Dept. Vet. Med.
Fac. vet. Med. Edfina, Alexandria University
Head of Dept. Prof. Dr. F.R.A. Slem

SEROLOGICAL DIAGNOSIS OF BRUCELLOSIS IN CATTLE AND SHEEP USING MONOCLONAL ANTIBODIES

(With 2 Tables & One Fig.)

By

A. ZAGHAWA; S.A. HATTAB; I. GRIESER WILKE and V. MOENNIG

(Received at 9/3/1993)

التشخيص السيرولوجي للاجهاض المعدى في الماشيه والأغنام بإستخدام الأجسام المناعيه المونوكلونال

أحمد زغاوة ، سيد حطب ، ارينا جريسرفلكا ، فولكر مينج

تم تركيز الأجسام المناعيه المونوكلونال المنتجه فى الجرذان ضد ميكروب البروسيلا المالطيه ١٦ م ، هذه الأجسام المناعيه المونوكلونال تتفاعل فقط مع الأنواع المختلفه من البروسيلا. تم إدماج هذه الأجسام المناعيه بانزيم البيروكسيديز حيث أستخدم المدمج الكشاف فى التشخيص السيرولوجى للإجهاض المعدى فى الماشيه والأغنام بطريقة الأليزا التسابقيه. تم تجميع ٥٤ عينه سيرم من الماشيه و٣٨ عينة سيرم من أغنام معروفه بأنها مصابه بالإجهاض المعدى. فحصت هذه العينات بطريقة الأليزا التسابقيه بإستخدام الأجسام المناعيه المونوكلونال وكذلك بطريقة روز بنجال. أسفر اختبار الأليزا التسابقيه عن نسبة ٧٤ ٪ إيجابيه فى الماشيه و٧٦٫٣ ٪ إيجابيه فى الأغنام بينما كانت نسبة الإيجابيه فى اختبار روز بنجال ٥٧٫٧ ٪ فى الماشيه و ٦٥٫٧ ٪ فى الأغنام. برهنت طريقة الأليزا التسابقيه على درجه عاليه من التخصص حيث وصلت ٩٥٫٤ ٪ كذلك فإن الاختبار له درجه عاليه من الحساسيه حيث أمكن بهذه الطريقه أكتشاف حالات إيجابيه بها كميه صغيره من الأجسام المناعيه. أوضحت الدراسه أن اختبار الأليزا التسابقيه يمكن إستخدامه فى برنامج المقاومه للأجهاض المعدى وذلك للحساسيه وكذلك التخصصيه العاليه للاختبار.

SUMMARY

Concentration of monoclonal antibodies raised in mice against Brucella melitensis 16 M has been made. These monoclonal antibodies reacted only with Brucella species and not with other genera of Gram-negative bacteria. The monoclonal antibodies had been conjugated with horse radish peroxidase in order to use in the competitve Enzyme Immunosorbent Antibody (c ELISA) for the serological diagnosis of brucellosis in cattle and sheep. Fifty four and 38 serum samples were collected from cattle and sheep with previous history of brucellosis. The samples were tested with the c ELISA and the ROSE Bengale test. The percentage of positive with c ELISA were 74% and 76.3% for cttle and sheep respectively, while the percentages of positive cases with Rose Bengale test were 53.07% and 68.3% for cattle and sheep respectively. The specificity percent of the cELISA was 97.6% and the test was found to be very sensitive to detect very small amounts of antibodies in the sera. The competitive ELISA test can be used for the eradication program for brucellosis as it is very sensitive and specific for such purpose.

INTRODUCTION

Brucellosis is a widespread disease and of major economic importance in most countries of the world. Losses due to decreased milk production as well as the common sequel of abortion can be of economic importance concerning brucellosis. From the viewpoint of human health, the disease is important because the causitive organism can cause undulent fever in man. The possibility of infection occurring by the drinking of infected unpasteurized milk, veterinarians and butchers. The importance of the disease in humans is an imprtant justification for its eradication (Blood and Radostitis, 1989). The eradication program of the disease depend on serological testing and culling of positive cases.

