International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; 9(1): 857-859
© 2024 VET
www.veterinarypaper.com
Received: 02-11-2023
Accepted: 03-12-2023

Suman Verma
Ph.D., Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Sanjay Kumawat
Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Mayank Rawat
Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Corresponding Author:
Suman Verma
Ph.D., Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Determination of IgM and IgG titers of anti- *B. abortus* murine serum by Indirect Enzyme Linked Immunosorbent assay

Suman Verma, Sanjay Kumawat and Mayank Rawat

Abstract
Brucellosis is the worldwide commonest bacterial re-emerging anthropozoonosis that create a serious public health issue along with significant economic burden on animal industries and poor rural communities. Despite being endemic in many developing countries, this devastating multi-organ disease is often neglected remains as under-diagnosed and under-reported. The economic loses occurs in terms of abortions, temporary infertility and weak offspring and is a major impediment for trade and export. The complete eradication of this disease would be unpractical due to wide host range that includes humans as well as domestic and wild type mammals. However, the strategy of "test and segregation" in conjunction with vaccination is perhaps the only method which is practical and feasible in most developing countries. Public awareness about its zoonotic importance is also a key point to throw out this drastic disease from many endemic areas. Enzyme Linked Immunosorbent assay is the most common assay that use catalytic properties of enzymes to detect and quantify the immunologic interactions. In our research study, we have raised the anti- *B. abortus* murine serum and immune response in terms of IgM and IgG titers was quantified by Indirect Enzyme Linked Immunosorbent assay on various days post immunization. There was a gradual increase in IgM titer from 2nd week which peaked at 4th week and then declined from 5th week. A constant increase in IgG titer was observed which peaked at 5th week. Hence, as expected in any post immunization antibody titer the same classical immune response was observed in our study too.

Keywords: *Brucella*, ELISA, Immune response

1. Introduction
Brucellosis is an infectious sub-acute or chronic zoonotic disease with worldwide distribution that infects wide range of animals, as well as humans. It was initially named as "Mediterranean fever", "Malta fever", "undulant fever", "Gibraltar fever" "thousand face disease", "raging fever" or "Melitococci disease" (Christopher *et al*., 2010) [3]. Its modern name *Brucella melitensis* was given to the honor of Sir David Bruce, the military physician who isolated and identified the organism in culture from spleen tissue of British soldiers. Presently, brucellosis has garnered the increased importance because of its consequences in human and livestock population as it is responsible for considerable economic loss in gross production and a major public health issue mainly in developing countries and persons living in resource limited settings. Recently the incidence of 500000 human cases per year was reported around the globe. Also, it is classified under category B bioterrorism agent because of its aerosol ease of transmission (Laine *et al*., 2022) [9]. Bovine brucellosis is endemic disease in India as significant sero-positivity was found in almost in every state of India (Chand and Chhabra, 2013) [2]. Bovine brucellosis is primarily caused by infection with Gram negative bacilli *B. abortus* that belong to alfa-2 subdivision of proteobacteria. However, infection with *B. melitensis* and *B. suis* are also reported. *B. abortus* is facultative intracellular, nonspore forming and modified acid-fast coccobacilli that lack classical virulence factor like capsule, endospore and naïve plasmids. An accurate diagnosis of *Brucella* infection is important for the control of the disease. The direct method of diagnosis includes bacteriological isolation and identification of the organism which is considered as gold standard for *Brucella* diagnosis because of its specificity and allows biotyping also (Dahouk *et al*., 2003) [7]. However, despite its high specificity, culturing of *Brucella* is challenging task as it is a fastidious bacterium

