International Journal of Integrative sciences, Innovation and Technology
(A Peer Review E-3 Journal of Science Innovation Technology)
Section A – Basic Sciences; Section B –Applied and Technological Sciences; Section C – Allied Sciences
Available online at www.ijiit.net.

Research Article

DETECTION OF ANTI-BRUCELLA ANTIBODIES IN GOATS USING 84 KDA SOLUBLE ANTIGENS OF B. MELITENSIS IN DOT-ELISA

DEEPAK KUMAR VERMA*
**Assistant Professor, Department of Biotechnology, Faculty of Natural and Computational Sciences, University of Gondar, P.O. Box. 196, Gondar, Ethiopia*
**Correspondence Author email: deepakbiotek@rediffmail.com, deepakvermabiotek@gmail.com*

ABSTRACT
The present study was designed to detect the presence of anti-Brucella antibodies in goat serum samples using dot ELISA. To achieve our objective dot-ELISA was optimized using 84 KDa soluble antigen of goat origin. Total 185 serum samples of goats were collected from the nearby villages of Makhdoom and processed at Microbiology Laboratory, Central Institute for Research on Goats (ICAR), Makhdoom, P.O. Farah, Distt. Mathura, India. Out of 185 serum samples tested in dot-ELISA, 25 (13.5%) samples were found sero-positive for Brucella melitensis. Development of brown dot at the end of the reaction indicates the positive reaction. The advantage of this assay is that it does not need any sophisticated laboratory instrument; therefore this assay will become an excellent field based diagnostic tool to know the incidence of caprine brucellosis in farmers flock.

KEY WORDS: *dot-ELISA, Brucella melitensis, gold standard, caprine brucellosis*

ABBREVIATIONS USED: B., Brucella; ELISA; Enzyme Linked Immunosorbent Assay; ICAR, Indian Council of Agricultural Research, SA, Soluble Antigen; PBST, Phosphate Buffer Saline Tween-20; DTH, Delayed Type Hypersensitivity, LTT, Lymphocyte Transformation Test.

INTRODUCTION
Caprine brucellosis is primarily caused by the zoonotic bacterium *Brucella melitensis*, is an economically important cause of abortion in small ruminants. It is believed to be an ancient disease that was described more than 2000 years ago by the Romans. Sir David Bruce first isolated *B. melitensis* in 1887. Since then, brucellosis has become an emerging disease in many parts of the world, characterized by chronic infections in animals leading to abortion and infertility, and a systemic, febrile illness in humans (Paulsen et al. 2002). *B. melitensis* uses goats and sheep as its preferred natural hosts but other animal may also be infected. It is an important disease because it undermines animal health and productivity. It is transmitted from animal reservoirs, wastes or their products. In domestic animals brucellosis is mainly an abortive disease that results from a long lasting unapparent infection. *B. melitensis* (Biovar 1, 2, 3) is the main causative agent of Caprine and Ovine brucellosis causes abortion during the third trimester of pregnancy. Following an abortion the infected female excretes copious amount of bacteria in uterine exduates and milk. Unequivocal definitive diagnosis is made by bacteriological identification of the causative agent (Alton et al., 1975a).

However, this procedure is time consuming, expensive and weakly sensitive. Control eradication program are then generally based partly or totally on serology, and then success largely depends on the accuracy and reliability of the tests employed. Numerous serological tests have been described (Alton et al., 1975a; Alton et al., 1975b; Diaz and Bosseray, 1974). The antibodies against *B. melitensis* were detected using plate-ELISA to study sero-prevalence of brucellosis in goats and sheep in different regions of India (Verma and Alemayehu, 2012). A rapid PCR based method was developed for the detection of *B. melitensis* in milk, tissue and blood samples of goats elsewhere including India (Gupta et al., 2004; Gupta et al., 2006).

Goat is known as the poor man's cow, therefore small marginal farmers and poor peoples of India are used to farm goats for the sake of milk and meat. The economy of nations depends to a large extent upon a healthy and productive livestock; the disease like brucellosis which is related to the reproductive failure is one of the major problems in developing countries like India (Verma, 2013). Interest in *B. melitensis* antigens from veterinary point of view has been mainly directed towards developing better diagnostic test. At present there is no "gold standard" for diagnosis of brucellosis in goats. The major problem in achieving the objective is the lack of well standardized and specific antigens. This question may be solved by immunological evaluation of *B. melitensis* antigens. This research is one of the footing step to solve the problem of diagnosis of caprine brucellosis in field/flock.

