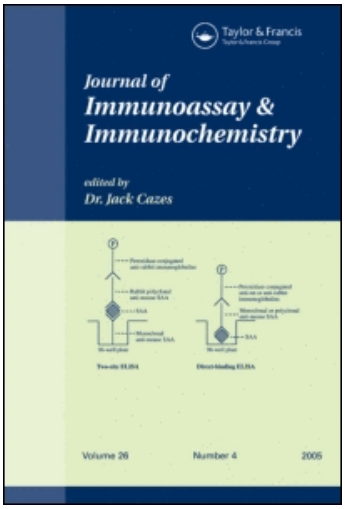

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QUANTITATIVE ROSE BENGAL TEST FOR DIAGNOSIS OF BOVINE BRUCELLOSIS

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□ *The Rose Bengal Test (RBT) is the most widely used screening test for brucellosis in both humans and animals. Owing to its apparent simplicity of reading, however, interpretations of the RBT results can be affected by personal experience. This study describes a simple way to improve the accuracy and uniformity of reading the RBT reaction by counting the number of agglutinated particles using transparent OHP film with Quantity One®, which was originally designed to count the bacterial colony numbers on agar plates. Using this system, the reactivities of three Rose Bengal antigens from different sources against international standard serum (1,000 units, VLA, UK) could be numerically measured: the intensity scale ranged from zero to around 1,600. This system enabled us to compare the antigenicity of Rose Bengal antigens from three different sources by using statistical analyses such as regression and mean intensity. Collectively, mathematical measuring of the reaction intensity used in this study may help interpret subtle test results by providing more reliable data and additional statistical information on the herd. In addition, the method would also be applicable to other agglutination test for other diseases.*

Keywords agglutination, Brucella, OIE, quantitative assay, Rose Bengal test, serological test

INTRODUCTION

Brucellosis is a worldwide emerging zoonosis caused by genus Brucella. The disease has diverse host range including human, cattle, hog, goat, dog, and even ocean animals. Though the major bacterial species infecting each host are different, the common antigens such as lipopolysaccharide

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(LPS) made from a brucella spp. have provided a possible way to diagnose brucellosis in multi-species including human. Especially, the agglutination based serological tests have been preferable in diagnosing several kinds of animals because of its simple application requiring no further reagent such as enzyme conjugated secondary antibodies. Of the agglutination test, the Rose Bengal Test (RBT) is widely used as a screening diagnostic test in veterinary medicine, and in the rapid diagnosis of human brucellosis in endemic areas.[15] The RBT is highly sensitive, easy to use, and results are obtained rapidly at minimal cost.[3,8,14] Because of its simplicity, however, the value of this test varies depending on standardization of antigen and accuracy of reading. Since its development by Dr. Pietz in 1965,[8,18] variable applications of the RBT have been reported using different samples, animals, pathogens, and methods.[2,10,21]

As yet, however, no quantitative methods are available to read and report agglutination intensity.

Currently, RBT results are reported as a dichotomous (positive and negative) outcome. There would be a number of benefits if the RBT results were reported on a quantitative scale. Reading discrepancies among laboratory workers could be reduced. It would also be possible to distinguish infected and non-infected individuals on the basis of a defined cut-off value. Currently, a serum sample could be considered positive if there is any evidence of agglutination, which can adversely affect test specificity. In addition, more accurate statistical analysis might be possible through quantification of intensity, based on a comparison of average intensity, regression analysis, and calculation of likelihood ratio. In this study, we describe a simple way to read and report the reaction intensity of RBT numerically by counting the number of agglutinated particles using transparent OHP film with Quantity One®. Using this method, we also compare the antigenicity of three different antigens sourced from different countries.