Serological cross reactions had been demonstrated between smooth Brucella strains and Escherichia coli, Francisella tularensis, Salmonella serotypes, Pseudomonas maltosida, Vibrio cholerea and Yersinia enterocolitica (*NILSON et al., 1981; CAROFF et al., 1984 and CORBEL, 1984).* Exposure to these bacteria by oral or parentral routes can provoke diagnostically

significant titers of antibodies cross-reacting with Brucella using agglunination or other serological tests.

The complement fixation test has been reported to be successful in the eradication of brucellosis. However the test is complex and has other disadvantages, such as prozone phenomenon, incompatability with haemolysed or anticomplementary sera and lack of sensitivity *(RIS et al., 1984 and SPENCER & BURGESS 1984).*

The Enzyme Linked Immunososbent Assay (ELISA) using a wide range of antigens has been evaluated for the diagnosis of brucellosis *(BERVOVICH & TAAIJKE, 1990; MARIN et al., 1989 and CHIN et al., 1991).*

The production of monoclonal antibodies against single Brucella specific antigens has been made to overcome these difficulties. *GRIESER-WILKE et al. (1985)* produced monoclonal antibodies against Brucella by immmunization of mice with acetone fixed *Brucella melitensis* 16 m. Two monoclonal antibodies reacted specifically with Brucella melitensis , Brucella abortus and Brucella suis. On the other hand they did not react with other species of Gram-negative bacteria.

The aim of the present work was planned to apply one of these monoclonal antibodies for diagnosis of brucellosis in cattle and sheep under field conditions in Egypt. The monoclonal antibodies were conjugated with horse radish peroxidase and finally the application of the more advanced type of ELISA (competitive ELISA) for the diagnosis of brucellosis in comparison with Rose Bengale test which is one of the field agglutination tests.

MATERIAL AND METHODS

Serum samples:

Buffered acidified *Brucella abortus* antigen was obtained from LTP, Department diagnostic laboratories, France. The test was applied by adding equal amounts (0.03 ml) of buffered antigen and serum on a glass slide shaking for 30 second and finally reading by observation of agglutination. The degree of affection was recorded according to the following schedule:

+++ = strong positive agglutination
++ = moderate positive agglutination
+ = slight positive agglutination
± = suspicious reaction

Production and Concentration of Serum Free-Monoclonal Antibodies:

Cloned antibody-producing hybridoma cells were prepared according to the method of *GRIESER-WILKE et al.,(1985).* The

cell suspension was transferred to roller bottles (Falcon 30327) and the pH was adjusted to 7.4, after which incubation was followed for 3-4 days in 5% CO2. The cell debris was removed and the antibody titer was controlled by testing. The supernatent containing antibodies was concentrated one hunderdfold by ultrafiltration using an Amicon PM 10 membrane.

Peroxidase labelling of monoclonal antibodies:

Concentrated monoclonal antibodies were conjugated with horse radish peroxidase (obtained from Sigma company) according to the method of *BOORSMA and STEFKREK (1979)*. The protein enzyme ratio was adjusted to be 3:2.

Competitive enzyme linked immunosorbent antibody (c ELISA):

Lipopolysaccharide extracted from *Brucella abortus* strain 99 by the hot phenol-hot water method was used to coat the U-shape microtiter plate in which 0.1 ml of the antigen was placed in each well and left oveneight at 4 C. After incubation the plates were washed with phosphate buffer saline (PBS)-tween. Serial twofold serum dilutions were made in PBS-tween (1:2-1:256). 0.1 ml of the diluted serum was placed in each well and immediatly 0.1 ml of a peroxidase conjudated anti Brucella monoclonal antibodies (mab BM40) in a dilution of 1:400 was added to all wells and the plates were incubated for 2 hours at 37 C. Negative and positive controls were setted in one plate for each test.

After incubation the plates were washed 3 times with PBS-tween (5 minutes intervals) and finally one time with distilled water. 0.1 ml of the chromogen (substrate) which is 2,2-Azido-di (3-ethylbezthiazolin-sulphoric acid) freshly prepared was added for each well and the plates were left for 15 minutes till the green colour appear. At that time stopping solution was added which was Citric acid 0.1 M to stop the reaction. The evaluation of the test was made by using an automatic ELISA reader from Behring company at a wave lengh 405. The lowest serum dilution showing an optical densty (OP) of more than 50% of the average OD of several control wells containing conjugate alone was considered end point.

