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Interpretation of tube agglutination test for bovine brucellosis with turbidimetric readings and international unit

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Abstract

The tube agglutination test has been used for bovine brucellosis diagnosis in Korea since middle 1950s. The reported high specificity was its value in eradication program. However, the reading of reaction mostly depends on personal experience, thus here we report a way to improve accuracy and uniformity of reading. The tube agglutination was conducted according to the protocol provided by Korean Ministry of Agriculture and Forestry. The intensity of reaction was measured by spectrophotometer. The relationship between turbidity and percentage clearing was generally in direct proportion and linear. The correspondent percent transmittance at 75, 50, and 25% clearing were 91, 82, and 73, respectively. Then, the degree of percentage of clearing at given international unit was measured. With about 1.5 unit of serum, the maximum percentage clearing was observed. The international unit showing 25, 50, and 75 percentage clearing were 0.61, 0.83 and 1.35, respectively. Based on the information obtained using international standard serum, the calculation of international unit of test serum was available. According to the protocol for bovine brucellosis diagnosis which provided by Korean Ministry of Agriculture and Forestry, the available range of detectable international unit was between 15 and 538. And the corresponding international units for suspicious case ranged between 42 and 127. Of the 35 sera from *B abortus* infected cattle, about half of them had more than 538 international units. Collectively, the reading of turbidity using spectrophotometer and application to international unit improved accuracy and uniformity of reading.

Key words: Tube agglutination test, Bovine brucellosis, Spectrophotometer

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Introduction

The first outbreak of bovine brucellosis in Korea was reported in Gyeonggi province in 1955[1]. Of the 124 dairy cattle imported from USA, 24 % (30 cattle) were identified as reactor. Additionally, 3.5% and 9.3% of Korean native cattle and dairy cow were also serologically identified, respectively in 1956[1]. Since then, most outbreaks have been reported in dairy cattle until 1999. Because of its huge impact on public health and tremendous increase in number of infected cattle, Korean government has conducted test and slaughter strategy for control. Since the first outbreak in 1955, the identification of reactor has been finally confirmed by tube agglutination test according to the protocol used in USA[1,2]. The identification of reactor was that showing complete agglutination at 100 times dilution of serum as shown in Table 1. And the suspicious were that showing more than 50% agglutination with serum diluted between 50 and 100 times. This method has been conducted more than 50 years in Korea without noticeable difficulties. It was expected, however, that inconsistency might occurred among different laboratories because the reading of agglutination intensity is largely dependent on personal experience. Thus, we provide here the criteria observed using spectrometric method. In addition, international unit of serum was calculated based on the information obtained.

Table 1. Interpretation of the Korean tube agglutination reactions.

Reaction at serum dilution of					Diagnosis
25	50	100	200	400	
-[1]	-	-	-	-	Negative
±[2]	-	-	-	-	Negative
+[3]	-	-	-	-	Negative
+	±	-	-	-	Suspicious
+	+	-	-	-	Suspicious
+	+	±	-	-	Suspicious
+	+	+	-	-	Positive
+	+	+	±	-	Positive
+	+	+	+	-	Positive
+	+	+	+	±	Positive
+	+	+	+	+	Positive

1. no agglutination, 2. agglutination over 50%, 3. complete agglutination

Materials and Methods

Tube agglutination antigens

The tube agglutination test antigens were produced with *Brucella abortus* 1119-3 according to the published procedure[3]. Briefly,

The bacterial seed was cultured 48 hours on potato infusion agar (potato infusion 36 L, Bacto agar 1,080g, Beef extract 180g, sodium chloride 180g, Bacto peptone 360g, glycerol 720 mℓ). Then the harvested cells were washed three times with 0.5% phenol saline and sterilized by heating at 100℃ for 45 min. Finally, the cell concentration was adjusted to be 4.5% by centrifugation in Hopkin's tube. The produced antigens were stored at 4℃ before use.

Cattle sera and international standard serum

The case definition of *B abortus*-infected cattle was the isolation of *B abortus* from internal organs. The identification of bacteria included growth characteristics on sheep blood agar and MacConkey agar in a 5% CO_2 incubator and biochemical tests such as the urease and oxidase tests[3]. Finally, every isolate was tested by PCR[4,5]. The number of cattle from which *B abortus* was isolated was 35. Most cattle from this group were slaughtered and sampled according to the serological results obtained in the process of the national control program using a series of tests including the milk ring test, the Rose Bengal test and the tube agglutination test. Each serum was inactivated at 56 ℃ for 30 min and stored at -70 ℃. The international standard serum (OIEISS, 1,000 unit/ mℓ) was purchased from Veterinary Laboratories Agency (VLA), UK.