EXPERIMENTAL

Test Sera

In this study, a case was considered a bovine animal where *Brucella abortus* had been isolated from internal organs. The cases were selected during 2005 and 2006 based on sequential laboratory submissions to the Bacteriology and Parasitology Division of the National Veterinary Research & Quarantine Service in Anyang, Korea. In most of these animals, it was initially suspected on the basis of results from a series of tests including the milk ring test, the Rose Bengal test and the tube agglutination test conducted as a part of the national brucellosis control and eradication

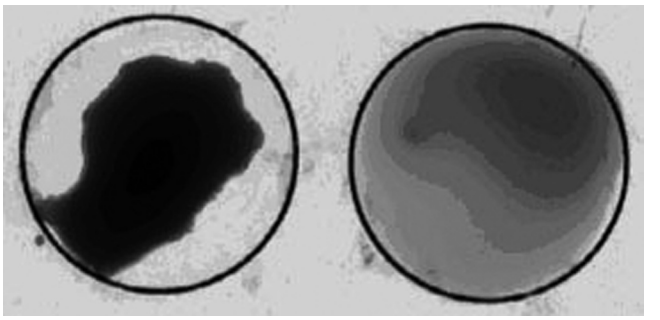

FIGURE 1 The reaction patterns observed with two different diluents, 0.5% phenol saline (left) and fetal calf serum (right).

program in Korea. Suspected bacterial isolates from these animals were identified using standard methods: growth characteristics on sheep blood agar and MacConkey agar in a 5% CO_2 incubator, biochemical tests such as the urease and oxidase tests, and strain identification and differentiation using PCR.[9,20]

During 2005 and 2006, *Brucella abortus* was isolated from 128 cattle as part of the national brucellosis control program in Korea. Sera collected from 128 cases and 734 uninfected cattle (our 'non-case' animals) during 2005 and 2006 were used in this study. Non-case cattle were selected from Jeju island, which has been proved to be free of bovine brucellosis since 2001. Each serum was inactivated at 56°C for 30 min and stored at −70°C. The international standard serum (OIEISS, 1,000 units/mL) was purchased from Veterinary Laboratories Agency (VLA), UK. In accordance with the protocol in the OIE manual, the standard serum was initially diluted with 0.5% phenol saline. This diluent is suitable for manual test interpretation, but not for numerical reading, as proposed here. Using this diluent, the reactant did not spread evenly on the OHP film (Figure 1). Thus, the OIESS was diluted using fetal calf serum prior to use in this study.

Test Antigens

The RBT antigen was prepared according to the protocol provided by OIE manual.[1] Briefly, a suspension of killed *B. abortus* S1119-3 cells was stained with 1% (w/v) Rose Bengal (Sigma-Aldrich, USA). Then, 1 g of stained cells was re-suspended in 7 mL of diluent (21.1 g of sodium hydroxide, 95 ml of lactic acid, and adjusted to 1,056 mL with phenol saline). The pH and packed cell volume (PCV) of the final product were 3.65 ± 0.05 and 8%, respectively. To measure the PCV of antigen, we added 4.5 mL of distilled water and then 0.5 ml of evenly suspended Rose Bengal antigen

to a Hopkins vaccine tube (Bellco Glass Inc., Vineland, N.J., USA). The tube was centrifuged at 2,500 g for 75 minutes. If the obtained volume was more than 8%, the original suspension was diluted to twice its original volume using distilled water, then PCV was measured in the same manner.

Two commercial *B. abortus* antigens were obtained, from Veterinary Laboratories Agency (VLA; product code PA0060 [batches 277], Weybridge, UK) and Institute Pourquier (Lot/Batch 320, Montpellier, France), and a commercial *B. melitensis* antigen from VLA (Product code PA2016, Batch number SG276). The expiry date of the commercial British and French products were January, 2031 and May, 2009, respectively. Experiments were conducted between late 2006 and early 2007.

