Assiut Vet. Med. J. Vol. 38 No. 76, January 1998

Division of Microbiology
Defence Research and Development Establishment
Gwalior 474002, India.

DETECTION OF BRUCELLA ANTIBODIES IN COW MILK WITH DOT-ELISA KIT

(With 1 Table)

By
H.V. BATRA, G.S. AGARWAL and KIRON BALA
(Received at 3/12/1997)

الكشف عن الأجسام المضادة للبروسيلا في لبن البقر باستخدام الإليزا النقطية

باتــرا ، أجــاروال ، كيرون بالا

تم أختبار عدد ١٤١ عينة لبن من أبقار تعاني من مشاكل تناسلية من مزرعة موبوءة بالبروسيلا وكذلك عدد ٥٠ عينة لبن من أبقار سليمة ظاهريا ومن مزرعة خالية من البروسيلا وذلك بإستخدام أختبار الإليزا النقطية الحديثة.كما تم إختبار عينات مصل نفس الأبقار بإختبارات تلازن المصل وتثبيت المتمم وإليزا الأطباق. وأظهر الأختبار سلبية الخمسين عينة التي جمعت من المزرعة الخالية من البروسيلا ، كما ظهرت سلبية عينات مصل نفس الحيوانات. كذلك تبين أن عينات لبن الأبقار من المزرعةالموبوءة كان بها ٩٥ عينة لبن إيجابية بالإختبار المذكور بينما ظهر أن ٩٣ عينة من مصل هذه الحيوانات كانت موجبة بإختبار إليزا الأطباق و٧١ عينة كانت موجبة بإختبار تلازن المصل. وكانت ٨٨ عينة من المصل موجبة بإختبار تثبيت المتمم من مجموع ١٣٤ عينة وأظهرت ٧ عينات نشاطا مضاداً لهذا الإختبار. كما لوحظ وجود إرتباط واضح للشكف عن الأجسام المضادة للبروسيلا في لبن البقر بإستخدام أختبار الإليزا النقطية مع نتائج أختبار المصل بإختبارىإليزا الأطباق وتثبيت المتمم. كما أمكن التوصل إلى أن بساطة وملاءمة إختبار الإليزا النقطية مقترنة بحساسيته وتخصصه توضح فائدته في الكشف الحقلي الروتيني لعينات لبن الأبقار في حملات أستبيان مرض البروسيلا.

SUMMARY

Milk samples from 141 cows with gynaecological problems in a brucellosis endemic farm and from 50 apparently healthy cows of brucella free farm were tested with a newly developed field dot-ELISA kit. Serum samples of the same animals were checked for antibody titres with serum agglutination

test (SAT), complement fixation test (CFT) and the plate enzyme linked immunosorbent assay (ELISA). The kit detected as negative all the 50 milk samples from brucellosis free farm. The corresponding serum samples of the cows were also found negative to the other serological tests. Of the 141 cows belonging to endemic farm, kit detected 95 as positive on milk testing, plate ELISA and SAT found 93 and 71, respectively, as positive, on sera testing. CFT was positive in 88 cases out of the 134 cows with 7 animals exhibiting anti-complementary activity. Detection of brucella antibodies in milk by the kit from individual cows appeared to correlate well with the serological results of the cows obtained by the plate ELISA and the CFT. The simple and convenient process of testing by the kit coupled to its high sensitivity and the specificity suggests its usefulness in routine field testing of milk samples from individual animals for the brucellosis disease surveillance programs.