RESULTS

The detection of antibodies to *Brucella species* in cattle and sheep with a previous history of brucellosis was made by the commercial Rose Bengale agglutination test. The same samples were subjected to titeration of antibodies to *Brucella species* using monoclonal antibodies in the advanced type of

ELISA (c ELISA). The results are shown in tables (1&2) as well as Fig. (1). From table (1), it is clear that a total of 29 samples from cattle were positive in Rose Bengale test from which 13 strongly positive, 8 moderate positive and 8 slightly positive. A total of 22 samples were negative and 3 samples were suspicious. Concerning the antibody titers using c ELISA, it was found that 14 samples were negative and 40 were positive from which 4, 5, 8, 5, 5, 5, 3 and 5 had antibody titers of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 respectively. Concerning sheep sera as shown in table (2) using Rose Bengale test, out of 38 samples 12 samples were negative, and 26 were positive. From the positive samples 9 were strong positive, 12 were moderate positive and 5 were slightly positive. On the other hand the results of c ELISA revealed that 9 samples were negative and 29 were positive from which 2, 5, 5, 3, 6, 5, and 3 had antibody titres of 1:2, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256 respectively.

Specificity of C ELISA:

A total of 86 serum samples collected from Brucella free sheep were collected. All the sera were negative when tested with tube agglutination test as obtained from the farm records. From these samples 84 were negative when tested the c ELISA and the specificity of the c ELISA was calculated according to *MARIN* et al. *(1989)* as follows:

$$\text{Specificity\%} = \frac{\text{Number of sera-ve from uninfected animal}}{\text{Total number of uninfected animals}} \times 100$$

$$= \frac{84}{86} \times 100 = 97.6\%$$

Percentage of agreement between c elisa and rose bengale test:

The percentage of agreement was calculated according to *NOON* et al. *(1980)* as follows:

Number of sera in each group	Serum reaction in result of C ELISA	Rose Bengale	End Result
20 (12 cattle & 8 sheep)	Negative	Negative	
55 (30 cattle &25 sheep)	Positive	Positive	Total agree = 75
1 (1 cattle)	Negative	Positive	
13 (9 cattle & 4 sheep)	Positive	Negative	
3 (3 cattle)	Positive	suspicious	Total disagree=17

$$\% \text{ of agreement} = \frac{\text{Total agree}}{\text{Total sera tested}} \times 100$$

$$= \frac{75}{92} \times 100 = 81.5$$

DISCUSSION

The indirect diagnosis of brucellosis depending on the detection of antibodies to Brucella remains a problem in this field. This is actually due to the high percentage of both false positive and false negative results obtained by different serological tests. The false negative results are probably due to the presence of small amounts of antibodies which could not be detected by the traditinoal serological tests. On the other hand the false positive results may be due to the cross antigenic reactivity of Brucella species and organism of other genera as Pasteurella species, Proteus species, Salmonella serotypes, *Bordetella bronchoseptica, Vibrio cholerae and Yersinia enterocolitica (STRAUT & CORBEL, 1982, NILSON et al., 1980 and CORBEL 1985).*

The results of antibody detection in serum samples of cattle and sheep with a history of brucellosis using Rose Bengale Test are summerised in tables (1&2). The percentage of positive were 53.7% and 68.3% for cattle and sheep respectively. From the same tables (1&2), using the cometitive ELISA the percentage of positive were 74% and 76.3% for cattle and sheep respectiviely. *GRIESER-WILKE et al. (1991)* examined 2 herds of cattle in Germany for brucellosis with the competitive ELISA and the serum agglutination test. They recorded the percentag of positive 43.4% and 45.6% using competitive ELISA and 28.4% and 32.6% using the serum agglutination test.

