

A new Superagglutination Test to minimize false negative and false positive results common with plate / slide agglutination tests for the diagnosis of infectious diseases

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ABSTRACT

Agglutination is the clumping of antigenic particles by antibodies. Several diagnostic kits for infectious diseases of animals and humans, based on slide agglutination / plate agglutination tests like the Rose Bengal Plate Test (RBPT) for diagnosis of brucellosis are used worldwide. False negative and false positive results are commonly encountered in these conventional agglutination tests. Simple, cost effective modifications can help in circumventing these problems. The novel Superagglutination Test reported here is a modified slide / plate agglutination test. False negative results due to smaller clumps formed by low titer of antibodies in serum are minimized by the addition of biotinylated antiglobulin followed by Avidin which forms easily detectable larger clumps. Similarly, prior staining of serum antibodies with a dye helps in differentiating a specific agglutinate formed by both antigen and antibody, from a non-specific aggregate of antigen alone that leads to false positive results. Superagglutination is more sensitive than the current agglutination tests and the agglutination based diagnostic kits. The modification steps are easy to perform and give a more accurate diagnosis without much increase in the cost of the test. The economic losses to livestock industry caused by the spread of infectious diseases like brucellosis due to false negative diagnosis and culling /slaughtering of productive animals due to false positive diagnosis can be avoided by employing the new test.

Keywords: False positive, false negative, agglutination, Brucellosis, RBPT, antiglobulin.

INTRODUCTION

The reaction of a particulate antigen with antibody leads to clumping of the antigen particles which is called agglutination. Agglutination test is used in the diagnosis of infections by demonstrating in blood plasma or serum the presence of antibodies in blood plasma or serum against antigens of infectious microorganisms. The agglutination test is useful when a quick screening result is required particularly in remote areas in the developing countries that lack the facilities for other advanced tests. The agglutination test is a simple and cheap method for diagnosis of infectious diseases. However, it has lesser sensitivity and hence has more chances of false negative results compared to other serological tests like ELISA. Earlier attempts to enhance agglutination have led to the development of Indirect Coomb's Test and Coagglutination Test. The Indirect Coombs' Test has been limited in use to blood typing by detecting anti-red cell antibodies and is not routinely applied for diagnosing infections. Coagglutination is a sensitive test for screening and rapid diagnosis of infectious diseases. However, it requires Protein A which binds to the immunoglobulins in a non – specific manner and is expensive or and cumbersome to prepare. In the present study, we have attempted to develop simple means to address the two major problems of false negative and false positive results obtained with the conventional slide / plate agglutination tests. The principle behind the innovative modifications is applicable not only to brucellosis but to other infectious diseases of animals and humans as well.

MATERIALS AND METHODS

Source and collection of samples

Forty three serum samples were collected from cattle and buffaloes suspected of having brucellosis on the basis of clinical symptoms and infection history of the herds they belonged to and forty six serum samples from non diseased animals were collected at the college veterinary clinics, and dairy farms and animal shelters in and around Ludhiana city of the Punjab state of India. All animals were older than two years. Thirty three of the above animals were suspected of having brucellosis on the basis of clinical symptoms and infection history of the herds they belonged to. Common serological tests i.e. the RBPT or conventional RBPT (cRBPT), Standard

Tube Agglutination Test (STAT) and Dot ELISA as well as the modified RBPT (Superagglutination Test) were applied on all the 89 serum samples included in the study.

Conventional Rose Bengal Plate Test

Equal volumes (5 µl of each) of RBPT coloured antigen (IVRI, Izatnagar, India) and test serum were mixed on a clean glass slide [1] with the help of a clean sterilized toothpick. The slide was observed after 2 min for the formation of clumps. The formation of clear clumps was considered a positive test while the absence of clear clumps was considered a negative reaction.

Superagglutination Test

Equal volumes (2.5 µl each) of RBPT coloured antigen and test serum stained with 1 µl of 0.1% Coomassie Blue dye, 1 µl of biotinylated anti-bovine IgG and 1 µl streptavidin were mixed on a clean glass slide thoroughly in the above mentioned sequence. The slide was observed for 4 mins for the formation of clumps. Ordinary hand lens was used occasionally for better visibility. The slides were viewed under a low power (4x or 10X) objective lens under a microscope to confirm the clumping in case of doubt. Formation of clear clumps, within which the blue colour (due to the Coomassie Blue dye staining the serum antibodies) and the pink colour (due to the RBPT coloured antigen i.e. the Rose Bengal dye stained killed *Brucella* organisms) could be differentiated on magnification, were considered as positive, while absence of clear agglutinates or aggregates of pink colour alone or blue coloured mass alone were considered as negative.