Quantitative Rose Bengal Test

We brought the relevant sera (case, non-case and international standard) and test antigens to room temperature 30 minutes before each experiment. The agglutination test was conducted on transparent OHP film (Figure 2) using 30 μL of each reactant and covering an area of approximately 2 cm in diameter. We used a densitometer carrying a transparent adapter (GS-800, Bio-Rad, Hercules, CA, USA) for scanning of the agglutination reaction and the intensity was quantified by Quantiity One® software (Bio-Rad, Hercules, CA, USA). The selected analysis options with software are X-ray film, linear, transmissive light and medium scan resolution (63.5×63.5). We measured the agglutination intensity using the colony counting menu with selected options of sensitivity 10 and

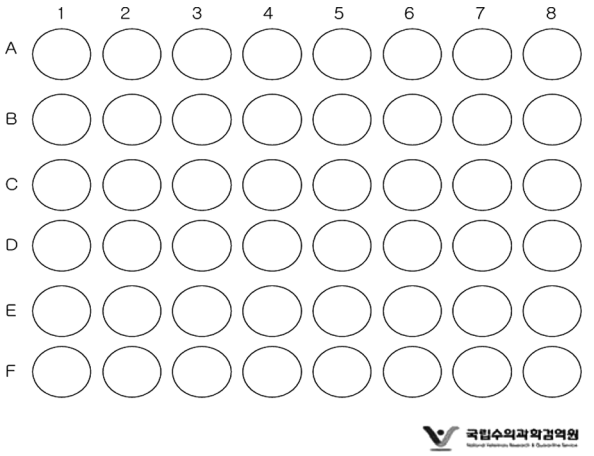

FIGURE 2 The transparent film used for the Rose Bengal test. The diameter of each circle was 2 cm.

averaging 3. The diameter of the circle for analysis was adjusted to fit the original circle on the transparent film; then, the same circle was moved without size modification from one well to another for counting.

Comparison of Antigenicity of RBT Antigens

The antigenicity of each antigen was compared by two different ways. Firstly, the OIEISS purchased from VLA was diluted and reaction with each antigen was measured according to the described quantitative RBT. For analysis, a non-linear regression line (a fourth-order polynomial equation) was created by forcing to intercept the origin using GraphPad Prism 4 (San Diego, CA, USA). The other way is testing all 128 case samples with antigens from different sources. The reproducibility of the test was determined using the produced in-house antigen, and the reactivity was compared with that of the commercial antigens. The antigenicity was compared by the calculated slope of linear regression line.

Reactivity of Cattle Sera from Case and Infection Free Herd

Quantitative RBT was conducted using sera from 128 cases and 734 non-cases. Average differences between paired results were analyzed using the Mann-Whitney test.

RESULTS

Test Validation and Antigenicity Comparison Using OIEISS

Results obtained in the quantitative RBT were compared with traditional RBT. As shown in Panel B of Figure 3, the agglutination intensity observed by naked eyes was well correlated with the number obtained by quantitative RBT. The number representing the agglutination intensity ranged from zero to around 1,600. To compare antigenicity, purchased OIEISS was diluted and each diluted serum was reacted with RBT antigens. The in-house *Brucella abortus* antigens made visible agglutination at a serum dilution of 1/45 but not at 1/55 (Panel A of Figure 3). A similar intensity was also observed with antigen A. However, the reaction with antigen B was clearly weaker than the others, showing no agglutination at 1/45. In addition, the reaction completed a little bit slowly.

The measured PCV using antigens A and B were 16% and 12%, respectively. As described previously, the PCV of in-house Rose Bengal test antigens prepared in this study was 8%.

