THE CONGLUTINATION COMPLEMENT-FIXATION TEST AS A SUPPLEMENTARY METHOD OF DETECTING ACTIVITY WITH BRUCELLA ABORTUS ANTIGENS

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Where as a general rule the results of the plate and tube tests with *Brucella abortus* antigens agree closely, an occasional sample of serum is encountered that shows a reaction in the former test but not in the latter. The converse, a negative result in the plate test and definite agglutination in the tube titration, occurs more rarely. When such occasions arise, a reliable supplementary method, even one too involved for routine purposes, might be helpful in the interpretation of the significance of these atypical results. A somewhat more sensitive method of detecting antibody at early stages of infection, or in animals infected for longer periods exhibiting a poor antibody response, would be of value particularly in testing known infected herds from which it is desired to remove animals as early as possible in the hope of limiting the spread of brucellosis. The sensitivity of the plate and tube tests cannot be increased beyond the present level without sacrifice of specificity. Only when definite reactions are obtained in these routine tests are they reported as "positive", weak reactions are reported as "questionable" for although they may represent a low degree of "specific" antibody, they may also be "nonspecific" in nature, traceable to antibody developed to some antigenically-related species of bacterium or dependent on changes in the relative proportion of serum globulins and albumin. Some other serological method which would augment the "specific" agglutination without increasing the "nonspecific" to a corresponding degree, would be highly desirable for retests of such questionable specimens.

Interest has recently been renewed in the conglutination test (1) which was developed many years ago and found to be of value in the diagnosis of a number of diseases including brucellosis and dourine. It was superseded by other methods, technically less difficult to perform and standardize. Hole and Coombs (1, 2) in searching for improved methods of diagnosing glanders, re-examined the conglutinating complement-absorption method, introduced a number of technical modifications corresponding to those used in the hemolytic-complement-fixation test and found it to be more sensitive than the latter in demonstrating antibody in sera of a number of other animal species in addition to the horse. The results of these investigators suggested that the method might be applicable in certain of our own serological problems, including the present one of providing a very specific, as well as highly sensitive, supplementary means of detecting brucellosis. Our preliminary studies showed the test to be considerably more sensitive than tube agglutination in the titration

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of activity with *Br. abortus* antigens in sera of known infected cattle (3). It remained to be determined whether it was equally as "specific", or more so than the routine agglutination tests.

The conglutinating complement-absorption test (CCA) was therefore carried out in parallel with the two agglutination tests, plate (PA) and tube (TA), and a hemolytic complement-fixation test (HCF), on some 3200 samples of blood obtained at one week to one month intervals, usually bi-weekly, over a two-year period, from a dairy herd of 239 cattle ranging in age from a few months to 14 years. An outbreak of brucellosis had occurred in this herd some months earlier and an attempt was being made to detect infected animals as early as possible. Almost all female calves were being vaccinated with the No. 19 strain of *Br. abortus* on reaching an age of 7 to 8 months; 65 of these vaccinated animals were tested at periodic intervals, some both before and after vaccination, others for more than two years beyond the date of vaccination. The serological results obtained in the non-vaccinated and vaccinated groups will be considered separately.

EXPERIMENTAL METHODS

Agglutination Tests. — The plate and tube agglutination tests were carried out in the routine manner. The group of serological reactions described as "questionable" has been subdivided into three, slightly questionable "SQ", questionable "Q", and very questionable "VQ", with a view to obtaining a more quantitative assessment of the relationship of CCA titres and agglutination results. A fourth group, showing mere traces of agglutination, that is 1+, with the largest amount of serum used was designated as very slightly questionable, "VSQ"; these reactions were considered too slight to be of diagnostic significance and reported as "negative" to the veterinarian.

Conglutinating Complement-Absorption Test. — The CCA tests were carried out as described in detail elsewhere (3). For this test five reagents are required; test serum, *Br. abortus* antigen, conglutinating complement (horse), heated bovine serum (supplying conglutinin and natural antibody for sheep red cells) and 1% sheep-red-cell suspension. The diluted test serum, complement (3 units), and Brucella antigen are mixed in 0.1 ml. amounts. After incubation for one hour at room temperature. 0.2 ml. of a mixture of equal parts of the 1% sheep-red-cell suspension and a 1:6 dilution of heated bovine serum, (the indicator system) is added. The tubes are shaken and incubated for 15 minutes at 37°C., centrifuged, and read by the re-suspension technique.