Brucella melitenis 16M culture used in this study for the preparation of antigen was isolated from the stomach content of goat (Rana et al., 2002). *B. melitensis* 16M antigens used in this study, because these antigens are twenty times more dominant than 'A' antigen which is dominant in *B. abortus.* To achieve our objective *B. melitensis* 16M extract prepared by ultrasonication, which was fractionated by using gel-filtration chromatography and further purified by anion-exchange chromatography. Molecular weight of the purified antigen was confirmed on polyacrylamide gel containing SDS. Serology was performed using 84 KDa antigen of *Brucella melitensis* of goat origin in dot-ELISA (Gupta et al., 2002).

MATERIAL AND METHODS

Total 185 serum samples from female goats were collected from the nearby villages of Makhdoom (India) and processed at Microbiology Laboratory, Goat Health Division, Central Institute for Research on Goats (ICAR), Makhdoom, P.O. Farah, Distt. Mathura, India.

For the preparation of soluble antigens logarithmic phase of *B. melitensis* 16M were sedimented by centrifugation at 10,000 rpm for 10 min. at 40C , washed twice with normal saline solution and resuspended in 20 ml NSS. The suspended culture was treated with ultrasonic waves in an ultrasonicator for 10 cycles, centrifuged at 10,000 rpm for 10 min. at 40C. Cell debris were removed and supernatant were collected, concentrated in vaccum concentrator, stored at 40C (Gupta et al. 1994). Protein concentration in the antigen was estimated by Lowry's method using Folin-Ciocalteu's phenol reagent (Lowry et al., 1961).

B. melitensis sonicated supernatants were fractionated by gel filtration on a 1.5 X 80 cm column bed of Sephacryl S-200 having a total volume (Vt) of 136 ml. Void volume (V0) of the column bed was 45 ml. The column was precalibrated with standard protein molecular weight marker viz. bovine serum albumin (MW 66 KDa) and cytochrome C (MW 12.5 KDa). 2 ml of concentrated sonicated antigen containing 80 mg total protein was eluted at the rate of 16 ml per hour with 30 mM Tris buffer (pH 7.5) containing 0.1 M sodium chloride. Absorbance of elutes was monitored at 280 nm. Fractions thus obtained in each region were pooled and were dialyzed against distilled water at 4o C. All the pooled fractions were concentrated by vaccum concentrator and filter sterilized through a membrane filter (0.22 µm), aliquoted and stored at -20oC (Gupta et al. 1994).

The Sephacryl S-200 fractionated region which was most intensely reacting in ELISA, DTH, LTT and CTL was subjected to anion exchange chromatography. The material (10 mg protein in 2 ml 30 mM Tris buffer, pH 8.7) was applied to DEAE-Sepharose CL-6B column equilibrated with 30 mM Tris buffer (ph 8.7), and then eluted with 30 mM Tris buffer (pH 8.7) containing 3M urea, using a linear gradient of sodium chloride from 0 to 0.3 M flow rate was 20 ml / hr and fractions of 5.5 ml were collected. All fraction were vacuum concentrated, filterd sterilized (0.22 µm membrane), alliquated and stored at -20oC (Gupta et al. 1994).

SDS – PAGE was performed as per the method of Laemmli 1970. First, the separating gel of 10% acrylamide was prepared by mixing acrylamide solution with buffers and ammonium per sulphate. This mixture was poured in between the two glass plates separated by spacer, which governs the thickness of the gel. The gel was overlayered with 1% isopropanol to ensure a flat surface and exclude air. After formation of separating gel, stacking gel solution was poured on the top of separating gel. Stacking gel solution mixture was prepared by mixing acrylamide solution with stacking buffer and ammonium per sulphate. Comb was inserted into the stacking gel solution for well formation into which the samples were loaded and allowed to set. Generally both the gels were taken 15-30 min. for polymerization. Before electrophoresis an equal volume of tracking dye were added to protein samples, boiled at 950C for 5 min. The gel with the glass plates were placed in the vertical electrophoresis tank filled with electrophoresis buffer, comb was removed from the gel for preparing the wells in the stacking gel. Prepared protein

samples were loaded into the wells and current was applied 60-80 volt. Protein molecular weight marker was also loaded into an adjacent lane along with the protein samples for molecular weight determination.