The higher sensitivity of other types of ELISA known as Dot ELISA in comparision to serum agglutination test was recorded by *LIU et al. (1991).* in which the percentage of postitive in Dot ELISA was 64% in comparision to 52.7% for serum agglutination test. These results agreed with those recorded by *GRIESER-WILKE et al. (1991)* and supports our results concerning the higher sensitivity of the competitive ELISA in comparision to Rose Bengale test. This higher sensitivity is very essential for detection of very small amounts of antibodies in infected animals in the eradication program for brucellosis. Regarding specificity percent of the competitive ELISA using the monoclonal antibodies, it was calculated as 95.4%. This high specificity of the test is

probably due to the specificity of the monoclonal antibodies used in this test which reacted only with Brucella species and not with other genera of Gram- negative bacteria (*GRIESER-WILKE et al., 1985*). From our results, it is clear that the antibody titers obtained by the competitive ELISA correlates very good with the legree of positivity (strong, moderate and slight) in Rose Bengale test. The strong positive (+++), moderate positive (++) and the slight positive (+) in Rose Bengale test crresponds antibody titers of 1:64-1:256, 1:16-1:32 and 1:4-1:8 in competitive ELISA respectively. These results are supported by *TIZARD (1992)* who stated that the antigen antibody reaction depended mainly on the concentration of both antigens and antibodies. Again the percentage of agreement between the competitive ELISA and the Rose bengale test was 81.5%. This agree with the result obtained by *JEMENEZ DE BAGUES et al. (1992)*.

It can be concluded that, the competitive ELISA using monoclonal antibodies can recognise the false negative as it detects very small amounts of antibodies. The test also excludes the false positive results by its higher specificity as the monoclonal antibodies reacted only with Brucella species and not with other genera of Gram-negative bacteria, although the production of monoclonal antibodies specific for each type of Brucella species is essentially needed in the field of Brucella diagnosis.

REFERENCES

Bercovich, Z. and Teaijke, R. (1990): Enzyme immunoassay using mouse monoclonal anti-bovine antibodies for the detection of Brucella abortus antibodies in cow milk. J. Vet. Med. B 37, 753-759.

Blood, D.C. and Radostitis (1989): Veterinary medicine, a textbook of the diseases of cattle, sheep, pigs, goats and horses. Seventh edition, ISBN, Great Britain.

Boorsma, D.M. and Strefkrek, J.G. (1979): Peroxidase or glutaraldehyde for preparing peroxidase conjugate? J. Immunol. Methods 30, 245-255.

Caroff, M.; Bunde, D.R. and Perry, M.B. (1984): Stucture of the O-chain of the phenol phase soluble cellular 8 lipopolysaccharide of Yersinia enterocolitica serotype O:9 EUr. J. Biochem. 139, 195-200.

Chin, J.C.; Pang, B. and Carrigon, M. (1991): Comparision of seroreactivity of rams with Brucellosis in complement fixation test, whole cell ELISA and by immunoblotting. Vet. Micrbiol. 26, 291-299.

Corbel, M.J. (1984): Evaluation of serological procedures for the differentiation of cross-reactions to Brucella. International Symposium on human and animal Brucellosis, Taipei, Taiwan. 2.

Corbel, M.J. (1985): Recent advaces in the study of Brucella antigens and their serological cross reactions. Vet. Bulletin 55, 927-942.

Grieser-Wilke I.; Moennig, V.; Thon, D. and Rauter, K. (1985): Characterization of monoclonal antiobodies against Brucella melitensis. J. Vet. Med. B. 32 616-627.

Griesser-Wilke, I.; MaacMillan, A.P. and Moennig, V. (1991): A competitive enzyme immunoassay with monoclonal antibodies for the analysis of sera from cattle of two herds with suspected Brucellosis. Tieraerztliche Praxis 19, 131-135.

Jemenez De Bagues; M.P.; Marin, C.P.; Blasco, J.M; Moriyon, I. and Gamazo, C. (1992); An ELISA with Brucella lipoplysaccharide antigen for the diagnosis of B. melitensis infection in sheep for the evaluation serological responses following subcutaneous or conjuntival B. melitensis strain Rev 1 vaccination. Vet. Microbiol. 30, 233-241.

Liu, Z.w.; Zhang, L.G; Yan, S.D.; Shen, Z.L.; Zhao, X.W.; Wang, X.Z.; Zhu, H.C and Liu, Z.T, (1991): Rapid detection of Brucella ovis infection using Dot ELISA. Chinese J. of Vet. Sci. and Technolo. 21, 36-36.

Marin, C.M. Jimenz, M.P; Blasco, J.M; Gamazo, C; Moriyon, I and Diaz, R (198(): Comparision of three serological tests for Brucella ovis infection of rams using different antigenic extracts. Vet. Rec., 125, 504-508.