The majority of sera were first tested in 1:5, 1:10 and 1:20 dilutions and only if found to be reactive were they tested in higher twofold dilutions: maximum 1:10.240. The titres were expressed in terms of the highest dilution showing 2+ agglutination in the presence of complement and antigen. These reactions were evaluated in relation to the results obtained in the tests of serum

without antigen. When a similar degree of inhibition of conglutination was observed in tests with and without antigen, the reaction was recorded as anti-complementary (Ac).

The brucella antigen preparation used in these studies was that employed in the tube agglutination test; it was however used in a dilution of 1:400 rather than the 1:200 adopted for the latter test.

Three complete conglutinative units of complement were added, rather than the 2 units originally recommended (3), because such a large proportion of the sera from the adult cattle proved to be anticomplementary with the lower unit dose, sometimes necessitating the repetition of one quarter of the tests. The sera of calves were very seldom anticomplementary. Practical experience indicated that fewer anticomplementary reactions were obtained when the natural antibody for sheep red cells in the heated bovine serum was supplemented by a suitable dilution of rabbit amboceptor. This amount of amboceptor is one fifth of that used in sensitizing the 5% sheep-red-cell suspension for the hemolytic complement-fixation test.

Hemolytic Complement-fixation Test. — All sera were also titrated by a hemolytic complement-fixation method (HCF). The same series of serum dilution were tested with 3 units (50% hemolysis of guinea-pig complement and a 1:200 dilution of *Br. abortus antigen*). The indicator system was a mixture of equal parts of 5% sheep red cells and anti-sheep-red-cell amboceptor prepared in rabbits. The period for fixation was 18 hrs. at 4-8°C., for hemolysis 30 min. at 37°C. The titres were expressed in terms of the highest serum dilution showing 50% hemolysis or less in the presence of antigen. Allowance was also made for anticomplementary properties, but these were noted much less often with guinea pig than with horse complement.

RESULTS

Non-Vaccinated Animals. — In all, 2191 samples from non-vaccinated animals were tested by the four techniques, the two agglutination tests, PA and TA and the two complement-fixation methods, CCA and HCF. Of the 174 animals, 48 became positive in the routine agglutination test for brucellosis. The distribution according to age and sex is given in Table I. The greatest number developing infection was in the 1947 age group.

When the antibody content of the serum was high, marked reactions were obtained in all four tests. Specimens that were definitely positive in PA and TA, had CCA titres of 1:160 to 1:5240, and HCF titres of 1:40 to 1:640. The plate agglutination and conglutinating complement-absorption tests were the most sensitive in detecting a low antibody concentration, the hemolytic complement-fixation test was the least sensitive. "Slightly questionable" agglutination PA reactions were usually accompanied by CCA titres of 1:10 to 1:40, "questionable" reactions by those of 1:20 to 1:80, and "definitely questionable" by CCA titres of 1:80, 1:160 or higher. In some

TABLE I

Summary of specimens from non-vaccinated cattle tested from March 28, 1950 to April 8. 1952.

Year of Birth	Number of Animals		Number of Sera Tested	Number of Animals Positive for Brucellosis
	Male	Female		
1936	1	2	55	1
1938	0	1	7	0
1939	0	1	33	0
1940	0	1	8	1
1942	0	3	38	1
1943	1	2	55	2
1944	0	8	176	4
1945	1	11	145	7
1946	0	13	166	9
1947	0	27	480	17
1948	1	25	515	4
1949	13	1	149	2
1950	23	3	239	1
1951	27	9	125	0
Total	67	107	2191	49

animals that subsequently became positive, slight reactivity was noted in the CCA test before any agglutination was apparent in the PA test; in other animals a trace of agglutination (VSQ), reported as negative diagnostically, was recorded earlier than inhibition of conglutination in CCA. The sera of several cows, eventually serologically "positive", were anticomplementary to a dilution of 1:20, 1:40, or even 1:160, on several occasions before definite reactions were observed. This anticomplementary effect was encountered frequently enough to suggest that it bore some relationship to the development of infection. It may have been traceable to changes in serum proteins during a febrile state or to some lipid imbalance resulting from the pathological process. Very slight traces of agglutinative activity that tended to appear and disappear from time to time were not usually accompanied by any CCA reaction. The findings for 8 of the more interesting of these cases are described below. The ages given are those of the animals at the time the first specimen

was collected. Data on the results obtained in ring tests on milk samples from the same animals has also been included for comparative purposes.

