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Growth agglutination and growth inhibition tests in the diagnosis of brucellosis

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SUMMARY

Growth agglutination and growth inhibition tests were established for the diagnosis of *Brucella abortus* infection. The former involves the agglutination of living organisms while the latter is a bactericidal test. Using mouse, guinea-pig, rabbit and bovine serum it was shown that the growth agglutination test is approximately ten times, and the growth inhibition test one hundred times, more sensitive than the conventional tube agglutination test. It is suggested that these techniques may be of assistance in diagnosing bovine brucellosis in situations in which the tube agglutination test results are suspected of being falsely negative.

INTRODUCTION

Although isolation and identification of brucella organisms from the infected host is the only incontrovertible evidence for brucella infection, this is not always practicable. Thus serological tests have necessarily played a major role in the routine diagnosis of brucellosis. The principal test used for this purpose is the serum tube agglutination (STA) test. The STA test is relatively simple and is thus particularly suitable for large scale use by relatively untrained personnel. It has been largely responsible for the progress achieved in the eradication of brucellosis in farm animals in Europe and North America. The STA, as conventionally performed, detects agglutinating antibodies which in cattle belong to the IgM and IgG2 subclasses. However, the major immunoglobulin produced by cattle in response to brucella infection is IgG1 and this immunoglobulin is a relatively poor agglutinator (Tizard, 1977). Consequently the STA is liable to produce false negative reactions. Secondly, low titres of agglutinating antibodies may be due to causes other than exposure to *Brucella abortus*, to sampling early in infection, or to the presence of persistent post-vaccinal titres.

Because of these problems it has been necessary to develop tests which can detect non-agglutinating antibodies. The most important of these is the direct complement fixation test (CFT) which is an effective diagnostic test for individual animals. Unfortunately, the CFT is complicated, requires rigorous controls, and must be performed by highly trained and competent personnel. There is thus a

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need for a specific and sensitive test for brucellosis which is both technically simple and does not depend upon agglutination.

Serum bactericidal activity generally requires both antibody and complement (Muschel & Jackson, 1963) and it is known that the reaction is very sensitive. Thus the quantity of antibody required to restore bactericidal activity to absorbed serum is extremely small (Muschel & Treffers, 1956). Because of this we decided to use this system, i.e. a growth inhibition (GI) test to measure anti-brucella antibody in serum.

Since the GI test requires that complement be added to the serum-bacteria mixture in order to cause growth inhibition, the effect of not adding complement was also investigated. Thus living organisms and antiserum were incubated together in a technique which we have termed the growth agglutination (GA) test.

In this paper the STA, GA and GI tests are compared.

MATERIALS AND METHODS

Bacteria

Five smooth strains of *Brucella abortus* were studied. Four of these (2308, 63/75, 544 and 319) were virulent strains obtained from stock culture collection of this laboratory. Strain 19 was obtained from the Institute Armand Frappier, Montreal.

Animal inoculation

Calves. Three castrated, Holstein, bull calves, 3 months of age were each inoculated with 3 ml of standard live *Br. abortus* strain 19 vaccine in the neck region by the subcutaneous route.

Guinea-pigs. Twenty 400 g male guinea-pigs were used. Ten received 1·0 ml of standard strain 19 vaccine subcutaneously. The other ten received 0·5 ml of *Br. abortus* strain 2308 in tryptic soy broth (TSB) containing 10^5 c.f.u./ml.

Mice. 132 outbred, male, white-Swiss mice weighing 18–20 g were used. 67 received 0·2 ml of strain 19 vaccine subcutaneously. The remainder (65) received 0·2 ml of strain 2308 in TSB containing 10^5 c.f.u./ml.

Rabbit anti-Br. abortus *serum.* Antiserum against *Br. abortus* S19 IAF was produced in two rabbits by six inoculations of heat-killed culture of *Br. abortus* S19 IAF in TSB at weekly intervals by the i.v. route. The rabbits were bled by intracardiac puncture 7 days after the last injection. The serum was separated with sterile precautions, pooled, and stored at $-20\,^{\circ}C$ until used.