requires rich media for primary cultures along with long cultivation periods (4 to 7 days up to 40 days) and requires biosafety level-3 laboratories (Lage *et al*., 2008) [8]. Another direct methods are immunohistochemistry, molecular approach such as real-time PCR, multiplex polymerase chain reaction typing, restriction fragment length polymorphism based technique (Geresu and Kassa, 2016) [4]. The diagnosis of brucellosis employing direct method is not always possible for a large herd, hence indirect diagnostic assays are frequently used. Indirect methods for diagnosis of brucellosis includes a battery of serological tests such as serum agglutination test, rose Bengal plate test (RBPT), complement fixation test (CFT), the indirect (iELISA) and competitive (cELISA) enzyme-immunoassays, the fluorescence polarization assay (FPA) and the lateral flow immunochromatography test that is used either single or in combination for screening of brucellosis. Since these tests can be performed in regular practice without biosafety level-3 laboratories requirement. Hence, these tests are most frequently implemented not only for sero-epidemiological surveys but also for confirmatory diagnosis.

Materials and Methods

Mice

All the experimental procedures was carried out on mice were approved by the Animals Ethics Committee (AEC) of Indian Veterinary Research Institute (IVRI), Izatnagar-243122 (India). Mice were procured from the Laboratory Animal Resource Section of IVRI, kept in AEC approved facilities and received water and food *ad libtum*.

Bacterial strains

B. abortus S19 and S99 were obtained from *Brucella* Reference Repository Centre, Indian Veterinary Research Institute. Both the strains were maintained at 4 ºC on *Brucella* agar slants after confirming the purity and identity by appropriate methods. Prior to experimentation, both strains were sub cultured and suspension of each strain having the desired viable count was prepared and stored at 4 ºC.

Anti -*Brucella abortus* murine serum

A group of twenty Swiss albino mice (6-8 weeks age, 20±2 g) were immunized with *B. abortus* strain 19 (10^5 CFU/ml), subcutaneously as per standard protocol (OIE, 2008). Blood samples were drawn at 2nd, 3rd, 4th, 5th week after inoculation. The pooled blood sample was processed to draw immue serum (IS) which was filtered (22 µm MICROPOR) and stored at -80 ºC till use.

Extraction of smooth lipopolysaccharide (S-LPS)

The SLPS was extracted from *B. abortus* S99 cells employing hot phenol-water extraction method of Westphal and Jann (1965) [5]. Briefly, 5 g of lyophilized cells of *B. abortus* strain 99 was resuspended in 170 ml of distilled water and heated to 66 ºC. Then, equal volume of phenol (90% v/v) was added with constant agitation for 20 min. After cooling, the suspension was centrifuged at 12000 g for 20 min at 4ºC. Later on, the phenol layer was filtered to remove cellular debris. Subsequently three parts of chilled methanol containing 1% (v/v) methanol saturated with sodium acetate was added and left it for 2 h at 4 ºC. The final precipitate was removed by centrifugation at 12000 g and resuspended in 80 ml distilled water and stored for overnight at 4 ºC with stirring. The crude SLPS was collected by centrifugation at 10,000 g for 15 min at 4 ºC. The resulting pellet was resuspended in 80 ml distilled water and stirred for 1 h. The supernatant was pooled, filtered (0.22 µm) and 50-100 mg each of ribonuclease, deoxyribonuclease and proteinase K was added. The final suspension was incubated at -20 ºC for 18 h and re-precipitated with methanol as above. Finally, the pellet was resuspended in 2 ml distilled water and dialyzed against water and freeze dried and stored at -20 ºC for future use in I-ELISA.

Indirect Enzyme Linked Immunosorbent assay (I-ELISA)

