Int.J.Curr.Microbiol.App.Sci (2017) *6*(11): 1881-1888

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 11 (2017) pp. 1881-1888**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.611.224

A Comparison of Antibody Titers in Brucellosis Affected Vaccinated Cattle, Unvaccinated Infected Cattle and Healthy Vaccinated Cattle by Agglutination Assays

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Keywords

Antibody titer, Brucellosis, Indirect Hemagglutination Assay, Microagglutination test, Standard Tube Agglutination Test.

Article Info

Accepted:
15 September 2017
Available Online:
10 November 2017

A B S T R A C T

The titers of anti-Brucella antibodies in naturally Brucellosis affected unvaccinated cattle, previously vaccinated infected cattle and normal healthy vaccinated cattle were estimated by common agglutination assays MAT, STAT, IHA. The mean antibody titers (log_{10}) were found to be 2.856+0.47 by MAT, 2.041+0.39 by STAT and 2.471+0.51 by IHA, respectively in case of Brucellosis affected cattle which had been vaccinated during calf hood. The mean titers in case of naturally infected cattle which had never been vaccinated were 3.759+ 0.35 by MAT, 3.408+0.32 by STAT and 3.508+ 0.32 by IHA, respectively. The mean titers in healthy unaffected cattle vaccinated during calf hood were as follows 1.401+ 0.31 by MAT, 1.401+0.15 by STAT, and 1.45+0.16 by IHA, respectively. It was interesting to find that the antibody titers in naturally affected cattle which had never been vaccinated previously were very significantly ($p<0.01$) higher than those of Brucellosis affected cattle which had been vaccinated during calfhood and normal healthy vaccinated cattle unaffected by the disease. The mean titer in Brucellosis affected cattle which had been vaccinated during calfhood was very significantly ($p<0.01$) higher than that of normal healthy vaccinated cattle unaffected by the disease.

Introduction

Brucellosis is an important re-emerging zoonotic disease caused by Brucella organisms. It is pathogenic for a wide variety of animals such as cattle, swine, goats, sheep and dogs. Brucellosis is endemic in India and is prevalent in all parts of the country. About 500,000 cases of human brucellosis are estimated to occur worldwide every year. It causes heavy economic loss to the animal industry through delayed conception, late-term abortions and retention of placenta and temporary or permanent infertility (Kollannur *et al.*, 2007) in females and orchitis and epididymitis in males, with excretion of organisms in semen, uterine discharges, and in milk (Godfroid *et al.*, 2013). It is estimated that due to Brucellosis, there is a loss of US$58.8 million per year in India (Kollannur *et al.*, 2007). The occurrence of this disease varies from 10% in marginal herds to 50% in organized farms. The socio-economic impact of the disease was estimated to run over

Rs.500 Crores annually (Jain *et al.,* 2013; Pandeya *et al.,* 2013). The vaccine against Brucellosis widely used for cattle is derived from the smooth live vaccine strain S19. It has proven to be very useful under most conditions but has some undesirable traits also (Moriyon *et al.,* 2004). Although vaccination with the live attenuated *Brucella abortus* strain 19 is believed to be effective in preventing Brucellosis in cattle and buffaloes, it is not uncommon to find adult cattle infected with Brucellosis which had been vaccinated in calf hood with S19 vaccine. In the absence of a marker vaccine and DIVA assay for bovine Brucellosis, it becomes difficult to assess whether the anti-Brucella antibody titers in an animal are due to vaccination or due to infection.

Although there is vast information on various aspects of bovine Brucellosis in the available literature, there is hardly any systematic study on the comparison of antibody levels between infected and vaccinated animals. We therefore compared the antibody titers of different categories of animals to investigate if titers alone could indicate whether the animal is infected or vaccinated or infected despite vaccination. The present study offers valuable insight into the status of humoral immune response in Brucellosis affected or vaccinated cattle which could help in devising appropriate control strategies for this dreaded zoonosis.

Materials and Methods

Permission of the Institutional Animal Ethics Committee (IAEC) was obtained and CPCSE guidelines were followed in all animal experiments.

Collection of serum

Blood samples were collected from naturally infected clinical cases of Brucellosis in cattle which had never been vaccinated in villages in and around Ludhiana district and from Brucellosis affected cattle which had been vaccinated during calf hood, normal healthy (uninfected) vaccinated cattle and healthy unvaccinated calves from an organized dairy farm from Ludhiana. Sera were separated from clotted blood and stored at -20^{o}C till further use for studying the antibody response of the animals.

Rose Bengal Plate Test (RBPT)

Equal volumes (25µl each) of RBPT colored antigen (Punjab Veterinary Vaccine Institute, Ludhiana) and test serum were mixed on a clean glass slide (Morgan *et al.,* 1978). The slide was observed till 3 min. for formation of clumps. Formation of clumps indicated a positive reaction while the absence of clear clumps was considered as a negative reaction.