Mouse anti-Br. abortus *tube agglutination test antigen.* Twenty adult, 20 g male mice were inoculated with 0·1 ml of undiluted test antigen by i/p route six times at 3-day intervals. The animals were anaesthetized and bled by intracardiac puncture 7 days after the last inoculation. The sera were pooled and stored at $-20\,^{\circ}C$ until used.

Serum collection

Blood samples were obtained by jugular venipuncture in the calves and by cardiac puncture in the laboratory animals. Individual samples were allowed to clot at room temperature for 1 h and kept at 4 °C overnight. The samples were then centrifuged, the serum removed using aseptic precautions and stored at −20 °C until tested.

Blood from groups of five mice was pooled as were samples from individual guinea-pigs of the same group.

Complement

A 200 ml pool of normal guinea-pig serum was employed as a source of complement. This material was stored at −20 °C in 5 ml volumes in stoppered tubes until required. Tubes were not refrozen once thawed.

Bacterial inoculum for tests

Organisms were grown in TSB for 2 days at 37 °C and the suspension standardized turbidimetrically.

Serology

Standard tube agglutination (STA) test. This was performed according to the method of Brinley-Morgan (1967).

Growth agglutination (GA) test. Test serum was filtered through a 0·45 nm Seitz filter in a syringe attachment and dilutions made in 0·5 ml volumes in TSB. One drop (0·025 ml) of an optimal dilution of 2-day-old culture of test strain organisms in TSB was added to each tube. The tubes were stoppered and incubated in a shaker water bath at 37 °C for 24 h. The tubes were permitted to remain at room temperature for a further 24 h. At the end of this time the tubes were examined for the presence of agglutinated growth. The growth agglutinin titre was expressed as the reciprocal of the highest dilution of serum showing distinct clumps of organisms and a clear supernatant. Control tubes showed no agglutination but were turbid as a result of growth of the organisms. A small round button of sedimented growth was present.

Growth inhibition (GI) test. For the purposes of the GI test, serum was first heat inactivated at 56 °C for 30 min and then filtered through a 0·45 nm millipore Seitz filter pad. Dilutions of this serum were then made in 0·5 ml volumes in TSB. One drop of optimal dilution of test culture and 0·1 ml of complement was added to each tube. (For determination of optimal dilution see below.) The tubes were then incubated at 37 °C for 24 h with mild shaking followed by further incubation at room temperature for 24 h. Control tubes included diluent containing neither test serum nor complement, test serum in the absence of complement, and complement alone. The growth inhibition titre was expressed as the reciprocal of the highest dilution of serum showing no growth.

Table 1. *Determination of optimal concentration of organisms in TSB for GA and GI tests using* Br. abortus *S19 IAF as antigen*

Test serum	Test	Antibody titres with different dilutions of 2-day-old growth in TSB: Undil.	1:10	1:20	1:40	1:80	1:100	1:200
Guinea-pig anti-*Br. abortus* tube test antigen	GA	6400	25600	25600	102400	102400	102400	102400
	GI	6400	25600	25600	102400	102400	102400	102400
Rabbit anti-heat killed *Br. abortus* S19 IAF	GA	25600	102400	102400	102400	102400	102400	102400
	GI	6400	409600	819200	819200	819200	819200	819200
Mice anti-live *Br. abortus* S19 IAF	GA	1600	3200	6400	6400	6400	6400	6400
	GI	< 800	12800	25600	51200	51200	51200	51200
Naturally infected bovine serum	GA	< 400	400	800	1600	1600	1600	1600
	GI	< 400	800	800	1600	1600	1600	1600
Serum from Calf no. 19 vaccinated with *Br. abortus* S19 IAF	GA	< 200	400	400	400	800	800	800
	GI	< 200	200	3200	3200	3200	6400	6400

Table 2. *Determination of optimal dose of guinea-pig complement for growth inhibition test*

