRT used for the detection of *Brucella* antibodies in bovine sera and milk

Tests RBPT	SAT	DIA_{serum}	MRT	DIA_{milk}	Aborted cows (n)	Healthy cows (n)	Total (n)
+	+	+	+	+	24	0	24
+	+	+	–	+	4	0	4
–	+	+	–	+	2	0	2
+	+	+	–	–	6	0	6
–	–	+	–	+	4	0	4
–	+	–	–	–	0	4	4
–	–	–	–	–	16	56	72
Total					56	60	116

DIA, dot-immunobinding assay; SAT, serum agglutination test; RBPT, Rose Bengal plate test; MRT, milk ring test.

aborted cows more efficiently than the MRT because 34 samples were positive in DIA but only 24 were positive with MRT. KERKOFS et al. (1990) also reported that ELISA detecting IgG1 in milk was 20 times more sensitive than MRT.

All the sera from healthy cows were negative with DIA and RBPT but four sera were found to be positive only with SAT. All the milk samples from healthy cows were negative with DIA and MRT. This shows that the specificity of DIA was comparable to SAT and RBPT and MRT. A high positive correlation between serological tests and ELISA on milk samples has also been described (HECK et al., 1980; KERKOFS et al., 1990). Four sera from healthy cows giving a positive reaction showed a low titre (125 IU/ml) of SAT and therefore the reaction

could be false positive. It has also been reported that 2% of the SAT positive cases could be nonspecific (VAN AERT et al., 1984/1985; CHAND et al., 1989b).

The sensitivity of the DIA detecting antibodies in sera appeared to be better than the DIA used for milk. Sera from six cows were found to be positive by DIA, SAT and RBPT but the milk samples were negative by DIA and MRT (Table 3). It is likely that these six milk samples gave negative results due to the lack of antibodies in the milk. However, serum and milk samples of four cows gave positive reaction only with DIA, but SAT, RBPT and MRT failed to detect antibodies in these samples. This could be due to the high sensitivity of DIA. But why DIA failed to detect antibodies in milk samples of six cows in which sera antibodies were detected by DIA, SAT and RBPT, is not clear, although IgG1 antibodies in milk could be detected by DIA as compared with MRT. The disagreement between MRT and serological tests was explained by the fact that MRT and other serological tests do not detect the same antibody classes. In a positive MRT, IgG2 and IgA play an active role, whereas IgG1 antibody is not directly implicated in the ring formation (SUTRA et al., 1986). It has been reported that the antibody titres to some viral infections (enzootic ovine leucosis, caprine arthritis encephalitis virus) in milk samples are $\approx$10% of those in sera collected at the same time (FLORENT et al., 1988; MOTHA JULIAN and RALSTON, 1994).

The results of this study showed that the specificity of DIA is comparable with SAT, RBPT and MRT for the detection of *Brucella* antibodies in bovine sera and milk. The DIA detecting antibodies in sera appeared to be more sensitive than SAT, and RBPT. In addition, DIA would be more suitable for the detection of *Brucella* antibodies in milk as compared with MRT, with which the usability is limited, e.g. chilling of the milk. It is concluded that DIA on NCM could be used in combination with SAT, RBPT and MRT for the detection of *Brucella* antibodies in both bovine sera and milk.

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