Estimation of antibody titers by Standard Tube Agglutination Test (STAT)

The standard method recommended by OIE (2004) was followed. Ten agglutination tubes were labeled and placed in rack.

Then 0.8 ml of 0.5% carbol saline was added to the first tube of the series. 0.5ml of carbol saline was added to the rest of the tubes except 8, 9 and 10 which were kept as controls, containing 1.25, 1.50 and 1.75 ml of carbol saline, respectively.

0.2 ml serum was added to the first tube and the contents were mixed. 0.5 ml of plain *Brucella abortus* antigen (Punjab Veterinary Vaccine Institute, Ludhiana) was added to the first tube and final dilutions were made in ratios of 1:10, 1:20, 1:40, 1:80 and so on. To the control tubes 8, 9 and 10, *Brucella abortus* plain antigen 0.75, 0.50 and 0.25 ml, respectively was added (Table 1). Tubes were incubated for 24 hours at 37^{o}C and results

were compared with the antigen control tube showing 50% agglutination. The highest serum dilution showing 50% agglutination was taken as the end point for the titer serum. A titer of 1:40 or above was considered positive.

Controls

Tube no. 8- 25% agglutination
Tube no. 9- 50% agglutination
Tube no. 10- 75% agglutination

Microtiter Plate Agglutination Test (MAT)

MAT was performed as per the method of William and Whittemore (1997)

Serum samples were serially two fold diluted in phosphate buffer saline to make final volume of 100µl in a 96 well U bottom microtiter plate (Tarsons).

Equal volume of 100µl of Plain *Brucella abortus* antigen (Punjab Veterinary Vaccine Institute, Ludhiana) was added to each well.

Negative control well containing 100µl Phosphate buffer saline and 100µl antigen was also marked.

The plate was sealed and mixed gently for 20 seconds and incubated at 37^{o}C for 24 hr followed by incubation at 4^{o}C for 1 hour.

Matt formation was considered as a positive reaction while button formation was considered as a negative reaction. Titers (log_{10} values) were expressed as reciprocal of the highest dilution of sera showing 50 percent agglutination.

Indirect Haemagglutination Test (IHA)

The Method of Sawada *et al.,* (1982) was followed.

Sheep Red Blood Cells (sRBCs)

Sheep blood was collected aseptically into Alsever's solution (1:1) by jugular vein puncture, and kept at 4^{o}C for 7 days before further processing. Blood was centrifuged at 1500-2000 rpm for 10 min to settle the RBCs. The packed RBCs were washed three times with 5-6 volumes of chilled normal saline solution (NSS) by centrifugation. The 10% suspension of RBCs prepared in chilled NSS was stored at 4^{o}C.

Fixation of RBCs and treatment with tannic acid

One percent solution of glutaraldehyde was prepared in NSS and stored at 4^{o}C for 30 min with intermittent gentle stirring.

The sensitized sRBCs were packed by centrifugation at 1500-2000 rpm for 10 min at room temperature followed by three washes in normal saline for removal of glutaraldehyde. This was resuspended in the same buffer containing 0.1 percent Sodium Azide to yield a 10 percent suspension of sRBCs. The glutaraldehyde fixed sRBCs (G-sRBCs) were stored at 4^{o}C.

10 percent suspension of G-sRBCs was mixed with equal volume of Phosphate Buffered Saline containing 0.005 percent tannic acid (w/v), and incubated at 37^{o}C with occasional shaking. The tanned G-sRBCs (TG-sRBCs) were pelleted by centrifugation at 650 x g for 10 min at room temperature and washed three times with PBS to yield a 10 percent suspension.

Preparation of antigen

The antigen prepared as described earlier was heated at 56^{o}C for 30 min in a water bath with frequent shaking. Heat treated suspension was centrifuged at 8000 rpm for 15 min at 4^{o}C.

The clear supernatant was separated and stored at -20^{o}C.

Sensitization of TG-s RBCs with antigen

One volume of packed RBCs and 15 volumes of the antigen, were mixed and incubated for 2 hrs at 37^{o}C in a water bath with frequent shaking. The sensitized cells prepared were washed three times with NSS by centrifugation at 2500 rpm for 10 min. After the final wash, the packed cells were resuspended in chilled NSS to obtain 1 percent suspension.

Adsorption of serum samples

To remove the heterophile antibodies, all the serum samples were adsorbed with packed sRBCs (1 volume) for 2 hours at 37^{o}C with periodic shaking before the test proper. The RBCs were removed by centrifugation.

The suspension was centrifuged at 600 x g for 15 min at 4^{o}C in a refrigerated centrifuge. The suspension was collected and used for the test.