Dot-ELISA was performed as per the method of Gupta et al. (2002). Nitrocellulose strips were coated with 84 KDa region of *B. melitensis* soluble antigen @ 0.2 µg/strip in carbonate bicarbonate coating buffer. These coated NCP strips were then blocked with 3%BSA in PBS containing 0.1% Triton X-100 with 200 µl of blocking buffer per strip. After washing these NCP strips with PBST dipped in 1: 100 dilutions of serum samples in dilution buffer. Known standard hyperimmune sera were also used for each test as positive control; combs were incubated at 370C for 1 hr. After washing the strips with PBST, NCP strips were incubated at 370C for 1 hr. with anti-goat peroxidase conjugate, 1: 5000 dilutions in PBST. Following incubation strips were again washed with PBST and color was developed by adding substrate solution containing 0.1 mg/ml of diaminobenzidine (DAB) and 1µl/strip of hydrogen peroxide in phosphate-citrate buffer.

RESULT AND DISCUSSION

After fractionating the soluble antigens by gel filtration chromatography on a 1.5 X 80 cm column bed of Sephacryl S-200, we found 6 peaks (Table 1 & Fig 1). For further purification of antigen of our interest which was intensely reacting in ELISA, DTH, LTT assay, peak II (SAG2) was again purified by anion exchange chromatography (Fig.2). To confirm the molecular weight of the purified antigen, which was purified by anion-exchange chromatography, it was loaded on a polyacrylamide gel containing SDS. After fractionating the antigen of our interest on SDS-PAGE, which separates protein on the basis of its molecular weight, we found that purified regions SAG2A2 corresponds to the molecular weight of 84 KDa.

This 84 KDa soluble antigen was used in dot-ELISA for the detection of anti-Brucella antibodies to know the seroprevelance of this disease in goats. In this study 185 serum samples from goats tested in dot-ELISA. Out of 185 serum samples tested in dot-ELISA, 25 (13.5%) were found seropositive, which was indicated by development of brown colour dot. The test samples were compared with positive control. The hyperimmune sera was also collected from goats which were injected with Brucella melitensis 16M soluble antigen with complete adjuvant. This hyperimmune sera was used as positive control (Fig. 4). This study also shows the same results with previous researches (Gupta et al., 2002; Singh et al., 1998).

Table 1: Table showing peaks, regions and molecular weight range of soluble antigen (SA)

Soluble Antigens (SA)

Peak	Region	Mol. Wt Range (KDa)
I	SAG1	0 – 80
II	SAG2	84 – 78
III	SAG3	78 – 66
IV	SAG4	66 – 45
V	SAG5	45 – 23.5
VI	SAG6	23.5 – 12.5

Fig. 1: Sephacryl S-200 of *B. melitensis* soluble antigen. Areas between arrows indicate the fractions pooled under each peak.

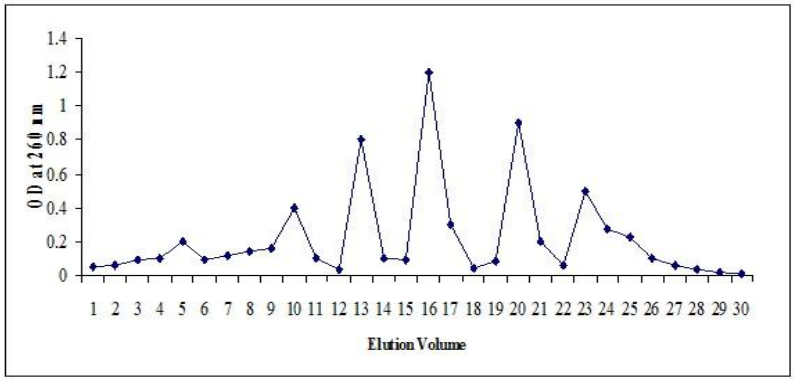


Fig. 2: DEAE –Sepharose CL-6B profile of Sephacryl S-200 elution peak-II of *B. melitensis* SA. Area between arrows indicate the fraction pooled under each peak. Buffer used – 30 mM Tris, pH-8.7, containing 3M urea and a linear gradient of NaCl from 0 to 0.6.