Test serum	Test strain	GI titres with different dilutions of guinea-pig complement							
		Undil.	1:2	1:3	1:5	1:10	1:20	1:40	1:80
Rabbit anti-heat-killed *Br. abortus* S19 IAF	S19 IAF	409600	51200	3200	<1600	<1600	<1600	<1600	<1600
	2308	51200	25600	3200	<1600	<1600	<1600	<1600	<1600
Mice anti-live *Br. abortus* S19 IAF	S19 IAF	51200	51200	25600	12800	<3200	<3200	<3200	<3200
	2308	25600	25600	25600	6400	<3200	<3200	<3200	<3200
Guinea-pigs anti-live *Br. abortus* 2308	S19 IAF	409600	409600	409600	204800	12800	6400	<1600	<1600
	2308	409600	409600	409600	204800	12800	6400	<1600	<1600
Naturally infected bovine serum no. 73	S19 IAF	1600	800	800	400	<400	<400	<400	<400
	2308	1600	800	800	400	<400	<400	<400	<400

Standardization of reagents

The optimal concentration of bacterial suspension was determined by using increasing dilutions of a 2-day-old culture of test strain organisms. A similar cross titration was performed in order to determine the optimal dilution of complement for use in the growth inhibition test (Tables 1 and 2). On the basis of the results obtained, the organisms were used at a dilution of 1/50 and the complement was used undiluted.

Specific absorption

Heat inactivated pooled guinea-pig or mouse serum, diluted 1/5 in saline, was mixed with washed, packed, heat-killed bacteria to a concentration of 10% by volume. After mixing, the suspension was allowed to stand at 4 °C overnight and the organisms removed by centrifugation.

RESULTS

Guinea-pigs

In both groups of guinea-pigs, those 'vaccinated' with strain 19 and those 'infected' with strain 2308, there was a marked serological response. In the 'infected' group the STA became positive 2 weeks after infection and rose to reach a peak titre of 1/25600 by 2 months. In contrast both the GA and GI tests became positive by 1 week and rose rapidly thereafter (Fig. 1) to reach titres of 1/819200, an increase over the STA titre of 32-fold. In the group of guinea-pigs vaccinated with strain 19, all tests became positive by 1 week but the GI test in this case was consistently higher than the other two. The GI results showed a biphasic response, peaking at 2 weeks at 1/6400 and at one month at 1/512000. The GA test, in contrast, reached 1/3200 at 1–2 months and the STA peaked at 1/800 three weeks after infection.

Mice

The results obtained using mice were essentially similar to those from guinea-pigs (Fig. 2). That is, the GI test generally gave much higher titres than either GA or STA tests. Mice infected with strain 2308 became GI positive by 3 days and rose to 1/102400. The GA and STA tests in these animals became positive at 2 weeks and rose eventually to reach 1/6400 and 1/400 respectively. A similar result was obtained with strain 19 vaccinated mice.

Cattle

Three calves vaccinated with strain 19 were tested serologically. In all three a similar pattern of responses occurred (Fig. 3). The STA took 4–6 days to become positive and the titres rose from 1/100–1/200 to reach 1/1600 between 1 and 3 weeks before declining. The GA test became positive 4 days after vaccination and peaked at 1–2 weeks with titres of 1/6400 to 1/12800. The GI test, in contrast, had reached these titres by 4 days and peaked 2 weeks later at 1/102400.