Key words: Brucellosis - Cow milk - Antibodies - ELISA

INTRODUCTION

Brucellosis is a widely prevalent bacterial zoonotic disease and infection to human can invariably result from consumption of raw milk from the infected animals (Schoerner et al., 1990 and Sutherland and Vet Bull, 1980). Dairy products prepared from unpasteurized milk also tend to be an important source of human infection (Sutra et al., 1986). Serological tests have widely been employed for identification of brucella infected individuals as isolation of the causative brucella organisms from cases is difficult to achieve and is also tedious and time consuming process (Corbel et al., 1984). Detection of brucella antibodies in serum samples has been accomplished by a variety of serological tests that include agglutination reactions, complement fixation test, fluorescent antibody test, enzyme immunosorbent assays and radio immuno assays (Havaldar et al., 1987; Young et al., 1975; Batra et al., 1989; Lawman et al., 1984; Keer et al., 1959; Kerkhofs et al., 1990 and Bercovich and Taaijke, 1990).

In cattle, testing of milk for brucellosis is more economical than serum testing, because milk is readily available in the dairy farms. Milk ring test (MRT) is the conventional test employed for antibody detection in milk for disease surveillance in many countries. In conditions when milk contains the colostrum, milk examined at the end of lactation period, milk from cows

with hormonal disorders and in milk where fat globules do not cluster, MRT tends to yield non-specific results and is not a favoured test for individual milk sample testing (WHO, Dhar et al., 1988; Bercovich et al., 1979 and Heck et al., 1980).

The ELISA meant to detect antibodies in the milk from individual cows is sensitive as well as specific (Araj et al., 1989; Sutherland and Vet Bull, 1980; Boraker et al., 1981; Alton et al., 1975 and Corbel et al., 1984) and will help in detecting antibodies 15 days to 6 months earlier than detection by MRT (Ray). For more practical utility, ELISA is suitable for field application and affordable. A new rapid and simple dot-ELISA kit for brucella antibody detection in milk was developed for routine field use and the test results with the kit were compared with SAT, CFT and plate-ELISA.

The present work was planned to develop field based simple, sensitive and specific ELISA system for brucella antibody detection in milk samples of individual cattle and to undertake the comparative evaluation of this kit vis a vis the known serological tests.

MATERIALS and METHODS

Serum and Milk samples. A total of 141 lactating cows from a brucellosis endemic farm having a history of abortion stillbirth, retention of placenta and/or infertility during the last two years period were tested for brucella antibodies. Serum and milk samples from 50 apparently healthy cows from a brucellosis free herd were also collected for testing. Blood samples and the milk samples from the cows were collected on the same day. Milk samples were tested by the kit and the corresponding serum samples were processed by SAT, CFT and plate ELISA.

Serum agglutination test. Antigen for serum agglutination test (SAT) was procured from the Indian Veterinary Research Institute, Izatnagar, Bareilly, India and the test was performed as described previously (Patterson et al., 1976) and sera showing a titre of 1:40 and above were considered positive in SAT.

Plate ELISA. Soluble antigens from sonic extract of *B. abortus* S-99 were coated on 96-well polyvinyl microtitre plates (Titertek) at concentration of 10 ug/ml in PBS, 50 ul per well. Two wells in each plate were kept as plate control with PBS added at 50 ul/well. Plates were incubated at 4°C overnight and then blocked with 2% bovine serum albumin in PBS containing 0.1% Tween 20 (PBS-T) with 200 uls of blocking buffer

per well. After rinsing in PBS-T three fold dilutions of individual serum samples i.e.,1:200, 1:600 and 1:1800 and 1:5400 were added into the plates coated with the antigen in 50 ul/well volumes. The same first three dilutions of a known standard hyperimmune sera were also included for each plate as high control. Plates were incubated at 37°C for 1 hour in an air tight humid chamber. Five washings were given in PBS-T and plates were incubated with anti-cow peroxidase conjugate (Dakopatts), 1:2000 dilution in PBS-T, in the amounts of 50 ul/well. The plates were incubated at 37°C for 1 hour in the humid chamber. Following incubation, plates were again washed for 6 times in PBS-T and the colour was developed by adding 50 ul/well of substrate solution containing 0.1mg/ml of orthophenylene diamine(Sigma) and 1 ul/ml of hydrogen peroxide (BDH) in citrate phosphate buffer (0.1M, pH 5.0). In substrate solution after 10 minutes the reaction was blocked with 2N sulphuric acid, 50 ul/well volumes. OD values were obtained at 490 nm in ELISA recorder (Microplate autorecorder, Dynatech). Fifty percent antibody binding of each group pooled sera to the high control of that plate was calculated by linear regression graph and this was expressed as ABT_{50} titres. Samples exhibiting ABT_{50} titres of 1:750 or above were considered positive.

