DIAGNOSIS OF BRUCELLOSIS*

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After the second World War in Japan, the outbreak of brucellosis has been so reduced as to be found only in a limited number of contaminated areas, with the decrease in the number of raised cattle. For the purpose of improvement of dairy farming after the War, more than 15,000 Jersey cattle were imported from the United States, Australia and New Zealand, and distributed to farmers during the eight-year period beginning in 1953. In spite of severe inspection by the quarantine service, it was not possible to eliminate all contaminated cattle which became the source of epidemics in many areas of Japan.

The examination method of bovine brucellosis at this stage consisted of the detection of the agglutinins, and the so-called "test and slaughter method" had been carried out for eradication of the disease. Many workers adopted the plate agglutination test, but the diagnosis was finally made after performing the tube agglutination test, in which the lower limit of the agglutinin content to be diagnosed as positive was set at about 83 I.U. per ml and which became the national diagnostic standard. As the examination network was consolidated and the number of examined cows increased, the discrepancy between the results of the plate test and those of the tube test became troublesome. However, the important problem was that *Brucella* organism could not be isolated from many cattle examined after slaughter, although they had been positive by sero-diagnosis.

After several attempts, a national standard test was established in 1968 to unify the results of various kinds of sero-diagnostic techniques applied.

Presently cattle considered as strongly positive in serological tests cannot been found except in the case of the imported ones, so that it may be said that Japan is free from *Brucella abortus*.

The present paper describes mainly recent progress in sero-diagnosis of bovine brucellosis including bacteriological studies and the differentiation of the *Brucella* antibodies.

During the period 1958-1967, we collected many sera of slaughtered cattle having had positive reaction in *Brucella* agglutination test in order to examine them serologically so as to determine which method would give results in agreement with bacteriological findings. Further, for the purpose of obtaining data for the improvement of the examination technique, a survey covering this period of time was planned.

Parts of the results of the survey of 1965 are shown in Figure 1. This figure illustrates "the distribution of antibodies according to four kinds of serological tests in 7,198 bovine serum samples". From these results, it is apparently impossible to consider that the results of the plate agglutination test correspond closely to those of the tube test. According to the results of the tube agglutination test, considerable number of cows ought to be diagnosed as infected with brucellosis. A similar tendency was observed in the next year's survey.

The sera most positive for agglutinin were negative in complement fixation(CF) test, and those cows were then observed for several months. A distinct increase in agglutinin titer was not observed in their sera and there was a decrease in agglutinin in the sera of most cows. Some of these were slaughtered, but *Brucella* organism could not be isolated from them. The cows which were known to have been in contact with contaminated ones or those whose sera were positive in CF test were slaughtered. From most of these cows, *Brucellae* were isolated and were all identified as *Br. abortus* type I, whereas until that time all *Brucellae* isolated in Japan had belonged to type II.

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* These studies have been carried out in collaboration with Drs. S. Shibata, T. Suto, Y. Isayama, T. Tanaka and K. Hashimoto at the National Institute of Animal Health.

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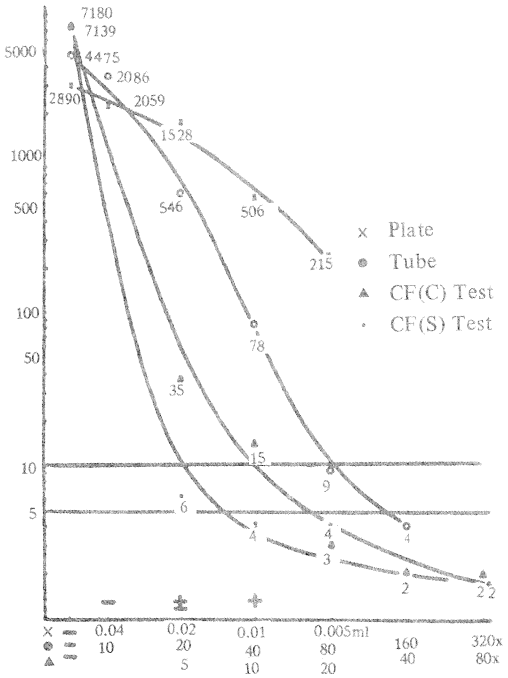