No. 4591 (Female, age 7 years) — 23 specimens tested 28/2/50 to 3/7/51. Aborted Oct. 1950. Of two guinea pigs injected with milk one month later, one became positive for *Br. abortus,* one remained negative for a period of 4 months. Previous to the time of abortion, 10 serum specimens had been tested: 4 of these were definitely anticomplementary in the CCA test but negative in all three other tests. It was not until June 13, 1951, that the serum showed "questionable" agglutination in PA and TA tests; it was "positive" July 3. The CCA titres on these dates were 1.20 and 1:80. Between Oct. 1950 and June 1951, 7 specimens had been anticomplementary or showed atypical CCA reactions. The HCF test was the last to become reactive; a 1:20 titre was obtained for the last specimen, 3/7/52. The milk ring test (MRT) was negative throughout.

No. 30708 (Female, age 6 yrs.) — 14 specimens collected from April 28, 1950 to Dec. 26, 1951. The last was positive in PA and TA tests and had titres of 1:160 and 1:80 in the CCA and HCF tests. The previous specimen, collected 5/12/51 had been "questionable" in the routine agglutination tests, was negative in the HCF test, and had a titre of 1:40 in CCA. Six earlier speciment had been anticomplementary to a 1:40 or 1:60 dilution in the CCA test.

A slight MRT reaction was obtained with undiluted milk 1/5/50. On 5/1/51 two guinea pigs were injected with cream, one became positive, one remained negative. The strongest MRT observed was 4+ with undiluted milk.

No. 93173 (Female, age 4 yrs.) — 19 specimens collected April 28, 1950 to October 4, 1951. Aborted at 7 months. Only the last two specimens showed definite agglutination; CCA titres 1:80 and 1:640 respectively. Three earlier specimens had been negative in PA and TA tests and had titres of 1:10 or 1:20 in CCA; the specimens of 11/7, 22/8, and 12/9/50 had been anticomplementary in the latter test.

No. 93162 — (Female, Age 4 yrs.) — 26 specimens tested between 28/2/50 and 4/9/51. Abortion occurred 21/8/51, that is two weeks before the last bleeding which was strongly reactive in all four tests. All previous specimens had been reported as negative. Four, those of 11/7 and 4/10/50, and 6/2 and 19/3/51, were anti-complementary in the CCA test. The MRT with undiluted and diluted milk was negative.

No. 93179 — (Female, age 4 yrs.) — 15 blood specimens tested 28/2/50 to 16/1/51. Fluctuating MRT reactions were obtained between 1/11/49 and 22/3/50 and again between 30/5/50 and 2/1/51. Milk collected 2/1/51 was injected into two guinea pigs; both became infected with *Br. abortus.* The agglutination tests were questionable 5/12 and 26/12/50,

and positive 16/1/51. The CCA titres of these three bleedings were 1:80, 1:160 and 1:320; the HCF titres were 1:10, 1:20, and 1:80. Many of the earlier specimens had shown a mere trace of agglutinative activity (VSQ); six had been anticomplementary in the CCA test.

No. 88 — (Female, age 3 yrs.) — 19 specimens tested 28/2/50 to 10/4/51. Aborted 31/5/51, at 8 months pregnancy. None of the serum specimens showed more than a trace of agglutinative activity except the one taken after the abortion had occurred; 3 previous specimens were anticomplementary in the CCA test. Fluctuating MRT reactions were recorded 5/12/50 to 14/2/51.

No. 24249 — (Female, 3 yrs) — 20 specimens tested 28/2/50 to 2/5/51. Aborted May 2, 1951. The previous four specimens had shown increasing agglutinative activity, from very slight on 6/2/51 to very questionable on April 4; the CCA titres increased from 1:20 to 1:320, the HCF titres from negative to 1:80. Three earlier specimens, 12/9, 24/10 and 5/12/50 had been anticomplementary in the CCA test. Blood drawn the day after the abortion was positive in the routine tests and had titres of 1:1280 and 1:640 in CCA and HCF tests. Slight reactions in the milk ring test were found on 23/10/50; negative reactions 30/10/50 to 6/3/51, a slight reaction 20/3/51, and finally a marked reaction 15/5/51.