Standard Tube Agglutination Test

Standard procedure was followed [2]. Plain antigen of *Brucella abortus* S99 (IVRI, Izatnagar) was used as described [2]. Two-fold serial dilutions (1:20 to 1:640) of the sera were prepared in phenol saline and equal volume (0.5 ml) of antigen was added to each tube. All the tubes were incubated at 37°C for 24 h. The results were compared with the antigen control tube showing 50% agglutination. A titer of 1:40 or above was considered positive.

Dot ELISA

ImmunoComb Bovine *Brucella* antibody test kit (Biogal-Galed Labs, Israel) was used for conducting Dot ELISA with the serum samples of cattle. The samples to be tested were mixed with the diluent in the first row of wells of a multichamber developing plate. The test spots on the

comb were then incubated with the serum samples in the developing plate. The comb was then transferred to a well containing buffer to wash the unbound antibodies from the antigen spots. The comb was then allowed to react with an anti-cattle IgG alkaline phosphatase conjugate. After 2 more washes, the comb was moved to the last well. Colour was developed by an enzymatic reaction. The intensity of colour of the test spots corresponded directly to the antibody level in the test sample.

RESULTS AND DISCUSSION

The modified plate / slide agglutination test described above is named as the “Superagglutination Test”. The test gave superior results in detecting anti-*Brucella* antibodies compared to the other serodiagnostic tests like the conventional RBPT, STAT and Dot ELISA. Sera from 30 animals suspected of brucellosis were analyzed with four serodiagnostic tests – RBPT, STAT, Dot ELISA and Superagglutination. Nine (30%) sera were positive by RBPT, 1 (3.33%) by STAT and 7 (23.33%) were found to be positive by Dot ELISA. All the 30 sera (100%) were found to be positive by Superagglutination (Table 1).

Table 1. Comparative efficacy of RBPT, STAT, Dot ELISA and Superagglutination in detecting anti-Brucella antibodies in cattle sera

Sample No.	RBPT	STAT	Superagglutination	ELISA
07-1222	+	-	+	+
07-1258	+	-	+	+
1/21(M1)	-	-	+	-
10-P	-	-	+	-
136P/J26	+	-	+	-
2-S	-	-	+	+
3-P	-	1:40	+	-
47	+	-	+	+
6	+	-	+	-
7-P	-	1:20	+	-
8-P	-	-	+	-
9-P	-	1:20	+	-
A2	-	-	+	-
J-15	+	-	+	+
J-4	-	1:20	+	+
M10	-	-	+	-
M12	-	-	+	-
M13	-	-	+	-
M15	-	-	+	-
M2	-	-	+	-
M24	-	-	+	-
M25	+	-	+	-
M28	-	-	+	-
M3	+	-	+	-

M30	+	-	+	-
M4	-	-	+	-
M5	-	-	+	-
M7	-	-	+	-
M8	-	-	+	-
M9	-	-	+	+

99

100 All the 37 sera from disease – free animals were negative by RBPT, STAT, Dot ELISA and

101 Superagglutination (Table 2).

102 **Table 2: A comparison of superagglutination with other tests in screening healthy animals**

103

Sample no.	RBPT	STAT	Superagglutination	ELISA
1				
M6				
M14				
M16				
M21				
M22				
A3				
D1				
52				
51				
2301B08				
2539B08				
2730B08				
1192B08				
2690B08				
2862B08				
2967B08				
2941B08				
2867B08				
2731B08				
2741B08				
2732B08				
2968B08				
2591B08				
1047B08				
2205B08				
143B09				
147B09				
62B09				
148B09				
141B09				
144B09				
142B09				
140B09				
145B09				
195B09				
246B09				

104

Superagglutination could identify all the 27 sera giving false negative results by RBPT (18), STAT (26) and Dot ELISA (8), respectively (Table 3).