Fig. 1 Potency distribution of 7198 bovine sera

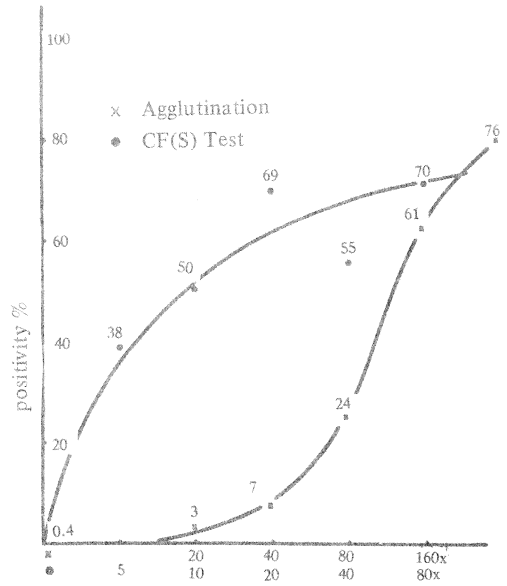

Fig. 2 Antibody titer and *Brucella* positivity

From these results, it was concluded that in Japan it is not sufficient to diagnose bovine brucellosis according only to the results of agglutination test.

As a result of the International Collaboration for the Standardization of *Brucella* CF test, it became known that in many laboratories the diluted tube agglutination antigen was used as CF antigen. On the other hand, in our institute, washed *Br. abortus* cells are extracted with 2% phenol saline in an incubator at 22°C for two weeks, the suspension is centrifuged and supernatant devoid of the cells is used as the antigen.

We compared the results obtained using both antigens in this survey. As shown in Table 1, CF(C; cellular) test indicates the results of the diluted agglutination antigen, and CF(S; soluble) test in the same table shows the results obtained using our soluble antigen, as summarized in Table 1 in which the origin of the sera is outlined. In the non-contaminated herd, the rate of CF(S) reactors is lower than that in contaminated herd, and all but one are suspected reactors, whereas those in contaminated herd, are mostly high reactors. The results of CF(C) in contaminated herds are not so

Table 1 Results of CF test using different antigens

Origin of serum	Antigen	Reaction			
		Neg.	x5	x10	x20 or more
Non-contaminated herd	Soluble	6726	6	1	0
	Cellular	6687	32	14	0
Contaminated herd	Soluble	454	2	1	8
	Cellular	452	3	2	8

different from those of CF(S) test. However, in the non-contaminated herds, the positive reactors increase in CF(S) test. When the sera of the slaughtered cows containing 50 I.U. or 100 I.U. of agglutinin are negative in CF(S) test, some of them give a positive reaction in CF(C) test.

The relations between the serological and bacteriological results are shown in Figure 2. The relation between the agglutination titers and the *Brucella* positivity assumes the shape of a sigmoid curve, and that of CF(S) test titers of an exponential one. From these results it may be deduced that the nature of the antibodies detected by both methods differs from one another. The cows which provide antibodies of high titer in their sera are certainly infected with *Brucella,* and therefore, *Brucella* can be isolated successfully from 80% of them. The problem arises when *Brucellae* are isolated from only one fourth of the cows which reacted in 1:80 dilution of the sera. On the other hand, when the organisms are isolated from about half of the cows whose sera are positive in 1:5 dilution in CF(S) test, such titer is generally considered as suspicious.

It has been stated that the sera of many cows are positive in the plate agglutination test. When the series of sera which contained the known amount of agglutinin were tested, agglutination of lower titer than expected was observed in the sera. The increase in the concentration of the antigen did not markedly improve the situation. It was therefore concluded that the plate agglutination test is suitable to select the sera to be tested further in the tube agglutination test.

The antigen for the plate agglutination test is made from *Br. abortus* St.125. The concentration is a hundred fold that of McFarland No. 1. The cells are suspended in 2% saline. It has been demonstrated that when 0.04 ml of the antigen is mixed with 0.02 ml of serum and some agglutination is observed, the serum contains more than about 30 I.U. per ml of agglutinin. If the agglutination is observed when 0.04 ml of serum is mixed, the serum contains more than about 15 I.U. and less than 30 I.U. per ml of it. To distribute such a small amount of liquid, it is more precise to use a dropper pipette than the usual graduated pipette. Therefore, the dropper pipette (one drop = 0.02 ml) was adopted.