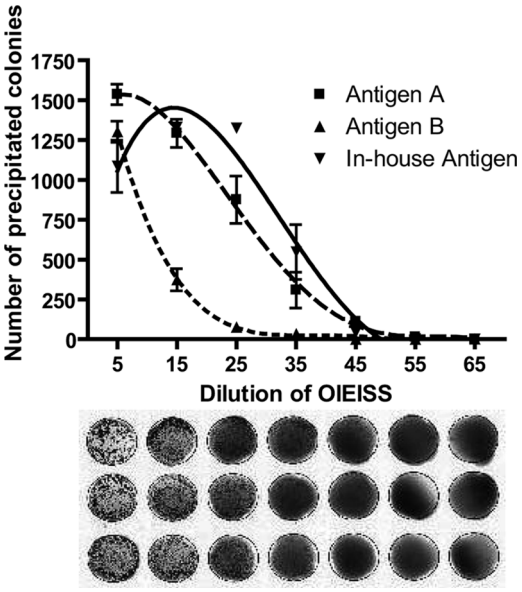

FIGURE 3 Agglutinations by Rose Bengal antigens from different sources and OIEISS diluted with fetal calf serum (Panel A). The three replicates of the Rose Bengal test using antigen produced in this study at different dilutions of OIEISS (Panel B).

Comparison of Antigenicity to Case Sera

In addition, the antigenicity of RBT antigens was also compared by observation of reactivity with 128 case sera. Firstly, reproducibility was measured by double trials of quantitative RBT using in-house antigen as shown in Panel A of Figure 4. The slope of the regression line was 0.9985 (95% CI 0.9634 ~ 1.034) and the standard deviation of the residual (Sy.x) was 176.8. Then, the antigenicity of the produced Rose Bengal antigen was compared with that of antigen B (Panel B of Figure 4). The slope of regression lines was 0.9556 (95% CI 0.9010 ~ 1.010) indicating less antigenicity of antigen B. The standard deviation of the residual (Sy.x) was 275.3, showing a slightly lower similarity than with the homologous antigen. Finally, antigenicity of in-house antigen was compared with the Rose Bengal antigen (Product code PA2016, Batch SG276, VLA, UK) that was made with *B. melitensis*. (Panel C of Figure 4). The slope of the regression lines was 1.020 (95% CI 0.9357 ~ 1.105) and the standard deviation of residual (Sy.x) was 426.9. Some sera showed a skewed response to one of the antigens.

Reactivity to Case and Non-Case Sera

The overall pattern of Rose Bengal reactions with case and non-case sera is presented in Figure 5. Among the non-case sera, no visible reactions

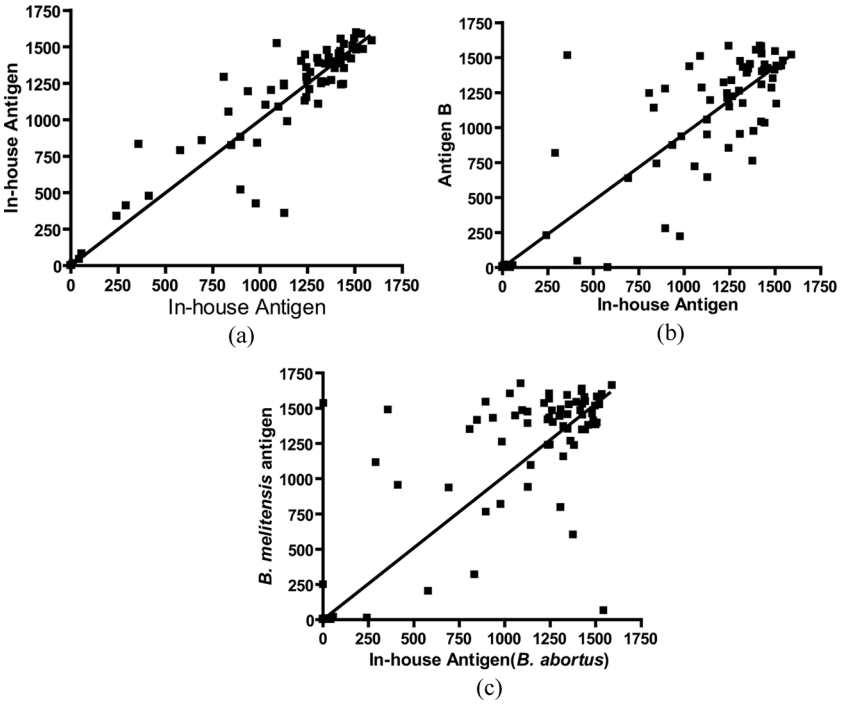

FIGURE 4 Antigenicity comparison among Rose Bengal antigens from VLA, Institute Pourquier and In-house antigen. The tested sera were from infected cattle in Korea.