Test protocol for IHA

Phosphate buffered saline (160µl) and inactivated adsorbed serum (40µl) was added to the first well (1 in 4 dilutions) and 100µl of PBS was added to all the wells. The plates were shaken and left at room temperature for 2 hr. Coarse agglutination of RBCs (matt formation) indicated a positive result and formation of small button of deposited cells was considered as a negative result.

Controls

Three controls were included in the test:

Antigen control: 100µl of sensitized and adsorbed RBCs.

RBC control: 100µl of 1:4 dilution of serum and 100µl of sensitized RBCs.

Serum control: 100µl of untreated erythrocytes and 100µl of test serum.

Statistical analysis

Data pertaining to serum antibody titers by STAT, MAT and IHA were statistically analyzed by ANOVA.

Results and Discussion

The titers of anti-Brucella antibodies in naturally Brucellosis affected unvaccinated cattle, previously vaccinated infected cattle and normal healthy vaccinated cattle were estimated by common agglutination assays MAT, STAT and IHA. The mean antibody titers ($\log_{10}$) were found to be 2.856+0.47 by MAT, 2.041+0.39 by STAT and 2.471+0.51 by IHA, respectively in case of Brucellosis affected cattle which had been vaccinated during calf hood (Table 2). The mean titers in case of naturally infected cattle which had never been vaccinated were 3.759+ 0.35 by MAT, 3.408+0.32 by STAT and 3.508+ 0.32 by IHA, respectively (Table 3). The mean titers in healthy unaffected cattle previously vaccinated during calf hood were as follows 1.401+ 0.31 by MAT, 1.401+0.15 by STAT, and 1.45+0.16 by IHA, respectively (Table 4, Fig. 1).

The mean titer in unvaccinated infected cattle was very significantly ($p<0.01$) higher than that of Brucellosis affected vaccinated cattle. The mean titer of Brucellosis affected vaccinated cattle was very significantly ($p<0.01$) higher than that of normal healthy vaccinated cattle. The mean titer of unvaccinated infected cattle was very significantly ($p<0.01$) higher than that of healthy vaccinated cattle. It was interesting to find that the antibody titers in naturally

affected cattle which had never been vaccinated previously were very significantly higher than those of Brucellosis affected cattle which had been vaccinated earlier and normal healthy vaccinated cattle unaffected by the disease by all the three assays (MAT, STAT, and IHA).

Assessment of immunological data from Brucella infected cattle can be helpful in charting the disease process, diagnosis and prognosis and may help in understanding the pathophysiology of disease. It has been reported earlier (Mohan *et al.*, 2016) that infected animals have very high titers of antibodies compared to the vaccinated animals. However, in the absence of an assay for differentiation of infected from vaccinated animals (DIVA assay), it is not possible to determine whether these antibodies are protective or not and are induced by the vaccine or due to response of the animal to acute infection and whether they are of any relevance to prognosis.