Tube agglutination test

For the tube agglutination test we used the procedural methods described in operation manual for prevention of tuberculosis and brucellosis which has been provided by Korean government. Instead of test tube, cuvette designed for spectrophotometer (UV2-100, UNICAM, England) was used. To make serum dilution of 1:25, 1:50, 1:100, 1:200, and 1:400, 80, 40, 20, 10 and 5 μℓ of test serum were delivered into cuvet, then 2 mℓ of prediluted antigens were added. The rack of cuvette were gently shaken and placed at 37℃ for 48 hours.

Measuring % transmittance using spectrophotometer

The agglutination intensity was measured at 600 nm using spectrophotometer. The % transmittance was better than absorbance because it provides direct proportion to % clearing.

Results

The relationship between % clearing and % transmittance

The % transmittance was measured after serial dilution of tube agglutination antigen using 5% phenol saline. It was observed that about 2% transmittance decreased when measured after 24 hours later (data not shown). As shown in Fig 1, the relationship looks like slightly curved shape. Thus there was minute difference in terms of the degree of r^2, however the linear regression was acceptable showing r^2 of 0.9976. The obtained equation was $Y = -185.5 + 2.804X$. The % transmittance at 25, 50, and 75% clearing were 73, 82, and 91%, respectively (Fig 1).

To see the effect of hemolysis, the % transmittance was measured after dilution

of normal and heavily hemolysed sera by 25, 50, 100, 200, and 400 times. The decrease of observed % transmittance was less than 2% with all application except for the heavily hemolysed serum at 25 times dilution. It was about 96% transmittance (data not shown).

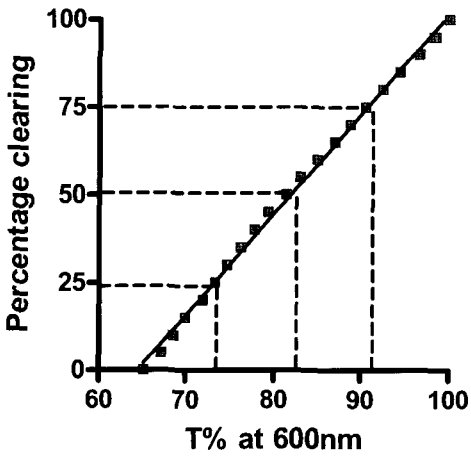

Fig 1. The % transmittance at 600 nm of bovine tube agglutination antigen at corresponding percentage clearing.

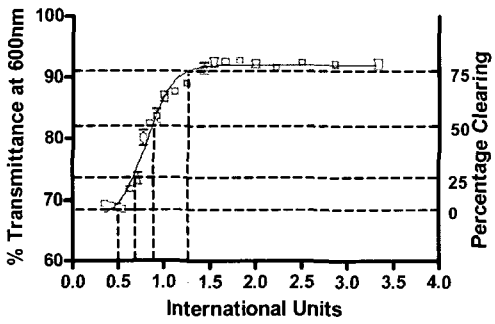

Fig 2. The agglutination intensity of bovine tube agglutination antigen at different concentration of international unit of serum. The intensity of reaction was marked as % transmittance (left) and percentage clearing (right).

Interpretation of tube agglutination result using international unit

The % transmittance ranged between 67 and 92 with international standard serum diluted with 5% phenol saline (Fig 2).

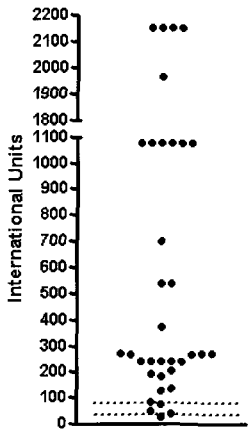

Fig 3. Distribution of bovine sera from *Brucella abortus* infected cattle showing international unit. The dotted lines indicate range of suspicious cattle, between 42 to 127.