Table 3. False negative samples identified by superagglutination test

Sample No.	RBPT	STAT	Super agglutination	ELISA
07-1222	+	-	+	+
07-1258	+	-	+	+
10-P	-	-	+	-
136P/J26	+	-	+	-
149B09	-	-	+	+
187B09	-	-	+	+
247B09	-	-	+	+
262B09	-	-	+	+
2697B08	-	-	+	+
2820B08	-	-	+	+
299B08	-	-	+	+
2-S	-	-	+	+
308B08	-	-	+	+
3-P	-	1:40	+	-
47	+	-	+	+
57B09	-	-	+	+
59B09	-	-	+	+
6	+	-	+	-
60B09	-	-	+	+
783B08	-	-	+	+
8-P	-	-	+	-
J-15	+	-	+	+
J-4	-	-	+	+
M25	+	-	+	-
M3	+	-	+	-
M30	+	-	+	-
M9	-	-	+	+

Superagglutination could also identify 9 sera giving false positive results by RBPT (2), STAT (2) and Dot ELISA (7), respectively (Table 4).

Table 4: False positive samples identified by Superagglutination combined with other tests

Animal No.	RBPT	STAT	ELISA	Superagglutination
J-20	+	1:40	-	-
M-17	-	-	+	-
M-18	-	-	+	-
M-19	-	-	+	-
M-20	-	-	+	-
5-P	-	1:40	+	-
J-6	-	-	+	-
J-19	-	-	+	-
A-4	+	-	-	-

The Superagglutination Test could detect all the 30 positive samples which went undetected by other tests (i.e., false negative) including 17 sera by a combination of RBPT, STAT and ELISA (17, Table 1), 17 by both RBPT and STAT (17), 7 by both STAT and ELISA (7) or and 3 by both RBPT and ELISA (3), respectively (Table 3). No false positives could be detected in case of Superagglutination when sera of disease free animals were tested and results were compared with those of RBPT, STAT, and Dot ELISA (Table 4). The sensitivity and specificity were determined to be 64.2% and 95.65% for RBPT and 100% each for superagglutination, respectively. Clumps of a larger size (1.8 times bigger) were formed in Superagglutination compared to RBPT (Table 5). The difference in clump size was statistically significant ($p < 0.05$).

Table 5: A comparison of the clump size in conventional RBPT and superagglutination test

Sample no.	Clump size	
	Conventional RBPT	Superagglutination test
1	1591	2275
2	878	1544
3	950	1540
4	981	1166
5	3461	5831
6	1181	4713
7	1696	2195
Mean $\pm$ SE	1534.00 $\pm$ 343.12	2752.00 $\pm$ 678.01

The difference is significant ($p < 0.05$).

In many countries, the standard Plate Agglutination Test is the routine test for human and animal brucellosis. However, it may give false negative results [3, 4]. RBPT is a variant of plate / slide agglutination test where killed *Brucella* organisms stained with Rose Bengal dye are used as antigen for detection of antibodies in the serum. The International Office of Epizootics has recommended the RBPT as one of the tests for the diagnosis of bovine brucellosis [5]. The comparative efficacy of three serodiagnostic tests (RBPT, STAT and Dot ELISA) in detecting anti

133 – *Brucella* antibodies in sera from brucellosis suspected and normal healthy (brucellosis
134 unaffected) cattle has been evaluated earlier [6]. Only 5.55% samples were found positive by
135 STAT and 50% samples were positive by RBPT, whereas Dot ELISA could detect antibodies in
136 all (100%) samples. RBPT could detect antibodies in 58.82% samples found negative by STAT.
137 All the samples positive by RBPT (100%) showed positive results with Dot ELISA also. Of the
138 RBPT negative samples, 11.25% showed positive and 88.88% showed negative results by STAT
139 while all the samples (100%) showed positive results with Dot ELISA. All the sera from normal
140 healthy animals were negative by RBPT, STAT and ELISA. It was suggested that in order to get a
141 better diagnosis of *Brucella* infection, a combination of RBPT and Dot ELISA should be used,
142 especially in case of samples found negative by either RBPT or STAT used alone or in
143 combination.