CFT. The CFT antigen was prepared from *B. abortus* S-99 and standardized as described previously[1]. The assay was carried out in 96-well microtiteration plates (Laxbro, India) with 3 units of fresh guinea pig complement. The serum samples showing a titre of 1:8 or above were taken as positive.

Testing by kit. Milk samples freshly collected from the individual cows at the time of milking were added to the individual wells of the 96-well plastic microtitration plate with the help of dropper straws provided in the kit. In each row of 12-wells, 10 wells were used for the samples and the rest 2 for internal positive and negative controls provided alongwith the kit. The wells were filled to three-fourth of the height. Further processing was done as per the instructions given in the kit. Briefly, the nitrocellulose tip plastic combs (NC combs) precoated with brucella antigens were numbered and immersed in each of the 12-well rows of the microtitration plate containing the milk samples. Incubation with NC combs was carried out for 30 minutes at room temperature. Washing of the NC combs was done in the plastic cup provided in the kit under the running tap water for 5 minutes. Contents of the conjugate vial were added to the bottle containing conjugate

dilution buffer and this was dispensed in fresh 4 rows of the wells. NC combs were then incubated in these wells for another 30 minutes at room temperature. Washing following this step was carried out for 10 minutes under the running tap water. Contents of the chromogen vials were dissolved in substrate and these were added to fresh rows of well making use of a new dropper cap. The washed NC combs were dipped in this for 5-10 minutes. Positive and the negative control samples show up results on their corresponding NC comb tips by this time. The reaction was stopped by washing the combs in tap water. Positive result is indicated by development of blue dot in the center of nitrocellulose membrane bound to the tip of NC comb.

RESULTS

The obtained results are presented in Table (1).

DISCUSSION

Serum samples from the 50 cows of brucellosis free herd were negative for brucella antibodies by the 3 tests employed. Milk samples from these animals were also found negative by the kit. Assessment of serological results from serum samples of the individual cows from brucellosis endemic farm with the 3 tests and results on their milk samples by the kit are shown in Table-I by constructing 16 possible combinations from the 4 tests.

Thirty-seven of the 141 cows were negative to all the 3 serological test on serum testing and the same were found negative by kit as well on their milk testing. All the 66 cows that were positive by the 3 serological tests showed positive reaction in their milk samples with the kit. There were 19 cows showing a positive titre to both the plate ELISA and the CFT but were negative to SAT. These 19 cows too were found positive by the kit on milk testing. Five additional cows were positive by the kit for brucella antibodies in the milk but were negative to all the 3 serological tests performed on the sera samples.

Seven samples showed anti-complementary activity in their sera. Of these, 3 samples had serologically positive titres to SAT and plate - ELISA and 2 among these were detected positive by the kit. The other 4 serologically negative cases to both SAT and plate - ELISA were also negative by the kit.