The maximum agglutination reached with 1.5 unit of serum. The measurable % transmittance ranges that change according to international units of serum were between 71 and 91. The corresponding international units were between 0.60 and 1.35 IU. Roughly it was between 10 and 75% clearing. The extended range showed plateau indicating beyond of measurable amount. The corresponding international units showing 25, 50, and 75% clearing were 0.61, 0.83, and 1.35 IU, respectively. Based on these values observed, the IUs at serum dilution from 25 to 400 with our tube agglutination system were calculated

(Table 2). The measurable range of IUs with our system were between 15 and 538. With interpretation of ± as 50% clearing, suspicious ranged between 42 and 127 IU (Table 2). Of the 35 sera from *B abortus* infected cattle, about half of them had more than 538 international units (Fig 3).

Table 2. The international units at given % transmittance and the application on calculation of international units of tested serum

% Transmittance	International Units	International units of serum tested at dilution factor				
		25	50	100	200	400
71	0.6	15	30	60	121	242
72	0.61	15	30	61	121	243
73	0.61	15	31	61	123	246
74	0.62	16	31	62	125	250
75	0.64	16	32	64	128	255
76	0.66	16	33	66	131	262
77	0.68	17	34	68	135	270
78	0.7	18	35	70	140	280
79	0.73	18	36	73	146	291
80	0.76	19	38	76	152	304
81	0.8	20	40	80	159	318
82	0.83	21	42	83	167	334
83	0.88	22	44	88	175	351
84	0.93	23	46	93	186	372
85	0.97	24	49	97	195	389
86	1.03	26	51	103	205	410
87	1.08	27	54	108	217	433
88	1.14	29	57	114	229	457
89	1.21	30	60	121	241	483
90	1.27	32	64	127	255	510
91	1.35	34	67	135	269	538

The shaded regions represent suspicious result according to interpretation protocol provided at Table 1.

Discussion

Traditionally, the degree of agglutination has been expressed qualitatively, positive, negative, and suspicious reaction[6]. Each reaction is based on the degree of clearance of serum-antigen mixture and the amount of precipitated antigen-antibody complex. Though degree of agglutination is continuous event, the available data was only reported with limited information. In addition, the reading was totally depend on personal experience, thus there was possibility of disaccordance between different laboratories. Thus, it was expected that expression of reaction using turbidimetric way will provide more information on the degree of agglutination. Additionally, it will minimize the possibility of disaccordance.

Since the development of tube agglutin-

ation test for *Brucella* infection, the degree of agglutination has been expressed in titer until a joint FAO/WHO expert committee on brucellosis recommends the unitage system in 1953[7]. The expression of unitage system indicated elevated level of harmonization between different laboratories and nations. As a standard of unitage system, serum was preferred to antigen because of its easy way to preserve and prolonged expiration date. Thus, the first international standard serum was established in 1952, then the second standard in 1969 to replace the shortage of the remaining serum. As shown in Fig 2, the measurable international units with our system were between around 0.60 and 1.35. And the most sensitive zone which is most variable according to international unit of serum was around % transmittance 82 corresponding to 50% clearing. It indicates that the reading of agglutination at 50% clearing was recommended for more accurate reading. Thus, the international units of 35 bovine sera shown in Fig 3 were determined with information obtained at dilution factor showing 50% clearing.

Both data shown in Fig 1 and 2 indicated that the concentration of tube agglutination reagent should be elevated. First, as shown in Fig 1, the T% at 600 ㎚ of 0% clearing was around 65% indicating below the level was not accessible with our system. Thus the available window for measurement of agglutination intensity was very limited. Second, the increased concentration of tube antigen will make the gradient in Fig 2 decrease allowing extended range of international unit before getting maximum percentage clearing. In addition, the decreased degree of gradient in Fig 2 will increase accurate calculation of international unit at corresponding percentage clearing. Collectively the increased amount of antigen will increase the range of available international unit that shown in Table 2. Considering the fact that about half of the *B abortus* infected cattle sera had more than 538 units, the extension of available range was required.

In interpretation of tube agglutination result using protocol provided, our data showed some problems. First, the 100 % clearing was not plausible with international standard serum though the protocol for interpretation mentioned + as complete agglutination. Thus, the complete agglutination might be interpreted as 76% percentage clearing. Second, the interpretation of ± as more than 50% was too much broad, thus it should be fixed at 50% clearing. Finally, when the criteria for positive is complete agglutination at 100 times dilution of serum, the minimum international unit for positive will be around 135 while the range for suspect in European and USA are 30 to 80 and 25 to 100, respectively. If we interpret suspect as 50% clearing, the range for suspicious will be between 42 to 83 IU. Thus, it is recommended to change the criteria of positive to 50% clearing at 100 times dilution of serum, then it will be around 80 international unit.

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