The titer of IgM and IgG antibodies in 2nd, 3rd, 4th, 5th week pooled mice serum was determined by followinging the modified Briggs and Skeeles method (1984) [1]. All the reagents used for ELISA were optimized by chequer board method. Pre-immunization mice serum was used as negative control. Briefly, polystyrene 96 well plates (NUNCR Thermo fisher) were passively coated with 100 ul of 1:64 diluted *B. abortus* S99 SLPS and incubated at 4 ºC overnight. Next day blocking was done by 5% skim milk prepared in PBS with 0.05% Tween-20 (PBST) for 1 h. Following incubation washing of the plate was done thrice with PBST and test antibody (1:64) dilution was added in duplicates in each well of test panel and control panel. Negative serum (1:64) was added in negative control panel. Following 1 h incubation the plate was washed thrice with PBST and goat anti-mouse IgG or IgM HRPO conjugate (1:6000) (Sigma Aldrich, USA) was added and incubated for 1 h. Substrate containing OPD and H_2O_2 was added. Soon after 10 min the reaction was stopped by using 1M H_2SO_4 and OD were taken by an ELISA Reader (ASYS Hitech, Austria) at 492 nm. The antibodies titer of individual serum sample was denoted in terms of positive/negative (P/N) ratio.

Results

Confirmation of identity and maintenance of *B. abortus* strains

Brucella abortus S19 (vaccine strain: non-virulent) and S99 were used for the investigations. Both the strains were maintained on several slants at 4 ºC after confirmation of their identity by morphological, cultural, biochemical and serological tests. Both strains were found to be Gram-negative, non-spore-forming, non-motile, coccobacillary rods. On *Brucella* agar, after 48 to 72 h of incubation at 37 ºC, *B. abortus* S19 produced smooth, round colonies with yellowish margin having diameter of ~1-2 mm, without added CO2 (Fig. 1).

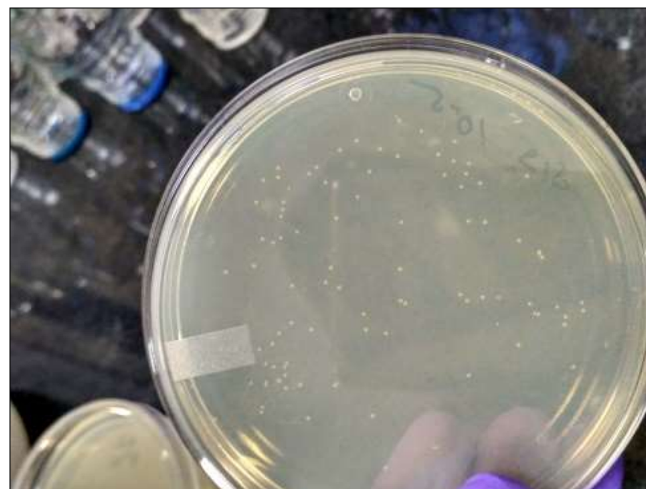

Fig 1: Colonies of *B. abortus* S19 on *Brucella* agar after 72 h incubation at 37 ºC

Standardization of viable counts *B. abortus* strains

Prior to the experiments, *B. abortus* S19 and S99 were sub cultured on *Brucella* agar (Difco) at 37 ºC in an appropriate atmosphere of CO_2 for 48 h. The pure growth of each strain was harvested in sterile phosphate buffer saline (PBS). The culture suspensions were washed thrice in sterile PBS by centrifugation. The final washed bacterial suspensions were matched to Brown's opacity standard No. 2 and the viable number of each strain was determined by plate count of serial tenfold dilutions. The stock suspensions were finally diluted to contain desired number of viable *Brucella* organisms for the experiments and stored at 4 ºC.

IgM and IgG titers of murine serum on various days post immunization

The *B. abortus* specific IgM and IgG titers of murine immune serum were determined by I-ELISA in terms of P/N ratio. There was a classical IgM immune response was observed as gradual increase in IgM titer from 2nd week which peaked at 4th week and then declined from 5th week. A constsnt increase in IgG titer was observed which peaked at 5th week (Table 1).

Table 1: Immune serum IgM and IgG level (titer in P/N ratio of OD at 492) in mice

Time of bleeding	IgM (Mean±SD)	IgG (Mean±SD)
2nd week	1.671±0.122^{a}	1.4140±0.095^{ab}
3rd week	2.0011±0.216^{b}	1.8268±0.119^{b}
4th week	2.0820±0.039^{bc}	3.3947±0.255^{c}
5th week	1.5600±0.076^{ad}	4.6148±0.399^{d}

*Mean value bearing different superscripts (a, b, c, d) in each column differ significantly ($p < 0.05$).