144 The RBPT is a quick, cheap and effective test for the diagnosis of brucellosis. However, many
145 factors affect the RBPT reactions and their reading. Some people are able to see the finer
146 agglutination while many others cannot. This causes variation in results. Some authors [7] have
147 reported unacceptable rate of false negatives with the RBPT. In RBPT, false positive reactions
148 may sometimes arise due to formation of fibrin clots. The acid pH diminishes agglutination by IgM
149 but encourages agglutination by IgG1. False positive results may also arise due to immune
150 response of the animal to other microorganisms that share epitopes with *Brucella* species.
151 Prozone may often lead to a false negative reaction in RBPT when sera of high antibody titers
152 are tested against it. I-ELISA has also been suggested as a screening test for routine
153 serodiagnosis of *Brucella* infection [8]. The intensity of the agglutination reaction is a good
154 indicator of the concentration of antibody in the serum. Very low concentration of antibodies may
155 not give visible agglutination. The lack of agglutination at high concentrations of antigen or
156 antibodies is called the Prozone effect. In Prozone, very small complexes are formed that do not
157 clump to form visible agglutination. These factors lead to false negative results.

158 In the modified RBPT described here, the test serum or plasma antibodies are prestained by
159 mixing with a protein stain of contrasting colour (like Coomassie Blue or Amido Black) to colour
160 the antibodies. Biotinylated anti-bovine IgG and streptavidin are added to the mixture of antigen

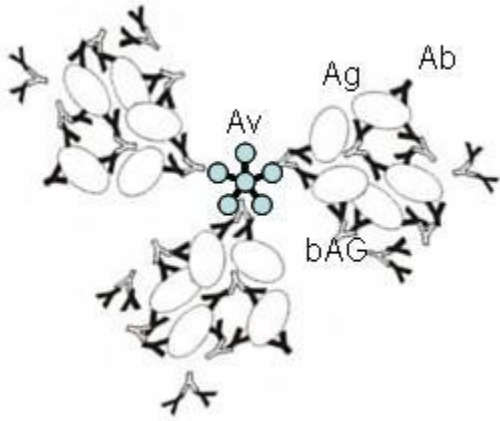
and antibodies to enhance the clump size by cross-linking the antibody molecules. Since Avidin has a strong affinity for Biotin, it will cross- link biotinylated antiglobulin bound to the antigen – antibody clumps making larger and more compact masses of clumps. The additional steps of staining the test antibody and adding Biotinylated Antiglobulin and Avidin are the our novel modifications to the conventional method of agglutination tests. The result is read out by the unaided eye or with the help of a hand lens or under low power of an ordinary light microscope after putting a cover slip on the slide. If visible clumps are formed, the test sample is positive for the antibody against that the microbial antigen. In antibody control (i.e., antigen, negative serum and species – specific antiglobulin), there will be no agglutination of antigen particles. In antiglobulin control (i.e., antigen, positive serum and unrelated antiglobulin), there will be normal agglutination but no enhancement of agglutination. The principle is illustrated with diagrams (Figs. 1, 2 & 3) and with the photographs of actual results of superagglutination for diagnosis of brucellosis (Figs. 4 to 6).



Figure 1. Normal agglutination with antigen and positive serum



Figure 2. Enhanced agglutination by antigen, positive serum and antiglobulin



182

183 Figure 3. Superagglutination of antigen (Ag) and antibody (Ab) complexes by

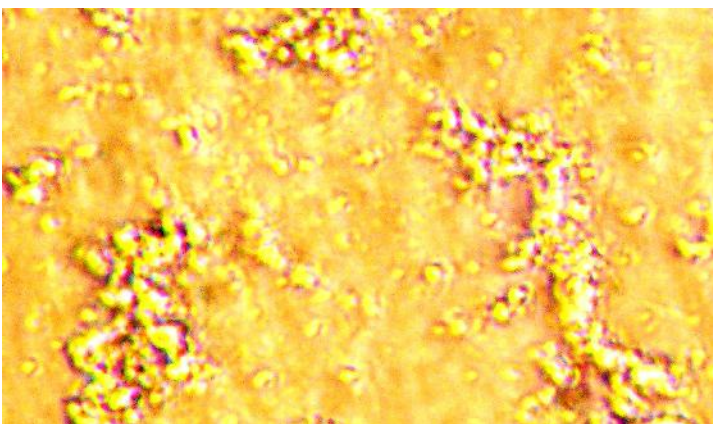
184 biotinylated antiglobulin (bAG) and avidin (Av)