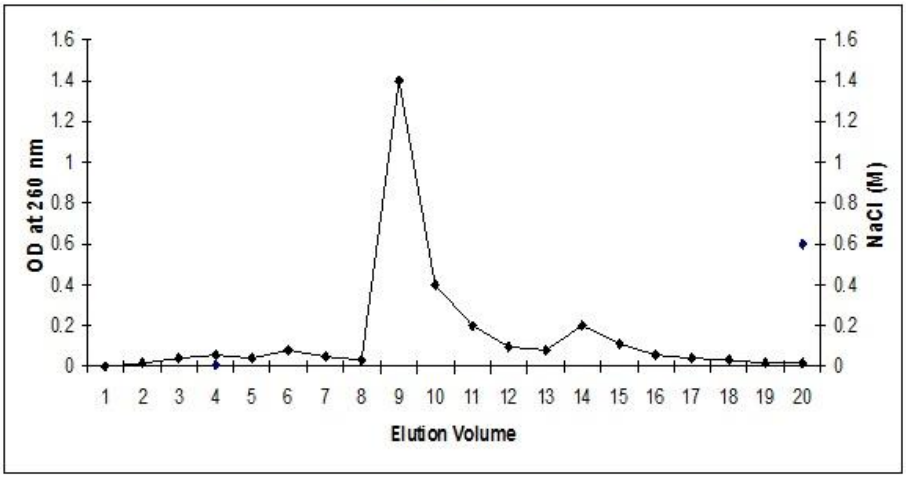


Fig. 3: Lane 4 SDS-PAGE analysis followed by coomassie blue staining of purified regions SAG2A2.

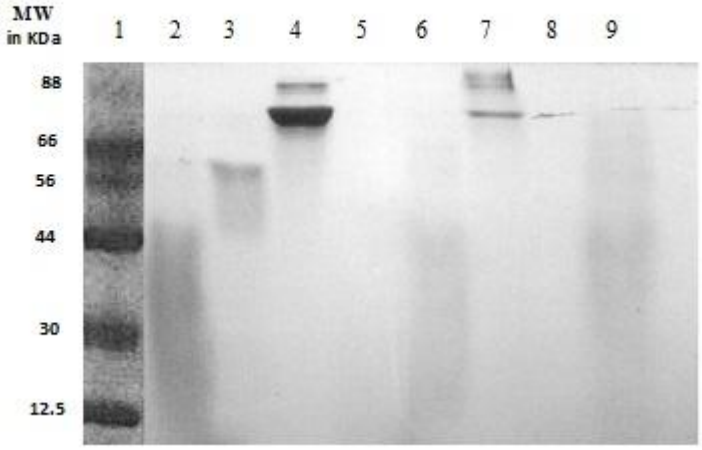
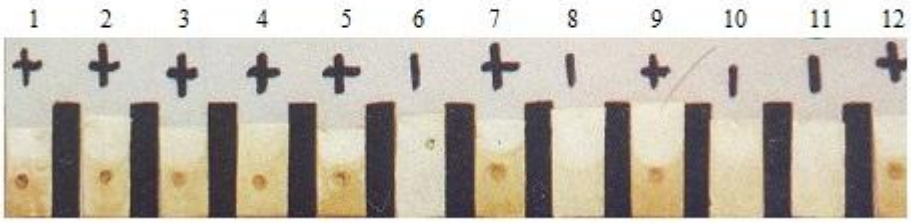


Fig. 4: *Brucella melitensis* 84 KDa soluble antigen based dot-Enzyme Linked Immunosorbent Assay (dot-ELISA). *S. No. 1-10: Test samples; S.No. 11: Negative control; S.No. 12: Positive control.* Antigen concentration used = 0.2 µg/strip, serum dilution used = 1:100. Development of brown dot indicate positive reaction



CONCLUSION

Dot-ELISA was found to be a rapid, handy and suitable screening test in the field for the diagnosis of brucellosis in goats. At the end of the test there is no need of any sophisticated instrument for the observation of results. We found a brown dot at the end of the reaction for the samples which were found seropositive for B. melitensis. It will serve as field based diagnostic tool for the diagnosis of caprine brucellosis. The technique will be applied on a large number of samples for better conclusion. It is possible to apply this technique for a number of diseases associated with other animals.