61788 (Male 2 mo.) — 25 specimens tested 25/5/50 to 30/10/50. The first 8 specimens were negative in all tests. The specimens of 14/11 and 15/12/50 showed weak agglutination and had CCA titres of 1:80. All specimens collected between 26/12/50 and 22/5/51 were negative throughout. A specimen of 13/6/51 was anticomplementary to a dilution of 1:20 in CCA. Five subsequent specimens 3/7 to 2/10/51 were questionable on PA and TA and had titres of 1:40 to 1:640 in CCA. The last specimen, 30/10/51, was positive in PA and TA; its CCA and HCF titres were 1:640 and 1:320.

On the basis of the later serological results, it would appear that all of the animals that showed very weak or anticomplementary reactions in CCA tests should have been considered potentially infected, isolated at that time, and observed closely for further developments. Such a policy would however, have necessitated the isolation of a number of other animals showing similar CCA reactions but which never developed more than "questionable" reactions and gave no clinical evidence of brucellosis. Four such cases are considered below.

24231 (Female, age 6 years) — 28 specimens of sera tested from 28/2/50 to 27/11/51. A slight MRT reaction was recorded 4/4/50; negative reactions were obtained 11/4/50 to 10/10/50 and 10/4/51 to 28/5/51, and a slight reaction again 23/7/51.

All of these sera showed some reactivity in both the CCA and HCF

tests and "questionable" reactions in PA and TA tests. No specimen gave sufficiently strong agglutination in either PA or TA to be reported as "positive". The animal was kept in isolation during the entire period from reporting of the first to the last test, after which it was slaughtered. Five specimens had titres of 1:160 or 1:320 in the CCA test, a level ordinarily encountered only in association with "positive" agglutination tests, and thus was in all probability evidence of brucellosis and not a "nonspecific" reaction.

41310 (Male age 7 years) — 24 specimens tested from 28/2/50 to 15/8/51. None showed more than a slight questionable reaction in the routine agglutination tests. Fourteen serum specimens reacted in the CCA test, titres 1:5 to 1:80; 10 showed a trace of activity in the HCF test, 1:5. These activities were transient, appearing and disappearing over the period of testing. They may have been "nonspecific" or the result of a very limited or transient infection.

No. 24264 (Female, age 3 years) — 28 specimens tested from 2/5/50 to 8/4/51. Slight MRT reactions were observed with undiluted milk on 21/8/50 to 23/7/51. All sera negative on PA, TA and HCF tests, 6 specimens were anticomplementary in CCA.

No. 30714 (Male, age 6 mo.) — 14 specimens tested. The first 8 specimens were weakly reactive in agglutination tests and had CCA titres of 1:20 to 1:80. The reactivity eventually disappeared in all tests from 27/11/51 to 15/1/52, but again appeared to a low degree in CCA; titres 1:10 and 1:20.

Vaccinated Animals. — A total of 1009 specimens from 65 vaccinated cattle were tested, 313 of these were collected before vaccination, 696 after vaccination with *Br. abortus,* strain No. 19, vaccine. In general reactivity with *Br. abortus* antigen was demonstrated by all four methods in bleedings taken 4 to 10 days after vaccination. In one animal a weak reaction in CCA and HCF tests was detected in serum collected the day after vaccination, which would suggest a secondary or anamnestic rather than a primary response to this antigen. Two weeks later high titres were observed in all 4 tests. The maximum serum dilution titre recorded in the CCA test was 1:10,240, in the HCF test 1:640. These were not usually attained until about 4 weeks after vaccination.

Individual animals varied very widely in the length of time their serological activity was maintained. Sera from 53 animals were tested approximately one year after vaccination. In the PA test, 4 were negative, 18 slightly questionable, 23 were questionable, 5 were very questionable, and 3 were positive. The reactions were usually slightly weaker in the TA test; negative 22, slightly questionable 14, questionable 12, very questionable 3, and positive 2. As was found in naturally-infected animals, the titres were higher in the CCA than HCF test and took longer to disappear. After about one year, in the 53 animals, the titre distribution in the CCA test was as follows:

11 negative, 3 with titre 1:10, 10 with titre 1:20, 15 with 1:40, 12 with 1:80, 2 with 1:160 and one Ac. The HCF titres were distributed as follows: 32 negative, 9 with 1:5, 3 with 1:10 and 9 with 1:20.