Discussion

Brucella abortus is an obligate parasite that causes zoonotic infections of worldwide importance. *B. abortus* infections in their natural hosts (commonly bovines; goat, sheep, pig are also affected) are rarely lethal and usually symptomless, though some minor clinical conditions such as infertility, undulant fever or arthritis may sometimes be observed. The most severe clinical manifestation of bovine brucellosis is abortion during first pregnancy. The organism can also infect humans. Since brucellosis of dairy animals, especially cattle, has a profound impact on socioeconomic status and livestock productivity, the control of the disease becomes a priority throughout the world. In India, "test and segregation" of infected animals combined with vaccination are the only preventive measures exercised to combat the disease and, vaccination has become the most important part of national control programme against bovine brucellosis. ELISA is the most widely used diagnostic to measure antibody titers for evaluating the post vaccination immunogenicity because of its reliability and less laborious than other methods. In our experimental design we got the classical immune response was observed where peak of IgM was found at 4th week after a gradual increment. On the other hand, the peak of IgG was found at 5th week. Currently, though the WHO recommended live vaccines (S19 and RB51) against bovine brucellosis are effective, they have several problems associated with production, transportation, keeping quality, field application, safety, and potency. The search for better vaccine candidates has therefore never been stopped during the last 75 years. But the attempts to develop better immunizing agents are adversely affected by poor understanding of pathogenesis of *B. abortus* infection in cattle. More significantly, our knowledge about the vaccine induced immune mechanisms that protect animals against abortion is also poor. As the organism is considered to be an intracellular pathogen, the role of cell mediated immunity (CMI) in protection has been conventionally overemphasized over the antibody mediated immunity (AMI). This has led to a dogmatic preference for the live vaccines over other formulations (Titball, 2008) [6]. Therefore, it's an urge to discover better vaccine candidate that focus both arms of immune system rather than solely targeting CMI considering it as obligate intracellular pathogen.

Acknowledgement

The authors are thankful to the Director, Indian Veterinary Research Institute for providing fund and necessary facilities to conduct the study.

References

1. Briggs DJ, Skeeles JK. An enzyme-linked immunosorbent assay for detecting antibodies to *Pasteurella multocida* in chickens. Avian Diseases. 1984;28:208-215.
2. Chand P, Chhabra R. Herd and individual animal prevalence of bovine brucellosis with associated risk factors on dairy farms in Haryana and Punjab in India. Tropical Animal Health and Production. 2013;45(6):1313-1319.
3. Christopher S, Umapathy BL, Ravikumar KL. Brucellosis: review on the recent trends in pathogenicity and laboratory diagnosis. Journal of Laboratory Physicians. 2010;2(2):55-60.
4. Geresu MA, Kassa GM. A review on diagnostic methods of brucellosis. Journal of Veterinary Science & Technology. 2016, 7(3). DOI:10.4172/2157-7579.1000323.
5. Westphal O, Jann K. Bacterial lipopolysaccharides extraction with phenol-water and further applications of the procedure. Methods in Carbohydrate Chemistry. 1965;5:83-91.
6. Titball RW. Vaccines against intracellular bacterial pathogens. Drug Discov Today. 2008;13(13-14):596-600.
7. Dahouk S, Tomaso H, Nöckler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis-a review of the literature. Part I: Techniques for direct detection and identification of *Brucella* spp. Clinical Laboratory. 2003;49:487-505.
8. Lage P, Poester P, Paixão A, Silva A, Xavier N, Minharro S, *et al*. Brucelose bovina: uma atualização. Revista Brasileira de Reprodução Animal. 2008;32:202-212.
9. Laine CG, Scott HM, Arenas GAM. Human brucellosis: widespread information deficiency hinders an understanding of global disease frequency. PLoS Neglected Tropical Diseases; 2022, 16.