ACKNOWLEDGEMENT

The author would like to acknowledge all the scientific and technical staff of Microbiology Laboratory, Goat Health Division and Director, Central Institute for Research on Goats (ICAR), Makhdoom, P. O. Farah, Distt. Mathura (U.P.)-281122, India, for providing permission, support and necessary facilities for conducting the present research.

REFERENCES

1. Alton GC, Jones LM and Pietz (1975a). Laboratory Techniques in Brucellosis. 2nd ed., W.H.O. Series No. 55, Geneva, Switzerland.

2.Alton GC, Rogerson J, Maw BA and Mopherson GG (1975b). The serological diagnosis of bovine brucellosis: An evaluation of the complement fixation, serum agglutination and Rose Benga Tests. Australian Veterinary Journal. 51: 57-63.

3. Diaz R and Bosseray N (1974). Estudio de las relaciones antigenicas entre Yersinia enterocolitica serotipo 9 y otras especies bacterianas gram-negativas. Microbiol. Esp. 27 : 1-14.

4. Gupta VK, Ram GC and Bansal MP (1994). Antigenic characterization of Mycobacterium bovis BCG soluble antigens. Vet. Microbiol. 38: 227-240.

5. Gupta VK, Rana R and Vihan VS (2002). Dot-ELISA for detection of Brucella melitensis antibodies in goats. Indian Journal of Animal Sciences. 72 (3): 237-239.

6. Gupta VK, Verma DK, Rout PK and Vihan VS (2004) Detection of Brucella melitensis in milk of goats by Polymerase Chain Reaction. Indian Journal Of Animal Sciences. 75 (10): 1163-1164.

7. Gupta VK, Verma DK, Singh K, Kumari R, Singh SV and Vihan VS (2006). Single step PCR for detection of Brucella melitensis from tissues and blood of goats. Small Ruminant Research. 66: 169-174.

8. Laemmli UK 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227 : 680-685.

9. Lowry OH, Rosenbrough NJ, Farr AL, Randall RL (1961). Protein estimation with Folin-Ciocalteu's Phenol Reagent. J. Biol. Chem. 193: 265-275.

10. Paulsen IT, Seshadri R, Nelson KE, Eisen JA, Heideberg JF, Read TD, Dodson RJ, Umayam L, Beanan MJ, DaughertyS C, Deboy RT, Durkin AS, Kolonay JF, Madupu R, Nelson WC, Ayodeji B, Kraul M, Shetty J, Malek J, van Aken SE, Riedmuffer S, Tetelin H, Gill SR, Boyle S M and Fraser CM (2002). The Brucella suis genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. USA 99: 13148-13153.

11. Rana R, Gupta VK and Vihan VS (2002). Isolation and characterization of Brucella melitensis from goats. Ind. Vet. Med. Jour. 26 : 257-259.

Singh SV, Singh N, Gupta VK, Shankar H, Vihan V S, Gupta VK and Tiwari HA (1998). Seroprevalence of brucellosis in a few important Indian goat breeds. Small Ruminant Research. 30 : 93-98.

12. Verma DK (April, 2013). Review Article: Brucellosis in animals and human beings with special reference to Indian sub-continent. Int. J. Int. sci. Inn. Tech. Sec.A, 2(2): 43-56.

13. Verma DK, Alemayehu A (Aug, 2012). Seroprevelance of Brucellosis in Goats and Sheeps in Different Regions of India, using B. melitensis Soluble Antigen in Plate-ELISA. Research And Reviews: A Journal of Biotechnology [STM Journal] 2(2): 46-50.