Tested 2 years after vaccination, 12 of 23 animals tested were negative or slight questionable in PA, and 19 TA. In CCA, 6 were negative, 8 had titres of 1:20 or less; in HCF, 15 were negative, the remainder had titres of 1:5 or 1:10. Two animals were still positive in PA and TA and had titres of 1:160 or 1:80 in the CCA test. One of these cows aborted. Further details in regard to them follows:

No. 41305 (Female) vaccinated 7/10/49. 19 specimens tested 28/2/50 to 24/7/51. Aborted 27/4/51. All specimens from this cow showed questionable reactions in the PA test. The last specimen was positive. The TA reactions ranged from questionable to positive. The CCA titres ranged from 1:40 to 1:160, the HCF titres were low 1:5 or 1:10; 9 specimens were negative. The titres in this animal, unlike those in the others did not continue to fall during the second year after vaccination.

No. 57296 (Female) — vaccinatied 7/3/50. 15 specimens tested 28/2/50 to 8/4/52. First specimen negative. First specimen taken 5 weeks after vaccination positive in PA and TA test, 1:640 and 1:80 in CCA and HCF tests. The agglutinative activity had decreased in a year to questionable, the CCA titre to 1:40. Specimens tested during the second year maintained this level. A specimen collected 8/4/52 showed increased activity in all tests, the CCA titre was 1:160.

Miscellaneous Specimens. — Seven specimens from three other herds, which showed atypical reactions in the routine PA and TA tests, were also tested by the CCA method. Of the three that were negative in PA and questionable in TA (4, 4, 1) (3, 1, 0) (3, 1, 0) the first had a high CCA titre (1:320), the other two were negative.

Conversely, 3 sera that were slightly reactive in PA and negative in TA were all non- reactive in CCA. One specimen that showed only a slight reaction in PA (4, 0, 0, 0) and was "questionable" in TA (4, 4, 2), had a CCA titre of 1:160. Thus the only 2 specimens of the 7 that reacted in the CCA, showed definite agglutinative activity in the tube test. In this small series the CCA test appeared to agree more closely with the tube agglutination test than with the plate test, whereas in tests of the much larger series of specimens from the known-infected herd there had been better agreement between the plate agglutination and the CCA tests.

SUMMARY

The conglutinating complement-absorption test (CCA) with *Brucella abortus* antigens has been used in parallel with the routine plate (PA) and tube agglutination (TA) tests and a hemolytic complement-fixation method

(HCF) in the titration of 2191 specimens of blood, collected at regular intervals over a period of two years from 174 non-vaccinated cattle in a dairy herd in which an outbreak of brucellosis had recently occurred. When strong agglutination reactions were recorded in both of the routine tests, the CCA titres ranged from 1:160 to 1:5120, the HCF titres from 1:40 to 1:640. The PA and CCA tests were the earliest to detect low degrees of activity with *Br. abortus* antigens in the sera of animals that later became definitely "positive". The HCF test was the least sensitive. In a few of the subsequently "positive" cows, weak or anticomplementary reactions were noted in the CCA test before evidence of serological change was noted in the PA or TA tests. Conversely, the first suggestion of infection was occasionally a mere trace of agglutination in the plate test.

The four tests were also carried out in parallel on 1009 specimens from 65 cattle vaccinated with *Br. abortus*, strain No. 19; 313 of these were collected before vaccination, 696 after vaccination. Marked reactions were recorded in all tests 4 to 10 days after vaccination and reached a maximum in 2 to 4 weeks. The CCA titres rose to 1:640 to 1:10,240. Individual animals varied widely in the length of time serological activity was maintained. In general HCF tests became negative first, PA tests last. A few animals showed "slightly questionable" reactions in PA tests and CCA titres of 1:20 at two years after vaccination.

The results of this investigation suggested therefore that the conglutinating complement-fixation test might be useful as a supplementary method in detecting possible early brucella infection in individual animals in known infected herds. Such weak reactions, although not of definite diagnostic significance, might serve as a guide in isolation procedures, and aid in eradication of the disease.

The number of atypically-reacting specimens from supposedly non-infected herds that have been examined in the CCA test, is too small to permit any conclusions to be drawn in regard to its specificity as compared with the routine agglutination tests.

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