

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. It 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 which is called agglutination. Agglutination test is used in the diagnosis of infections by demonstrating 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 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 ELISA as well as the modified RBPT (Superagglutination Test) were applied on all the serum samples.

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. 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.

82 The comb was then allowed to react with an anti-cattle IgG alkaline phosphatase conjugate. After
 83 2 more washes, the comb was moved to the last well. Colour was developed by an enzymatic
 84 reaction. The intensity of colour of the test spots corresponded directly to the antibody level in the
 85 test sample.

86 RESULTS AND DISCUSSION

87 The modified agglutination test described above is named as the “Superagglutination Test”. The
 88 test gave superior results in detecting anti-*Brucella* antibodies compared to the other
 89 serodiagnostic tests like the conventional RBPT, STAT and ELISA. Out of All the 19 sera giving
 90 false negative results with the RBPT, 19 were found to be positive by Superagglutination Test
 91 while 3 were positive by ELISA and only one was positive by STAT (Table 1).

92 **Table 1. Samples found to be negative with RBPT but positive by other techniques**
 93

Sample No.	RBPT	STAT	Super agglutination	ELISA
1/21(M1)	-	-	+	-
M2	-	-	+	-
M4	-	-	+	-
M5	-	-	+	-
M7	-	-	+	-
M8	-	-	+	-
M9	-	-	+	+
M10	-	-	+	-
M12	-	-	+	-
M13	-	-	+	-
M15	-	-	++	-
A2	-	-	+	-
3-P	-	1:40	+	-
8-P	-	-	++	-

10-P	-	-	++	-
7-P	-	1:20	+	-
9-P	-	1:20	+	-
2-S	-	-	+	+
J-4	-	1:20	+	+

94

95 Out of All the 26 serum samples giving false negative results with the STAT, 26 were found to be
96 positive by Superagglutination Test while 6 were positive by ELISA and nine were positive by
97 RBPT (Table 2).

98 **Table 2. Brucella positive samples found negative by STAT but positive by other**
99 **techniques**

Sample No.	RBPT	STAT	Superagglutination	ELISA
6	+	-	+	-
136P/J26	+	-	+	-
J-15	+	-	+	+
1/21(M1)	-	-	+	-
M2	-	-	+	-
M3	+	-	+++	-
M4	-	-	+	-
M5	-	-	+	-
M7	-	-	+/-	-
M8	-	-	+	-
M9	-	-	+	+
M10	-	-	+	-
M12	-	-	+/-	-
M13	-	-	+/-	-
M15	-	-	+++	-
M24	-	-	+	-

M25	+	-	+	-
M28	-	-	+	-
M30	+	-	+	-
07-1258	+	-	+++	+
07-1222	+	-	+	+++
47	+	-	+	+++
A2	-	-	+	-
8-P	-	-	+++	-
10-P	-	-	+++	-
2-S	-	-	+/-	+

100

101 Out of All the 18 sera giving false negative results with ELISA, all the 18 were found to be positive
102 by Superagglutination Test while one was positive by STAT and only two were positive by RBPT
103 (Table 3).

104 **Table 3. Brucella positive samples found negative by ELISA but positive by other**
105 **techniques**

Sample No.	RBPT	STAT	Superagglutination	ELISA
136P/J26	+	-	+	-
1/21(M1)	-	-	+	-
M2	-	-	+	-
M3	+	-	+++	-
M4	-	-	+	-
M5	-	-	+	-
M7	-	-	+	-
M8	-	-	+	-
M10	-	-	+	-
M12	-	-	+	-
M13	-	-	+	-

M15	-	-	+++	-
A2	-	-	+	-
3-P	-	1:40	+	-
8-P	-	-	+++	-
10-P	-	-	+++	-
7-P	-	1:20	+	-
9-P	-	1:20	+	-

106

107 All the 10 cattle found to be free from Brucellosis by RBPT, STAT and ELISA were also negative
 108 by Superagglutination Test (Table 4).

109 **Table 4. Serological evaluation of sera from Brucellosis – free cattle**

Sample no.	RBPT	STAT	ELISA	Superagglutination
1	Negative	Negative	Negative	Negative
M6	Negative	Negative	Negative	Negative
M14	Negative	Negative	Negative	Negative
M16	Negative	Negative	Negative	Negative
M21	Negative	Negative	Negative	Negative
M22	Negative	Negative	Negative	Negative
A3	Negative	Negative	Negative	Negative
D1	Negative	Negative	Negative	Negative
52	Negative	Negative	Negative	Negative
51	Negative	Negative	Negative	Negative

110

111 The Superagglutination Test could detect positive samples which went undetected (i.e., negative)
 112 by a combination of RBPT, STAT and ELISA (15), RBPT and STAT (18) or RBPT and ELISA
 113 (16), respectively (Table 5).