About 500 of sera collected from the field which were found to contain less than 200 I.U. per ml of agglutinin with a turbiometer were examined with the modified plate agglutination method. The results are shown in Figure 3. A dot represents one serum sample. The dots in the first and third quadrants represent the sera on which both tests give similar results. The rate of coincidence is about 76%. The sera which contain less than 30 I.U. of agglutinin per ml and give positive results in the plate test amount only to 2%. The sera which give negative results in the plate test irrespective of the demonstration of more than 30 I.U. of agglutinin in the tube test total 21%. However, about 85% of those which contain less than 50 I.U. of agglutinin per ml and the sera which contain more

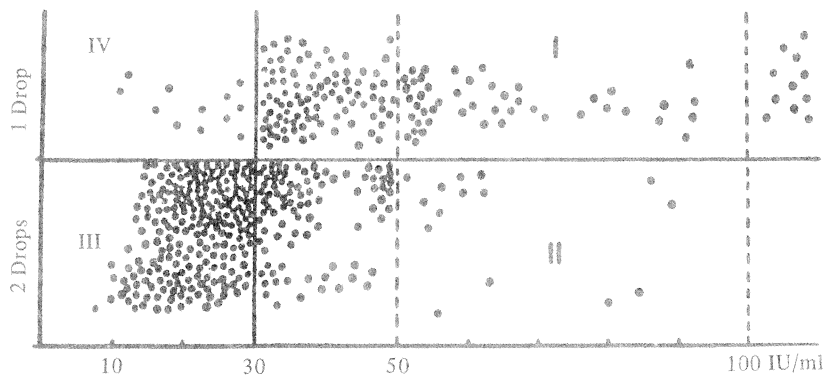

Fig. 3 Comparison of plate and tube tests

than 100 I.U. give a positive result. Thus, it was concluded that the plate test is to be kept as a screening method.

From the above results, we designed and proposed the following diagnostic system which was accepted by the Ministry and made public from the beginning of 1968. In this system, the sera of all dairy cows are examined in the plate test. All sera which have been found to contain more than 30 I.U. of agglutinin per ml are further examined in the tube test. The concentration of the tube antigen is increased to reach 50% agglutination in 20 fold diluted serum containing 50 I.U. of agglutinin per ml. The sera containing more than 50 I.U. are examined in CF test using soluble antigen. The cows raised in the same herd as the positively diagnosed ones are all regarded as suspicious and their sera examined in CF test irrespective of the agglutinin content.

The diagnostic standard of the serological and bacteriological results is shown in Tables 2 and 3. The cattle whose sera inhibit more than 50% of hemolysis in five fold dilution in CF test and contain more than 50 I.U. but less than 100 I.U. of agglutinin in the serum are regarded as free

Table 2 Diagnostic standard – I

Methods	Reading	Diagnosis
(1) Plate agglutination test	– 30 IU/ml	Negative = Healthy
	+ 30 IU/ml	To (2) Tube aggluti. test
(2) Tube agglutination test	– 50 IU/ml	Negative = Healthy
	+ 50 IU/ml	To (3) CF test
	+100 IU/ml	To (3) CF test
	+ 200 IU/ml	To (3) CF test; Diseased (Brucellosis)
(3) Complement fixation test by soluble antigen	– x5 dilution	To Table 2 Diagnostic standard – II
	+ x5 dilution	
(4) *Brucella* organism isolated: aborted fetus, milk or others		Positive = Diseased (Brucellosis)

Table 3 Diagnostic standard – II

Agglutinin	CF test x5 dilution	
200 IU/ml or more	+	Positive = Diseased (Brucellosis)
	–	Positive = Diseased (Brucellosis)
100 IU/ml	+	Positive = Diseased (Brucellosis)
	–	Suspicious
50 IU/ml	+	Suspicious
	–	Negative = Healthy
*Less than 50 IU/ml	+	Suspicious
	–	Negative = Healthy

Suspected animals should be retested at 2-week to one-month-interval 3 more times, and results suspicious until 3rd test should be considered as Negative = Healthy.
* Taken from the cattle in the same herd with the positive one.