Nilson, K., Samagh B.S., and Stemshon, B., (1980): Agglutination of Brucella abortus cells by sera from cattle experimentally infected with Escherichia coli. Vol. Microbiol. 5, 123-124.

Nilson, K.; Ruckerbauer, G.M., and Samagh, B.S., (1981): Serologic response to Brucella abortus of cattle and guinea pigs experimentally with a Yersinia enterocolitica serotype from milk. Comparative Immunol, and Microbiol, and Infectious Diseases 4, 59-64.

Noon, K.F.; Rogul, M.; Binn, L.N.; Keefe, T.J.; Marchwicki, R.H., and Appel, M.J., (1980): Enzyme-Linked Immunosorbent Assay for evaluation of antibody to canine distemper virus. Am. J. Vet. Res. 41, 605-609.

Ris, D.R., Hamfi, K.I. and Iong, D.I., (1984): Comparison of an Enzyme-Linked immunospecific (ELISA) with the cold

coplement. New Zealand Vet. J. 32, 18-21.

Spencer, T.I. and Burgess, G.W., (1984): Enzyme-Linked Immunosorbent Assay for brucella ovis antibody in ram sera. Research in Vet. Sci. 36, 164-167.

Straut, F.A. and Corbel, M.J., (1982): Identification of a serological cross-reaction between Brucella abortus and Escherichia coli O: 157. Vet.Rec. 110, 202-203.

Tizard, I.R. (1992): An introduction to veterinary immunology. ISBN Saunders publicher, Philadelephia, USA.

Table (1): Detection of Brucella antibodies in sera of cattle with a previous history of brucellosis using competitive ELISA and Rose Bengale Tests.

Serial Number	Antibody titre # with C ELISA	Rose Bengale	Serial Number	Antibody titers with C ELISA	Rose Bengale
1	2	-ve	28	2	-ve
2	128	+++ve	29	4	+ve
3	0	-ve	30	0	±ve
4	0	-ve	31	64	+++ve
5	16	++ve	32	64	+++ve
6	32	++ve	33	8	+ve
7	2	-ve	34	0	-ve
8	4	+ve	35	8	++ve
9	8	-ve	36	16	+ve
10	256	+++ve	37	0	-ve
11	256	+++ve	38	0	-ve
12	256	+++ve	39	32	++ve
13	16	+ve	40	16	++ve
14	0	-ve	41	256	+++ve
15	0	-ve	42	256	+++ve
16	32	++ve	43	0	-ve
17	32	++ve	44	64	+++ve
18	4	-ve	45	0	±ve
19	16	+ve	46	64	+++ve
20	0	-ve	47	0	-ve
21	8	+ve	48	4	±ve
22	2	-ve	49	4	-ve
23	0	-ve	50	128	+++ve
24	0	-ve	51	128	+++ve
25	32	++ve	52	8	+ve
26	8	-ve	53	8	+ve
27	64	+++ve	54	8	-ve
Total +ve	40	29	% of +ve	74%	53.7%
Total -ve	14	22			
Susp.	-	3			

Expressed as reciprocal serum dilution

cELISA = Competitive Enzyme Linked Immunosorbant Antibody.

+++v = Strong positive agglutination

++ve = Moderate positive agglutination

+ve = Slight positive agglutination

± = Suspicious reaction.

Table (2): Detection of Brucella antibodies in sera of sheep with a previous history of brucellosis using competitive ELISA and Rose Bengale Tests.

Serial Number	Antibody titre * with C ELISA	Rose Bengale	Serial Number	Antibody titers with C ELISA	Rose Bengale
1	16	-ve	20	128	+++ve
2	8	+ve	21	0	-ve
3	0	-ve	22	2	+ve
4	0	-ve	23	16	++ve
5	64	++ve	24	8	-ve
6	0	-ve	25	8	-ve
7	0	-ve	26	64	++ve
8	0	+ve	27	64	+++ve
9	128	+++ve	28	64	++ve
10	2	-ve	29	32	++ve
11	16	+ve	30	32	++ve
12	64	++ve	31	0	-ve
13	256	+++ve	32	0	-ve
14	8	+ve	33	0	-ve
15	8	++ve	34	256	+++ve
16	16	++ve	35	128	+++ve
17	16	++ve	36	256	+++ve
18	128	++ve	37	64	+++ve
19	128	+++ve	38	32	++ve
Total +ve	29	26	% of +ve	76.3%	68.3%
Total -ve	9	12			
Susp.	-	-			

* =Expressed as reciprocal serum dilution
cELISA = Competitive Enzyme Linked Immunosorbant Antibody.
+++ve = Strong positive agglutination
++ve = Moderate positive agglutination
+ve = Slight positive agglutination
± =Suspicious reaction.