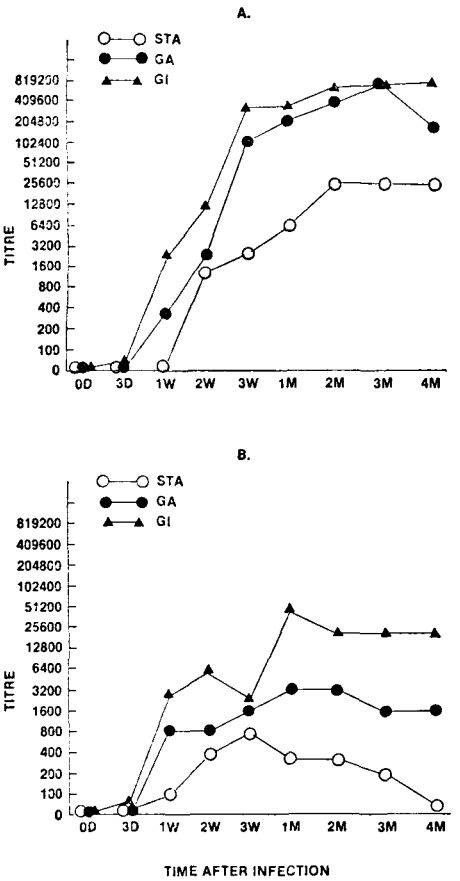

Fig. 1

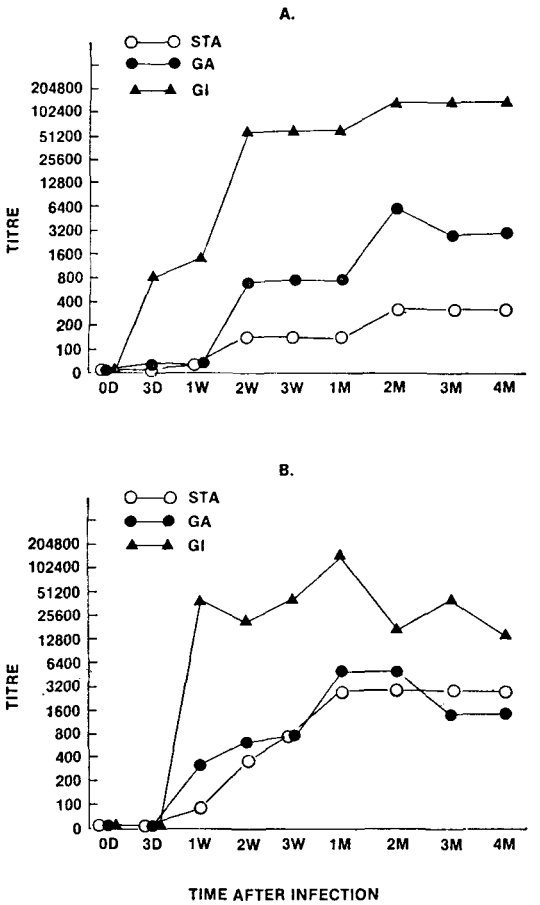

Fig. 2

Fig. 1. The serological responses of two groups of ten guinea-pigs to (A) 'infection' with *Br. abortus* strain 2308 or to (B) 'vaccination' with *Br. abortus* strain 19.

Fig. 2. The serological responses of two groups of mice (each point is the result of testing pooled serum from five mice). Group A were infected with *Br. abortus* strain 2308 while group B were infected with strain 19.

Sensitivity

Using a number of rabbit, guinea-pig, mouse and bovine sera, the GI test consistently gave titres almost ten times higher than the GA test, while the GA test in turn gave titres up to ten times higher than the STA technique (Table 3).

Specificity

There was very little variation in titres when rabbit, guinea-pig, mouse and pooled bovine sera were tested against the following strains of *Br. abortus*: S19 IAF, 2308, 544, 319 and 63/75. All detectable antibody activity to *Br. abortus* could also be absorbed from positive sera using these strains.

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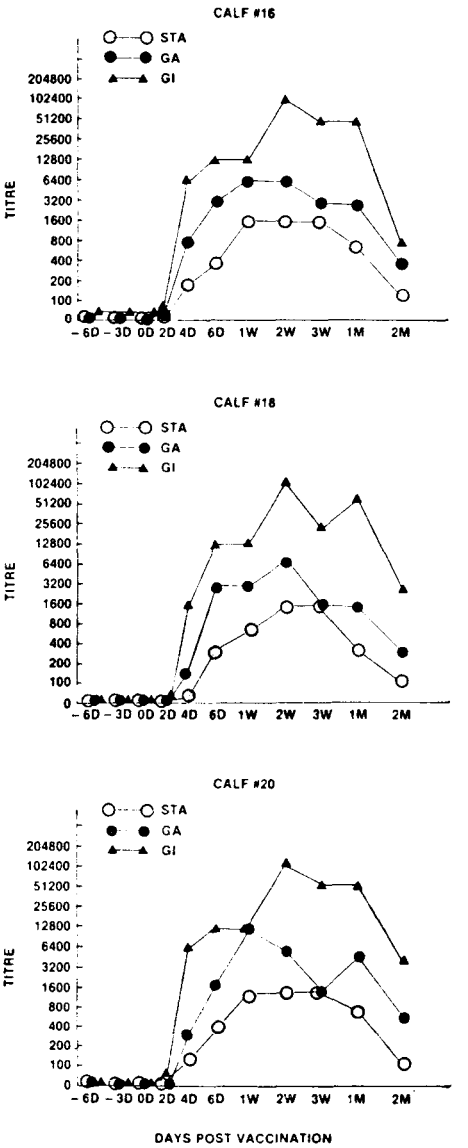

Fig. 3. The serological responses of three calves following vaccination with *Brucella abortus* strain 19.