(healthy), but the cattle with sera containing 100 I.U. are diagnosed as suspicious and are observed successively for several months. The cattle with sera containing more than 200 I.U., irrespective of the CF titer are diagnosed as positive for brucellosis without reserve. However, it is expected that the accumulation of data on such cattle may require a change in their treatment.

In 1965, cattle in several herds were found to be infected with *Brucella* in a village where about 20,000 head were examined in conformity with the above diagnostic system.

After undertaking three examinations (1965 - 1967), no cattle could be diagnosed as being apparently infected. In this case, the diagnostic standard was strengthened and the cattle which were in the same herds as the suspected ones were all examined, but no positive cattle could be detected.

It may be expected that the number of cattle slaughtered on account of brucellosis has remarkably decreased to one fourth of that before the adoption of the system. Until now, cattle which are strongly positive in CF test have not been found except in the case of imported animals inspected by the quarantine service, so that it may be considered that Japan is free from bovine brucellosis.

In taking into account the policy of importation of beef cattle breed for the purpose of improvement of domestic breed and enhancement of production of beef herd, strict safeguards with respect to brucellosis should be enforced since some of the animals are being imported from areas contaminated with *Brucella*.

As brucellosis is a chronic and systemic disease, it has been thought that the various kinds of antibodies appear according to the process of the disease, and many workers have reported the detection of the various antibodies against *Brucella.* We have carried out serological examination of the sera obtained from dairy cows diagnosed as infected with brucellosis, and we have often observed that, while the sera are distinctly positive in the agglutination test, the cows from which the sera had been obtained were negative for the isolation of *Brucella* organisms after their being slaughtered. Subsequently, an attempt to correlate serological findings to *Brucella* negativity was made. The formula of CF test using soluble antigen extracted from *Brucella* organisms is considered to be effective in their differentiation.

Table 4 shows the results obtained in 26 sera with high titers which contained more than 200 I.U. of the agglutinin. It is worth noting that among them there are some cattle from which *Brucella* could not be isolated. Most of these cattle were raised in the districts where no outbreaks of brucellosis had been reported. The common characters of these sera are that they are not distinctly positive in CF(S) test though some are positive in CF(C) test, and after gel filtration no antibody can be detected in IgG fraction. The antibodies of about half of the *Brucella* positive cattle which were sampled from the contaminated herds were distributed only in IgG fraction, whereas in the sera of the test, a small amount of antibodies was detected in IgM fraction.

Table 5 shows the results of 27 sera containing about 100 I.U. of agglutinin. In only 2 of the 27 head of cattle, was *Brucella* isolated. The antibodies in the sera were distributed only in IgG fraction. Most of the sera of the cattle from which *Brucella* could not be isolated were negative in CF(S) test, however, it is worth noting that some were positive in CF(C) test.

The 67 sera with low titers contained less than 50 I.U. of agglutinin detected in our laboratory and these titers were not obtained when the cattle were diagnosed as infected in the various veterinary centers. The organisms could not be isolated from any of them and their antibodies were distributed in IgM fraction.

One serum which originated from an animal sampled from contaminated herd on account of CF(S) positivity drew our attention. While no agglutinins were detcted in the serum, CF antibodies could be detected. From this animal, *Brucella* could be isolated after slaughter. From the above results, it may be considered that CF(S) test is useful to detect infected animals and that it prevents cattle from being slaughtered unnecessarily to confirm the presence of antibodies in IgG fraction.

When findings on the field sera are analysed, it appears that CF(S) test is more specific than CF(C) test, and it is almost impossible to isolate *Brucella* from the cattle whose antibodies are

Table 4 Molecular size pattern of antibodies in the sera of slaughtered cattle with 200 and more I.U./ml