Testing of milk for brucella antibodies is more convenient, economical and rapid than the testing of blood or serum. MRT, the conventional test employed for this purpose though is simple and quick to perform has the problems of both sensitivity and specificity. MRT chiefly detects gluteal IgA and IgM immunoglobulins bound to fat globules and is, therefore, dependent upon conditions that favour clustering of fat globules (Chand et al., 1989; Heck et al., 1980 and Morgan et al., 1978). Testing of individual milk samples is not recommended by MRT as lack of the factors responsible for clustering of fat globules or the low levels of IgA and IgM tends to yield false negative results (Corbel et al., 1984). Detection of antibodies in milk by ELISA is independent of these factors. Using lipopolysaccharide (LPS) as antigen in an indirect ELISA for antibody detection in milk, a sensitivity of 95.2% and a specificity of 99.95% was recently reported (Rogers et al., 1974). Conventional ELISA though sensitive and specific, is a laboratory based test. The ideal test should be sensitive, specific, inexpensive and field based. The dot-ELISA kit for antibody detection in milk correlated well with the serum results obtained with the 3 serological tests. From the brucellosis free herd, all the cows had negative serology to brucella antibodies and were also found negative by the kit on milk testing. In the brucellosis endemic farm, the kit, beside detecting all the cows that exhibited positive titres to the 3 tests, was able to identify additional 19 cases found positive to plate-ELISA and CFT but negative to SAT. Low sensitivity of SAT in comparison to ELISA and CFT is well documented (Batra et al., 1989). Five cows showed brucella antibodies in the milk by the kit but had no titres in serum samples. Whether these reactions were as a result of early appearance of antibodies in the milk or were false positive, remains to be ascertained.

The success of any brucellosis control programme depends on the precise case mapping and accurate single case finding. Owing to its high sensitivity and specificity, this field kit can have important role in identifying accurately the true reactor animals for segregation and culling, to keep the herd free from brucellosis. Utility of the kit in detection of brucella antibodies in the milk samples of individual cows merits its application not only to locate reactor animals in a farm but also in separating milk from infected cows from that of non-infected cows at the time of pooling of milk at the milk collection centers.

The application of this field kit is very simple and convenient and does not require the use of any outside accessory. The shelf life of the kit is over 9 months at 4°C and one month at 37°C.

ACKNOWLEDGMENT

Authors are grateful to Dr. R.V. Swamy, Director, Defence Research and Development Establishment, Gwalior, India for the encouragement provided to undertake this study.

REFERENCES

Alton, G. G., Jones, L. M., Pietz, D. E. (1975): Laboratory Techniques in Brucellosis, 2nd edn, WHO Monograph Series No. 55.

Araj, G.F., Kaufmann, A.F. (1989): Determination by enzyme-linked immunosorbent assay of immunoglobulin G (IgG), IgM and IgA to *Brucella melitensis* major outer membrane proteins and whole-cell heat-killed antigens in sera of patients with brucellosis. *J. Clin. Microbiol.* 27, 1909-1912.

Batra, H. V., Chand, P., Ganju, L., Mukherjee, R., Sadana, J. R. (1989): Dot-enzyme linked immunosorbent assay for the detection of antibodies in bovine brucellosis. *Res. Vet. Sci.* 46, 143- 146.

Bercovich, Z., Moerman, A. (1979): Non-specific positive milk ring test in tank milk and Estrumate in the treatment of cattle. *Tijdschr Diergeneesk* 104, 713 -716.

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

Boraker, D. K., Stinebring., W. E., Kunkel, J. R. (1981): Bruc ELISA: An enzyme-antibody immunoassay for detection of *Brucella abortus* antibodies in milk: Correlation with the *Brucella* ring test and with shedding of viable organisms. *J Clin Microbiol* 14, 396-403.

Chand, P., Sadana, J. R., Batra, H.V., Khanna, R.N.S (1989): Comparision of the dot enzyme-linked immunosorbent assay (dot-ELISA) with other serological tests for the detection of *Brucella* antibodies in bovine sera. *J. Vet. Med.* 36, 346-352.

Collin, J. C. (1976): The role of the different immunoglobulins in the milk ring test used for the detection of brucellosis. *Ann. Microbiol* (Inst. Pasteur) 127 B, 117 - 187.

Corbel, M. J., Stuart, F. A., Brewer, R. A. (1984): Observations on serological cross-reactions between smooth *Brucella* species and organisms of other genera. *Develop. Biol. Standard.* 56, 314 -348.

Dhar, R., Lastimoza, J. L., Hira, P. R. (1988): A modified indirect fluorescent antibody test for the diagnosis of brucellosis. *Diagn. Microbiol. Infect. Dis.* 11, 189-194.