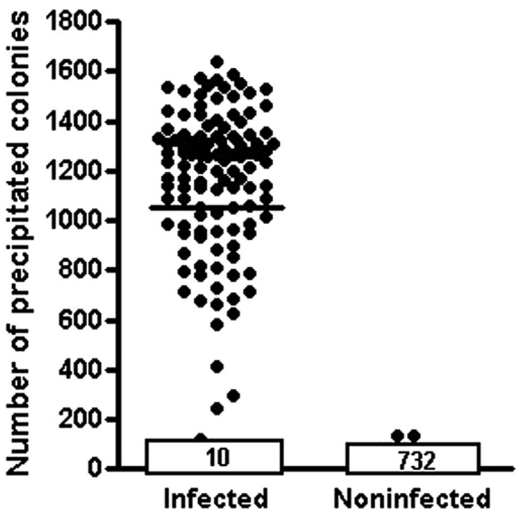

FIGURE 5 Rose Bengal reaction patterns with cattle sera from infected, and infection-free regions. The number within box indicates the number of cattle showing agglutination intensity less than 50. The bar in each group indicates the mean of the observed values. The Rose Bengal antigen produced in this study was used.

were observed, and only a small number of agglutinations were detected with two sera by the developed system. The sensitivity and specificity at cut-off 53.5 were 92.19% (118/128; 95% confidence interval: 86.10, 96.19) and 99.73% (732/734; 99.02, 99.97), respectively.

DISCUSSION

Numerous difficulties are inherent in evaluating serological tests for bovine brucellosis. First, the infected animals used for the estimation of a test's sensitivity cannot exactly represent the population in which the diagnostic test will be used, because bacterial culture results are often negative for an infected animal. It is assumed that the animals in an acute stage are more likely to have positive culture results than those with a chronic infection. Secondly, class of antibodies detected by one assay might be different from those detected by the others. Some inconsistencies have been observed in the results given by various tests.[6,7] Thus, a series of tests has been conducted in most countries to diagnose bovine brucellosis. Usually, it has been recommended that the Rose Bengal test is adequate for screening and tube agglutination and the complement fixation test are good for confirmation.[8] Recently, some quantitative tests such as iELISA and FPA were recommended as single screening tests.[17] The tests used in bovine brucellosis can be categorized into two groups according to the form of the reported data: quantitative and qualitative. Quantitative test methods include the serum agglutination test, Rose Bengal test, and complement fixation test. As qualitative assays, the immunofluorescence test and enzyme-linked immunosorbent assay are available for brucellosis.

There are several advantages in assays that report results with quantitative values. First, when a series of tests is used, an overall probability can be calculated using the likelihood ratio for each test result.[11] The likelihood ratio is defined by the probability of that test result with disease divided by the probability of the result without disease. It expresses how many times more (or less) likely a test result is to be found in a disease case. However, if it is calculated with the dichotomous results (positive or negative), the discrimination power with animals showing weak or strong antibody titers will be minimized. Thus, the numerization of each test is essential for a correct decision on disease status. The information obtained by each level of the test results will increase the probability of a correct diagnosis. As shown in Figure 5, however, the discrepancy between the infected and infection-free groups was so clear that most values in each level are ruled in/out. We hypothesized that this effect is due to the use of the wrong standard of infected animals, as described before. Though it was not applicable

to the likelihood ratio, the advantage of numerization was still clear with the presented data.