	No.	Whole serum			IgM			IgG			*Brucella*
		Agg.	CF		Agg.	CF		Agg.	CF		
			S	C		S	C		S	C	
400 I.U. or more	1	640	640	1280	0	0	0	170	398	540	+
	2	320	160	320	0	0	0	312	176	444	+
	3	320	640	1280	0	0	0	382	364	660	+
	4	160	80	160	0	0	0	94	46	87	+
	6	2560	640	1280	34	3	8	934	1029	1999	+
	7	1280	640	1280	6	2A	2A	282	294	451	+
	8	640	320	640	24	0	12	268	184	350	+
	9	640	320	640	8	0	0	304	172	360	+
	10	640	320	640	10	1A	1A	480	246	450	+
	11	320	40	80	36	0	10	100	56	106	+
	12	320	160	320	8	0	0	162	68	156	+
	13	160	160	320	12	0	4	132	51	168	+
	14	160	80	160	4	0	0	106	68	107	+
	22	320	40	80	14	0	0	278	53	104	+
	N70	160	0	0	20	6	16	0	0	0	–
	N48	160	40A	20A	52	22A	22A	0	0	0	–
200 I.U.	5	80	80	160	0	0	0	28	41	50	+
	15	80	80	160	0	0	0	46	44	52	+
	16	80	160	320	0	0	0	94	80	174	+
	26	80	0	5	32	3	6	0	0	0	–
	N52	80	0	10	30	3	9	0	0	0	–
	N51	80	0	10	41	0	5	0	0	0	–
	N32	80	0	5	37	2	2	0	0	0	–
	N69	80	0	5	57	8A	8A	0	0	0	–
	N17	80	40A	40A	53	0	0	0	0	0	–
	N62	80	0	0	41	0	0	0	0	0	–

N = Non contaminated herd; A = Anti-complement

detected only in IgM fraction, while isolation is easy from the cattle whose antibodies are detected in IgG fraction.

Furthermore, the sera obtained serially from artificially infected cows were examined in the same way using as inoculum, the avirulent strain *Br.abortus* St.99, St.19 as intermediate or weakly virulent strain, and St.544 as highly virulent strain. About 10^9 of the viable cells were injected to a cow intravenously. Two cows received the same strain. The sera were taken serially at intervals of about one week. Six weeks after the first inoculation, the same amount of cells was injected.

These results are summarized as follows: it could be demonstrated that there is a correlation between the virulence of *Brucella* inoculated and the trend of the antibodies in the serum of the inoculated cattle. However, we could not find a similar correlation in the case of the field sera which contained no IgG antibodies. It is therefore necessary to determine what type of antigen is responsible for those IgM antibodies and how the cattle are inoculated.

Table 5 Molecular size pattern of antibodies in the sera of slaughtered cattle with about 100 I.U./ml

	No.	Whole serum			IgM			IgG			*Brucella*
		Agg.	CF S	CF C	Agg.	CF S	CF C	Agg.	CF S	CF C	
100 I.U	17	40	40	80	0	0	0	24	24	47	+
	18	40	40	80	0	0	0	26	29	45	+
	24	40	10	20	20	15A	14A	0	0	0	–
	27	40	0	10	25	0	2	0	0	0	–
	28	40	0	5	34	2	5	0	0	0	–
	N4	40	0	10	15	9A	9A	0	0	0	–
	N18	40	0	5	20	0	4	0	0	0	–
	N65	40	0	5	22	5A	5A	0	0	0	–
	N43	40	0	5	19	2A	7A	0	0	0	–
	N30	40	0	5	23	0	0	0	0	0	–
	N11	40	0	0	29	2	3	0	0	0	–
	30	40	0	0	16	0	0	0	0	0	–
	31	40	0	0	19	0	0	0	0	0	–
	32	40	0	0	17	0	0	0	0	0	–
	N12	40	0	0	33	0	0	0	0	0	–
	N13	40	0	0	14	0	0	0	0	0	–
	N21	40	0	0	10	0	0	0	0	0	–
	N23	40	0	0	6	0	0	0	0	0	–
	N24	40	0	0	19	0	0	0	0	0	–
	N28	40	0	0	7	0	0	0	0	0	–
	N34	40	0	0	19	0	0	0	0	0	–
	N49	40	0	0	18	0	0	0	0	0	–
	N60	40	0	0	27	0	0	0	0	0	–
	N63	40	0	0	107	0	0	0	0	0	–
	N64	40	0	0	22	0	0	0	0	0	–
	N64	40	0	0	3	0	0	0	0	0	–
	N6	40	0	0	26	0	0	0	0	0	–

N = Non contaminated herd; A = Anti-complement

Discussion

Joseph, P.G. (Malaysia): How is the complement fixation test (CFT) antigen standardized?

Answer: The standardization of CFT antigen is made by using the Japanese national standard anti-*Brucella abortus* serum for CF test.