Fig.1 Antibody titers in brucellosis infected or vaccinated cattle

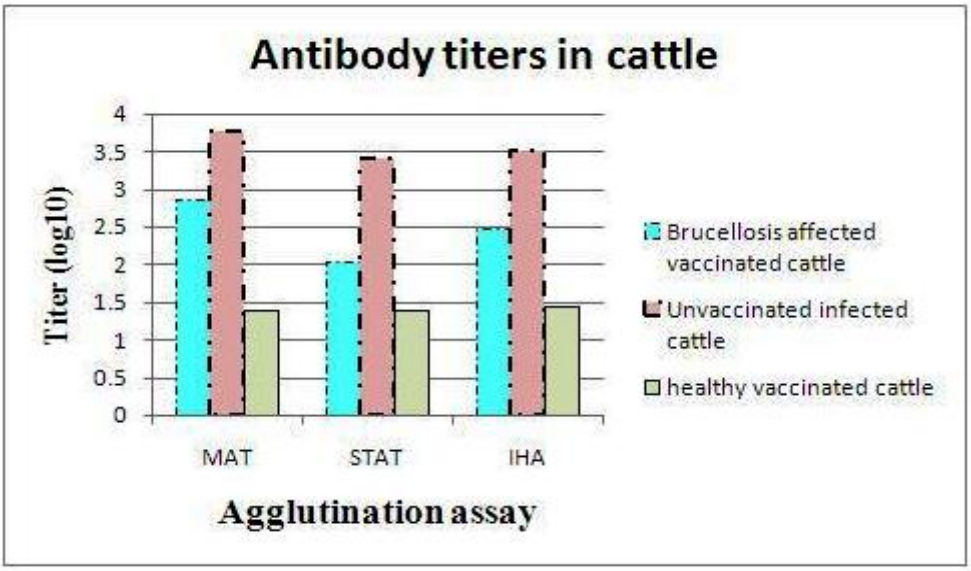

Table.1 STAT protocol

Tube no.	Carbol saline (ml)	Test serum (ml)	*B. abortus* plain antigen (ml)	Final dilution
1	0.8	0.2	0.5	1:10
2	0.5	Serial dilution was performed after thorough mixing. 0.5 ml of the contents was transferred from tube no.1 to the next tube up to tube no.7.Finally 0.5 ml of the contents was discarded from tube no.7	0.5	1:20
3	0.5		0.5	1:40
4	0.5		0.5	1:80
5	0.5		0.5	1:160
6	0.5		0.5	1:320
7	0.5		0.5	1:640
8	1.25		0.75	
9	1.50		0.50	
10	1.75		0.25	

Table.2 Antibody titers in brucellosis affected vaccinated cattle by agglutination assays

S. No	Animal number	Antibody titer		
		MAT	STAT	IHA
1	B1	2.505	1.602	2.205
2	B2	2.806	1.903	2.204
3	B3	2.505	2.204	1.903
4	B4	2.505	1.602	2.505
5	B5	2.806	1.903	2.204
6	B6	3.408	2.204	3.408
7	B7	3.709	2.204	3.408
8	B8	2.505	1.602	2.204
9	B9	2.806	1.903	2.806
10	B10	3.408	2.505	2.806
11	B11	2.505	1.602	2.505
12	B12	2.204	1.903	1.903
13	B13	2.505	1.602	2.404
14	B14	2.204	1.602	2.204
15	B15	3.107	2.505	2.806
16	B16	3.709	2.505	3.408
17	B17	2.505	1.602	2.404
18	B18	3.107	1.903	3.107
19	B19	2.806	1.903	1.903
20	B20	2.505	2.505	2.204
21	B21	3.408	2.806	3.107
22	B22	2.505	2.204	1.903
23	B23	3.709	2.806	1.903
24	B24	2.806	1.903	1.903
Mean ± SD		**2.856 ± 0.47**	**2.041 ± 0.39**	**2.471 ± 0.51**

Table.3 Antibody titers in brucellosis infected unvaccinated cattle

Animal No.	MAT	STAT	IHA
A I	3.408	3.107	3.107
A II	3.709	3.107	3.408
A III	4.010	3.709	3.709
A IV	3.709	3.709	3.408
A V	3.408	3.107	3.408
A VI	4.311	3.709	4.010
Mean ± SD	**3.759 ± 0.35**	**3.408 ± 0.32**	**3.508 ± 0.31**

Table.4 Antibody titers in healthy vaccinated cattle by agglutination assays

Animal no.	MAT	STAT	IHA
C1	1.602	1.301	1.301
C2	1.301	1.301	1.602
C3	1.301	1.602	1.602
C4	1.903	1.301	1.602
C5	1.000	1.301	1.301
C6	1.301	1.602	1.301
Mean + SD	**1.401 + 0.31**	**1.401 + 0.15**	**1.451 + 0.16**

Numerous outer and inner membrane, cytoplasmic, and periplasmic protein antigens of Brucella have been characterized, yet the antigen that dominates the antibody response is Lipopolysaccharide (LPS). Some antigens are recognized by the immune system during infection and are potentially useful in diagnostic tests. The L7/L12 ribosomal proteins are important in stimulating cell-mediated responses (Oliveira and Splitter, 1994).

The role of humoral immunity against intracellular bacterial infections is limited and may not be protective. Antibody mediated opsonization by immunoglobulins (IgM, IgG1, IgG2a and IgG3) enhances phagocytic uptake of bacteria, limiting the level of initial infection with Brucella but has little effect on intracellular course of infection (Bellaire *et al.*, 2005; Baldwin and Goenka, 2006).

Since the infected animals in the first group were the ones who had already been vaccinated during calf hood, the infection in these animals may suggest that the vaccine was unable to induce adequate protective levels of antibody. Secondly, the heightened antibody response after infection in vaccinated animals may indicate a secondary immune response to Brucella antigens.

The anti-Brucella antibody titers in naturally affected unvaccinated cattle were very significantly higher than those of Brucellosis affected vaccinated cattle and normal healthy vaccinated cattle unaffected by the disease as determined by three agglutination assays (MAT, STAT, and IHA).

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

The study was funded by a grant under RKVY scheme (PI: H M Saxena)

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How to cite this article:

Sugandha Raj, Hari Mohan Saxena and Singh, S.T. 2017. A Comparison of Antibody Titers in Brucellosis Affected Vaccinated Cattle, Unvaccinated Infected Cattle and Healthy Vaccinated Cattle by Agglutination Assays. *Int.J.Curr.Microbiol.App.Sci*. 6(11): 1881-1888.
doi: https://doi.org/10.20546/ijcmas.2017.611.224