THE USE OF A SINGLE COMPLEMENT FIXATION TEST TECHNIQUE IN BOVINE BRUCELLOSIS, JOHNE'S DISEASE, DOURINE, EQUINE PIROPLASMOSIS AND Q FEVER SEROLOGY

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ABSTRACT

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The same techniques may be used in the complement fixation test (CFT) for the serological diagnosis of bovine brucellosis, Johne's disease (paratuberculosis), dourine, equine piroplasmosis and Q fever (caused by *Coxiella burnetii*). The reproducibility of results is excellent, falling for the most part within the twofold range and never exceeding the fourfold range. Agreement with other laboratories is excellent (i.e. within twofold) in the case of brucellosis and equine piroplasmosis antibody titres. A good correlation between the occurrence of the disease and serological reactions is found on circumstantial evidence in the cases of dourine, Johne's disease and Q fever. A standard unitage system is used to report the antibody titres found in all the tests. To simplify laboratory protocols, laboratories required to employ the CFT for the diagnosis of these diseases are advised to use a single proven technique in all the tests.

Problems experienced with transient false-positive Johne's disease antibody titres in cattle following on tuberculin (bovine and avian) testing make it advisable to take specimens for the Johne's disease test prior to performing the tuberculin tests.

INTRODUCTION

That the complement fixation test (CFT) is recognized as a reliable test for bovine brucellosis by no means implies that the test is done in the same way by laboratories worldwide (Morgan, Davidson & Herbert, 1973).

At the Veterinary Research Institute, Onderstepoort, the CFT technique used by the Central Veterinary Laboratory, Weybridge (Morgan, MacKinnon, Gill, Gower & Norris, 1978) has been adapted to the microtitration system as used in Australia (Anon, 1977) (Herr, Bishop, Bolton & Van der Merwe, 1979; Herr, 1982). When the CFT is applied in other diseases, such as Johne's disease (paratuberculosis), dourine equine piroplasmosis and Q fever (caused by *Coxiella burnetii*), the particular techniques used by the laboratories concerned are often reported in unpublished laboratory manuals. As these techniques may affect the sensitivity of the test, other laboratories which introduce these tests are reluctant to change these protocols. This leads to a very complex system, where a laboratory doing the 5 CFT's mentioned might have a variety of red blood cell (RBC) suspensions, different periods and temperatures of inactivation, different doses of complement and other variables for each test. When this laboratory, which is mainly involved in the bovine brucellosis CFT, was requested to undertake the other tests as well, the feasibility of using the same CFT technique in all the tests was investigated.

MATERIALS AND METHODS

Techniques of the CFT

The tests were done in microtitration plates, using an 8-channel microdilutor[1], which works on the peristaltic pump principle, for both the dilution of serum and the dispensing of reagents. Complement was prepared, using Richardson's preservative (Alton, Jones & Pietz, 1975) and was titrated spectrophotometrically to determine the 50 % haemolytic dose (C'H$_{50}$). Three-and-one-eight C'H$_{50}$'s were used in the test (Herr, Pieterson &

Boshoff, 1981). The quality of the complement used in the test allowed for a dilution of between 1/60 and 1/72. Haemolysin was produced in rabbits and had a titre of at least 1/2000. Five minimum haemolytic doses (MHD's) of haemolysin were used in the test which was done on doubling serum dilutions with the 1/2 dilution used as an anticomplementary control, and the test proper was confined to the 1/4 to 1/128 serum dilutions. Results for bovine brucellosis were reported in International Units (IU/ml), according to the interpretation as set out in Table 1. This is based on the International Unitage system for brucellosis so that a standard serum containing 1000 complement fixing units/ml would give a 50 % end-point reading at a 1/220 serum dilution. For ease of reporting, the other test results are reported in the same system, referring to the units as South African Units/ml (SAU/ml).

Only bovine, equine and caprine sera were used, and these were inactivated undiluted in a waterbath for 30 min at 58 °C. A 3 % red blood cell (RBC) suspension was used and was stendardized spectrophotometrically (Anon, 1977). The haemolytic system consisted of equal parts of the 3 % RBC suspension and veronal buffer with (5 MHD's) haemolysin, giving an effective 1,5 % RBC suspension in the test. The total volume of the test was 0,125 ml made up of equal portions of antigen, complement and diluted serum (0,025 ml each) and a double portion (0,05 ml) of haemolytic system. Warm fixation for 30 min at 37 °C was used. After the addition of the haemolytic system the test plates were incubated for a further 30 min at 37 °C on a shaker. The plates were centrifuged for 1 min at 200 g before being read over a mirror.