Table 5. Comparative efficacy of RBPT, STAT, Dot ELISA and Superagglutination in detecting anti-Brucella antibodies in cattle sera

Sample no.	RBPT	STAT	ELISA	Superagglutination
1/21(M1)	Negative	Negative	Negative	Positive
M2	Negative	Negative	Negative	Positive
M4	Negative	Negative	Negative	Positive
M5	Negative	Negative	Negative	Positive
M7	Negative	Negative	Negative	Positive
M8	Negative	Negative	Negative	Positive
M9	Negative	Negative	Positive	Positive
M10	Negative	Negative	Negative	Positive
M12	Negative	Negative	Negative	Positive
M13	Negative	Negative	Negative	Positive
M15	Negative	Negative	Negative	Positive
A2	Negative	Negative	Negative	Positive
3-P	Negative	1:40	Negative	Positive
8-P	Negative	Negative	Negative	Positive
10-P	Negative	Negative	Negative	Positive
7-P	Negative	1:20	Negative	Positive
9-P	Negative	1:20	Negative	Positive
2-S	Negative	Negative	Positive	Positive
J-4	Negative	1:20	Positive	Positive

In many countries, the standard Plate Agglutination Test is the routine test for human 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

121 RBPT as one of the tests for the diagnosis of bovine Brucellosis [5]. The RBPT is a quick, cheap
122 and effective test for the diagnosis of Brucellosis. However, many factors affect the RBPT
123 reactions and their reading. Some people are able to see the finer agglutination while many
124 others cannot. This causes variation in results. Some authors [6] have reported unacceptable rate
125 of false negatives with the RBPT.

126 The intensity of the agglutination reaction is a good indicator of the concentration of antibody in
127 the serum. Very low concentration of antibodies may not give visible agglutination. The lack of
128 agglutination at high concentrations of antigen or antibodies is called the Prozone effect. In
129 Prozone, very small complexes are formed that do not clump to form visible agglutination. These
130 factors lead to false negative results.

131 In the modified RBPT **described here**, the test serum or plasma antibodies are prestained by
132 mixing with a protein stain of contrasting colour (like Coomassie Blue or Amido Black) to colour
133 the antibodies. Biotinylated anti-bovine IgG and streptavidin are added to the mixture of antigen
134 and antibodies to enhance the clump size by cross-linking the antibody molecules. Since Avidin
135 has a strong affinity for Biotin, it will cross- link biotinylated antiglobulin bound to the antigen –
136 antibody clumps making larger and more compact masses of clumps. The additional steps of
137 staining the test antibody and adding Biotinylated Antiglobulin and Avidin are **the our** novel
138 modifications to the conventional method of agglutination tests. The result is read out by the
139 unaided eye or with the help of a hand lens or under an ordinary light microscope after putting a
140 cover slip on the slide. If visible clumps are formed, the test sample is positive for the antibody
141 against **that the microbial** antigen.

142 In antibody control (i.e., antigen, negative serum and species – specific antiglobulin), there will be
143 no agglutination of antigen particles. In antiglobulin control (i.e., antigen, positive serum and
144 unrelated antiglobulin), there will be normal agglutination but no enhancement of agglutination.

145 The principle is illustrated with diagrams (Figs. 1, 2 & 3) and with the photographs of actual
146 results of superagglutination for diagnosis of **brucellosis** (Figs. 4 to 6).



149

150 Figure 1. Normal agglutination with antigen and positive serum

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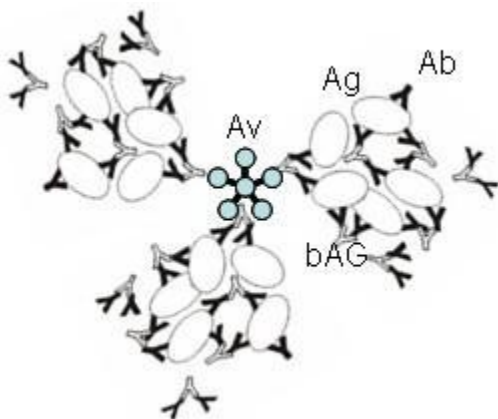


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154 Figure 2. Agglutination by antigen, positive serum and antiglobulin

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158 Figure 3. Superagglutination by biotinylated antiglobulin and avidin