Havaldar, P.V., Vijay Kumar, S.Y., Desai, A.S., Siddibhavi, B.M. (1987): Brucellosis in children. *Ind. Pediatr.* 24 (11) , 995 - 1006.

Heck, F.C., Williams, J. D., Pruett, J., Sanders, R., Zink, D.L. (1980): Enzyme-linked immunosorbent assay for detecting antibodies to Brucella abortus in bovine milk and serum. Am. J. Vet. Res. 41, 2082-2084.

Keer, W. R., Pearson, J. K. L., Rankin, J. E. F. (1959): The bovine under and its agglutinins. Brit. Vet. J. 115, 105-119.

Kerkhofs, P., Botton, Y., Thiange, P., Dekeyser, P., Limet, J. N. (1990): Diagnosis of bovine brucellosis by enzyme immunoassay of milk. *Vet. Microbiol.* 24, 73 - 80.

Lawman, M.J.P., Thurmond, M.C., Reis, K.J., Gauntlett, D.R., Boyle, M.D.P. (1984): Solid phase radioimmunoassay for the detection of immunoglobulins against bovine *Brucella abortus. Vet Immunol Immunopathol* 6, 291-305.

Morgan, W. J. B., MacKinnon, D. J., Gill, K. P. W., Gower, S. G. M. Norris, P. I. W. (1978): Brucellosis: Diagnosis: Standard Laboratory Techniques, Ministry of Agriculture, Fisheries and Food, London.

Oliver, D. G. Cooper, R. S. (1981): Comparative study of enzymme-linked immuno assay (IgG and IgM) with standard serological tests for diagnosis of brucellosis in cattle. Proc. Annu. Meet US Anim. Health. Ass. 85, 90 - 140.

Patterson, J. M. Deyoe, B. L. (1976): Effect of physical properties of milk fat globules on *Brucella* ring test sensitivity. *J. Dairy Sci.* 60, 851-856.

Ray, W. C. : Brucellosis (Due to *Brucella abortus* and *B. suis*). In: Handbook Series in Zoonosis. Ed., J.H. Steel, Vol. 1, pp. 99-185. CRC Press, Inc., Baca Raton , Florida,

Rogers, T. B., Keir, P. M., Henderson, W. G. (1974): Acute brucellosis with unexpected source. Br. Med. J. 2 (5921), 706.

Schoerner, C., Wartenberg, K., Rollinghoff, M. (1990): Differentiation of serological responses to *Yersinia enterocolitica* serotype 09 and *Brucella* species by immunoblot or enzyme-linked immunosorbent assay using whole bacteria and *Yersinia* outer membrane proteins. J. Clin Microbiol 28, 1570-1574.

Sutherland, S.S. (1980): Immunology of bovine brucellosis. *Vet. Bull.* 50, 359-368.

Sutra, L., Caffin, J. P., Dubray, G. (1986): Role of milk immunoglobulins in the *Brucella* milkring test. *Vet. Microbiol* 12, 359-366.

Thoen, C.O., Pietz, D.E., Armbrust, A.L., Harrington, R. (1979): Enzyme immunoassay for detecting Brucella antibodies in cow's milk. *J. Clin. Microbiol.* 10, 222 - 225.

WHO (1981): A guide to the Diagnosis, Treatment and Prevention of Human Brucellosis. Geneva, World Health Organisation.

Table-I

Serological results on serum/milk of 134 cows from brucellosis endemic farm by kit, CFT, plate ELISA and SAT.

	Results in different combination															
Test Employed	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SAT on serum	-	+	-	-	-	+	+	+	-	-	-	+	+	-	+	+
Plate-ELISA on serum	-	+	+	+	-	+	-	+	+	+	-	-	-	-	+	.
CFT on serum	-	+	+	-	-	+	+	-	-	+	+	-	-	+	-	+
Dot-ELISA kit on milk	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+
Total	37	66	9	1	5	0	0	0	1	1	2	0	0	0	2	0