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186 Figure 4. Absence of agglutination with antigen, negative serum and antiglobulin

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188

189 Figure 5. Normal Agglutination caused by antigen and positive serum

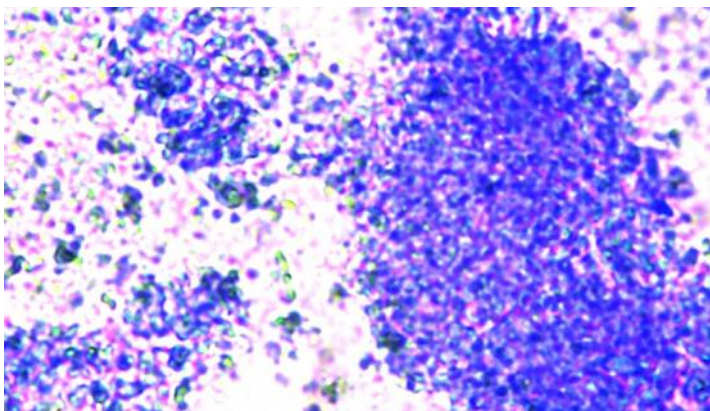


Figure 6. Superagglutination caused by pink colored antigen, blue stained antibody from positive serum and antiglobulin

These simple and easy modifications very significantly enhance the sensitivity of the agglutination test because of formation of compact and larger clumps without compromising specificity and hence minimize the chances of false negative results occurring due to low titer of antibody in the serum. The staining of antibody in the serum / plasma eliminates the false positive results arising due to non – specific aggregates of antigen particles caused by improper mixing mistaken for an agglutinate. The real agglutinates will be of two colours due to the coloured antigen and the stained antibody whereas aggregates of antigen particles alone or antibody alone will be of one colour only.

The new modifications in the agglutination test proposed here do not require any extra equipment and they do not cost much since only a few microlitres of the stain, the diluted biotinylated antiglobulin and diluted Avidin are required for each test. Furthermore, the additional steps do not require more than five minutes. The antiglobulin of IgG isotype, being bivalent, can give two – fold enhancement in agglutination whereas, the antiglobulin of IgM isotype, due to its ten binding sites, can give upto ten – fold enhancement in agglutination. The binding of Avidin with Biotin on the biotinylated antiglobulin makes the clumps larger and compact due to four binding sites for biotin on each avidin molecule and hence they are easily detectable.

CONCLUSIONS

The novel superagglutination test minimizes false negative results of conventional slide / plate agglutination test (like RBPT) by employing biotinylated antiglobulin with avidin which enhances

clump size and as well as false positive results by staining the antibody enabling differentiation of true agglutinates from non specific aggregates of antigen alone.

COMPETING INTERESTS

The authors do not have any competing interests.

AUTHORS' CONTRIBUTIONS

H M Saxena generated the innovative idea behind the study. He designed the study, wrote the protocol, performed the analysis, and wrote the draft of the manuscript. P Kaur did the experimentation under his supervision. All authors read and approved the final manuscript.

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Figure legends

(1) Figure 1. Normal agglutination with antigen and positive serum.

(2) Figure 2. Enhanced Agglutination by antigen, positive serum and antiglobulin

(3) Figure 3. Superagglutination of antigen (Ag) and antibody (Ab) complexes by biotinylated antiglobulin (bAG) and avidin (Av).

(4) Figure 4. Absence of agglutination with antigen, negative serum and antiglobulin.

(5) Figure 5. Normal Agglutination caused by antigen and positive serum.

(6) Figure 6. Superagglutination caused by pink colored antigen, blue stained antibody from positive serum and antiglobulin.

Table legends

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Table 2: A comparison of superagglutination with other tests in screening healthy animals.

Table 3. False negative samples identified by Superagglutination test.

Table 4: False positive samples identified by Superagglutination combined with other tests.

Table 5: A comparison of the clump size in conventional RBPT and superagglutination test

Abbreviations

RBPT = Rose Bengal Plate Test

STAT = Standard Tube Agglutination Test

ELISA = Enzyme Linked Immunosorbant Assay