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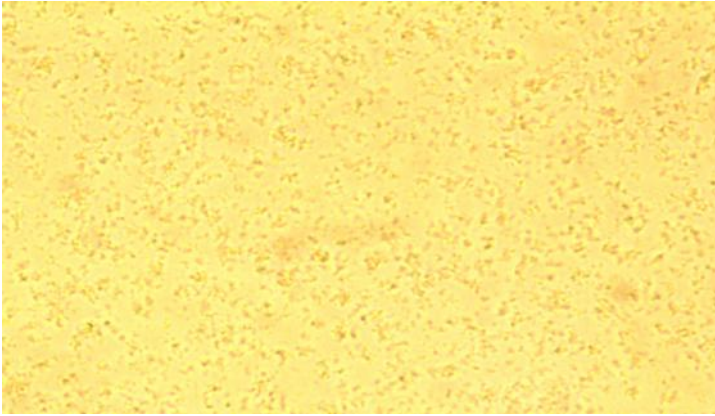


Figure 4. Absence of agglutination with antigen, negative serum and antiglobulin

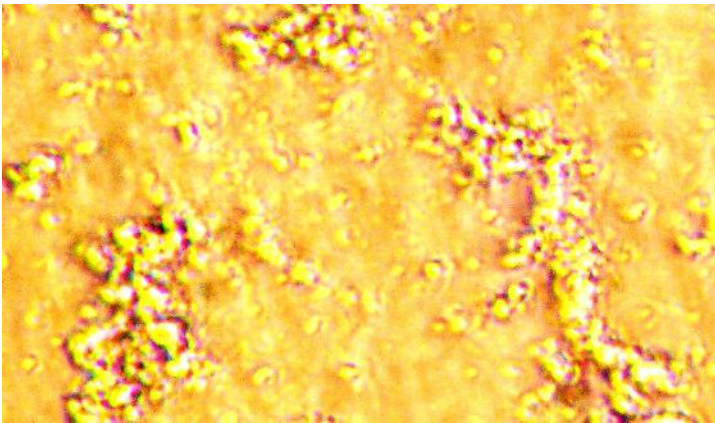


Figure 5. Normal Agglutination caused by antigen and positive serum

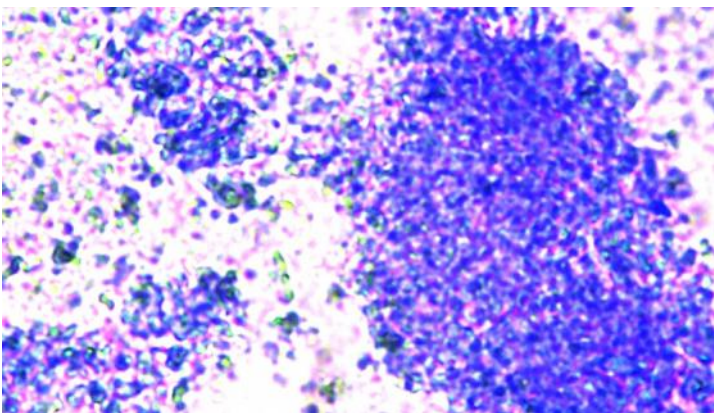


Figure 6. Superagglutination caused by antigen, positive serum and antiglobulin

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

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

186 **CONCLUSIONS**

187 The novel superagglutination test minimizes false negative results of conventional slide / plate
188 agglutination test (like RBPT) by employing biotinylated antiglobulin with avidin which enhances
189 clump size **and as well as** false positive results by staining the antibody enabling differentiation of
190 true agglutinates from non specific aggregates of antigen **alone**.

191 **COMPETING INTERESTS**

192 The authors do not have any competing interests.

193 **AUTHORS' CONTRIBUTIONS**

194 H M Saxena generated the innovative idea behind the study. He designed the study, wrote the
195 protocol, performed the analysis, and wrote the draft of the manuscript. P Kaur did the
196 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. Agglutination by antigen, positive serum and antiglobulin
- (3) Figure 3. Superagglutination by biotinylated antiglobulin and avidin.
- (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 antigen, positive serum and antiglobulin.

Table legends

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225 Table 5. Comparative efficacy of RBPT, STAT, Dot ELISA and Superagglutination in
226 detecting anti-Brucella antibodies in cattle sera.

227 **Abbreviations**

228 RBPT = Rose Bengal Plate Test

229 STAT = Standard Tube Agglutination Test

230 ELISA = Enzyme Linked Immunosorbant Assay