As shown in Figure 3, the intensities measured by machine were well matched with those observed by naked eyes. The *numerization* (counted numbers of intensity) ranged from zero to around 1,600. All three antigens were agglutinated well, showing more than 1,000 precipitated particles. However, the antigen B was different, showing a steep downward slope after dilution with fetal calf serum. Thus, the reaction was not visible at 1/35 dilution. To explain the unexpected result, first we compared the packed cell volumes (PCV) of three tested antigens. As mentioned in the results section, the PCVs of those purchased from VLA and Institute Pourquier were 16% and 12%, respectively, though the manual published by OIE mentioned that 8% of packed cell volume will react with OIEISS at 1/45, but not at 1/55. The relationship between the amounts of antigen and intensity might be affected by the bacterial strain used, temperatures for saving or transportation, and periods passed after production. Blasco *et al.* (1994) also reported that the Rose Bengal antigens from several countries and batches were different in their PCV and pH 3. In all, our results implied that for the harmonization of Rose Bengal antigens from different countries or batches, an agglutination test using OIEISS is an essential prerequisite.

The antigenicity of each Rose Bengal antigen was also compared with cattle sera isolated from infected cases (Figure 4). The reproducibility of the test results was indicated by the slope of the regression line and standard deviation of the residual (Sy.x) obtained from two different trials using in-house antigen. We postulate even spread of reactant within circle is one of the important steps affecting on standard deviation of the residual (Sy.x). Then, it was compared with the data obtained with Rose Bengal antigen B. The slope of the regression was lower than twice trials of homologous antigen in Panel A of Figure 4, indicating weaker antigenicity with antigen B as observed in Figure 3. Though the slope of regression line in Panel C of Figure 4 was not much deviated from the result shown in Panel A, some sera showed much stronger reactivity to one of the two antigens. It might indicate bovine brucellosis caused by *B. melitensis*, however, further investigations are required. Otherwise, it is accredited to antigenic difference of the two RBT antigens made with *B. abortus* and *B. melitensis*. Though lipopoly saccharide (LPS) play a key role in diagnosis of brucellosis and the O-antigenic chain of LPS is shared between *B. abortus* and *B. melitensis*, we think different proteins exposed to surface also take part in the RBT reaction with lesser intensity, thus affecting on the reactivity.[4,13,19] A series of antigenic proteins including Omp 25, Omp 36, Omp 10, IalB, AcvB, FrpB, and FliC are reported.[5,13]

Our results showed that the difference between the infected and infection-free groups was clear (Figure 5). One of the advantages we can

take of the developed test was the ability to compare the intensity of test results using group averages. There was a minute difference in averages between infected and cohabitated cattle (data not shown). The selection of cattle for bacterial isolation had been based mostly on antibody titers obtained through tube agglutination tests. Thus, the antibody titer in the cohabitated group was almost similar to that of the infected group. However, the agglutination intensities of both groups were significantly different from the infection-free group.

Regarding the specificity of the Rose Bengal test, 86.3% was reported with bovine brucellosis.[12] The factors affecting specificity included cross-reaction with the LPS of bacteria including *Yersinia enterocolitica, Salmonella* group N, *Pseudomonas maltophilia* and *Escherichia coli* O157. Specifically, the *Yersinia enterocolitica* O9 has been noted to be a major microorganism causing cross-reaction to serum from brucella-infected cases.[16] As shown in Figure 5, however, our data using bovine sera from Jeju Island, an isolated area of brucellosis free in Korea, revealed that the possibility of cross-reaction to other bacterial infection mentioned above was very low in this specific area.

CONCLUSIONS

Collectively, the numerization of agglutination intensity was possible using transparent film and software providing a colony-counting menu. Several advantages are expected and observed with numerization of the intensity of the Rose Bengal test. These include reduction of discrepancy between laboratories in interpretation of agglutination and application of statistical analyses such as regression and comparisons of means between different groups. The same advantages are expected with other qualitative tests if numerization is available.

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