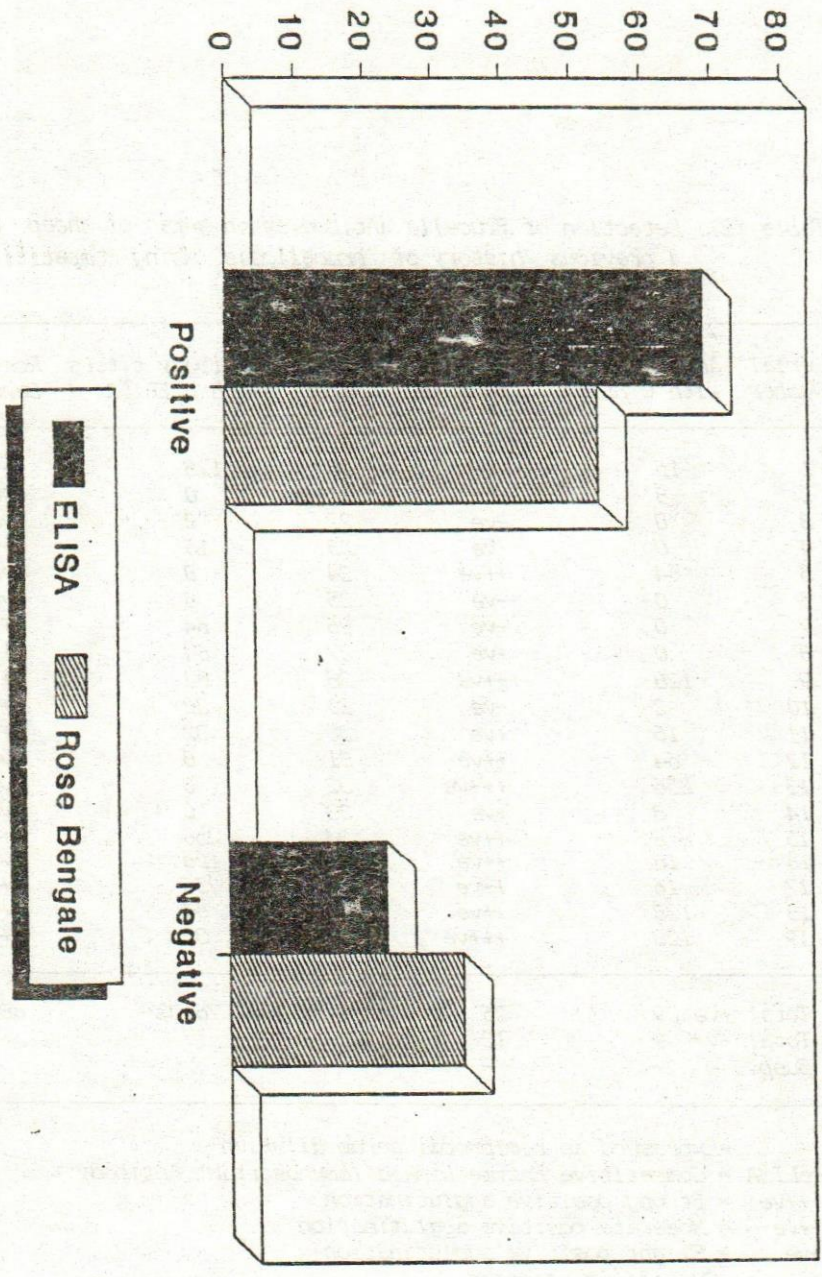

Fig. (1): Total Brucella positive and negative serum samples collected from cattle using the competitive ELISA and Rose Bengale tests.