DISCUSSION

A comparative study of three tests, tube agglutination, growth agglutination and growth inhibition tests has been made on rabbit, guinea-pig, mouse and bovine sera to *Br. abortus*. The growth agglutination test, from the results obtained here, is approximately ten times more sensitive than the STA. There are two probable reasons for this. Firstly, the standard antigen suspensions used in the STA consist of heat-killed organisms suspended in phenol-saline. Such an inactivation process is relatively severe and undoubtedly causes destruction of brucella surface antigens. Secondly, the standard antigen suspensions are relatively dense,

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Table 3. *A comparison of the sensitivity and specificity of the STA, GA and GI tests using four different antisera and five different strains of* Br. abortus

Antiserum	Test	Test strain: S19 IAF	2308	544	319	63/75
Mice anti- *Br. abortus* tube test antigen	STA	3	3	3	3	3
	GA	4	4	4	3	4
	GI	5	6	6	5	5
Guinea-pigs anti-live *Br. abortus* 2308	STA	3	3	3	3	3
	GA	4	4	4	3	4
	GI	5	5	4	4	4
Rabbit anti-heat killed *Br. abortus* S19 IAF	STA	3	3	3	3	3
	GA	4	4	4	4	4
	GI	5	5	5	5	5
Pooled positive bovine sera	STA	2	2	2	2	2
	GA	3	3	3	3	3
	GI	4	4	4	4	4

being standardized at 0·045% by volume (Alton, Jones & Pietz, 1975). It is known that the sensitivity of the STA may be increased by decreasing the antigen concentration. However, this makes the test very difficult to read. In the GA technique, very little antigen is employed thus significantly increasing the sensitivity of the test, while the eventual growth of organisms renders the test easily read.

The brucellicidal activities of serum have been studied previously, but primarily with the intention of determining mechanisms of protective immunity. Thus Irwin, Beach & Bell (1936) attempted to determine whether the bactericidal activities of serum correlated with protection against brucella. While such a correlation was not detected, Irwin and Ferguson did show (Irwin & Ferguson, 1938) that the level of serum brucellicidins did rise in cattle following recovery from infection. Huddleson *et al.* (1945) also studied this bactericidal activity and went on to suggest that this phenomenon may be of assistance in diagnosis. These workers also demonstrated that appreciable titres of brucellicidins could develop in the absence of agglutinins. A similar observation has been made with respect to the Enterobacteriaceae (Kenny & Herzberg, 1968).

The failure to employ growth inhibition techniques on routine serology has been caused by two prime factors. Firstly, in the case of the enterobacteria and the vibrios, the replicative power of the organisms has been greatly in excess of the bacteriostatic ability of the serum. Thus although growth may be considerably slowed, overgrowth of organisms ultimately occurs. In contrast, the slow growth of brucella *in vitro* permits serum activity to be fully expressed and enables end-points to be clearly seen.

As described in the Materials and Methods section, both the GA and GI techniques are extremely simple. They require little more equipment or expertise than the conventional STA. They are considerably less complex than the CFT, a

test which they rival with respect to sensitivity. However their major disadvantage is the requirement for living organisms. This obviously precludes their use in routine laboratories. Nevertheless, their exquisite sensitivity indicates that they could be used to advantage in certain problem areas. Thus in the serodiagnosis of bovine brucellosis the ability of the STA to detect very early infections is limited. In general, the time at which animals appear to seroconvert is a direct function of the sensitivity of the serological technique involved. Thus a very sensitive test will be able to detect serum antibodies earlier than an insensitive one. We would suggest therefore that the GI test, especially, may be employed with advantage in situations in which early detection of brucella infection is essential.

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