Brucellosis test

The antigen used was the locally produced CFT antigen[2] which was standardized to give a 50 % end-point reading with the 2nd International Standard anti-*Brucella*

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[1] Micro Compu-Pet, General Diagnostics, Div. Warner-Lambert Co.,Morris Plains, New Jersey 07950, USA

[2] *Brucella abortus* complement fixation antigen, Veterinary Research Institute, Onderstepoort 0110, Republic of South Africa

TABLE 1 The conversion of CFT end-point reactions to IU/mℓ[a] or SAU/mℓ[b] on a scale where 50 % haemolysis in a 1/220 serum dilution is equivalent to 1 000 IU/mℓ or SAU/mℓ

Serum dilution[c]	Final dilution[d]	End-point reading		IU/mℓ or SAU/mℓ
		% haemolysis	Reaction	
1/4	1/20	75	1-+	15
		50	2-++	18
		25	3-+++	21
		0	4-++++	24
1/8	1/40	75	1-+	30
		50	2-++	36
		25	3-+++	43
		0	4-++++	49
1/16	1/80	75	1-+	60
		50	2-++	72
		25	3-+++	86
		0	4-++++	98
1/32	1/160	75	1-+	120
		50	2-++	145
		25	3-+++	172
		0	4-++++	196
1/64	1/320	75	1-+	240
		50	2-++	290
		25	3-+++	344
		0	4-++++	392
1/128	1/640	75	1-+	480
		50	2-++	581
		25	3-+++	688
		0	4-++++	784
1/220	1/1 100	50	2-++	1 000

[a] IU/mℓ = International Units per millilitre
[b] SAU/mℓ = South African Units per millilitre
[c] Serum dilution = dilution factor with veronal buffer only
[d] Final dilution = final dilution factor after all reagents are added

abortus serum[3] (1 000 IU/mℓ) at a 1/220 serum dilution. To check the reproducibility of the test, a standard serum was diluted with veronal buffer to the point where it would contain 240 IU/mℓ. It was tested on 489 occasions (Herr, Roux & Pieterson, 1982). Cattle which had never been inoculated against brucellosis and which were part of an infected herd were selected for slaughter and attempted isolation of *Brucella* when their CFT titres were 24 IU/mℓ or higher. The methods employed in the isolation techniques were described previously (Herr *et al.*, 1982).

Dourine test

The antigen[4] was produced by harvesting the buffy-coat layer from the blood of rats 3 days after infection with a laboratory strain of *Trypanosoma equiperdum*. The antigen was titrated in chequer-board pattern in microtitration plates using a standard positive serum diluted with veronal buffer to an effective 240 SAU/mℓ. The dilution of antigen giving the highest titre with this serum was selected as the optimal dilution. The wells in the test showing negative reactions were closely examined for trace reactions indicative of anti-complementary activity present in the antigen. The reactions in these wells were compared with those of the anti-complimentary control wells. Only an antigen satisfactory in this respect was used.

Before the test was adopted as routine all the sera received from the field during a 3-month period were subjected to 2 tests. The first was done in tubes according to a protocol established over many years by the Section of Protozoology, Veterinary Research Institute, Onderstepoort. The second was the test as described above in microtitration plates. During this period, 166 sera from the field were tested in parallel. These results were interpreted as negative if below 15 SAU/mℓ, as suspicious between 18–98 SAU/mℓ and as positive if 120 SAU/mℓ or higher. In the animals showing titres of 18–98 SAU/mℓ a retest was done after 1–2 months. The animals whose antibody titres had returned to negative status on retest were classified as negative. Those that showed no rise in antibody titre to above 120 SAU/mℓ were classified as negative for dourine, provided there were no further grounds for suspecting dourine in the stud. Titres are expected to develop within 1–3 weeks of infection (Soulsby, 1968). The standard serum diluted to contain 240 SAU/mℓ was included daily as a positive control serum at all times. In the subseqeunt routine use of this test, animals with positive results were checked for the presence of clinical signs wherever possible.

Johne's disease test

The antigen used was the paratuberculosis antigen[5], supplied by the Centraal Diergeneeskundig Instituut, Rotterdam, Holland, at the recommmended dilution of 1/150. A standard serum with an end-point reading of 240 SAU/mℓ was used as positive control. Wherever possible, positive results were correlated with the demonstration and culture of *Mycobacterium paratuberculosis* by the Section of Bacteriology at the Veterinary Research Institute, Onderstepoort. The sera tested originated either from herds where suspected cases of Johne's disease had been seen or from herds where no signs of this desease were present. An end-point titre of 30 SAU/mℓ equivalent to a +/8 reaction or greater was considered as positive, as other authors had regarded as positive reactions between 4+/5 and 4+/8 (Gorrie, 1959; Wilks, Taylor & Russell, 1981).

Mycobacterium paratuberculosis *demonstration and isolation techniques*

Specimens from the field included faecal material, intestinal mucosa from the iliocaecal valve area and adjacent mesenteric lymph nodes. Smears were made from faecal material, from the intestinal mucosa and the adjacent lymph nodes. All the smears were stained by the Ziehl Neelsen method (Kleeberg, Koornhof & Palmhert, 1980). The isolation methods used were those of Merkal, Kopecky, Larsen & Thurston (1964).

The mycobactin incorporated into Herrold's medium was prepared as described by Williams Smith (1953).

Q fever test

A commercial antigen and positive control serum were used[6]. The sera tested originated from cattle or

[3] Central Veterinary Laboratory, New Haw, Weybridge, Surrey, England

[4] Section of Protozoology, Veterinary Research Institute, Onderstepoort 0110, Republic of South Africa

[5] Antigeen voor de C.B.R. Para-tbc (251179), Centraal Diergeneeskundig Instituut, Posbus 65, 8200 AB Lelystad, Nederland

[6] Q fever antigen (ORAS 04/05) and Q fever control serum positive (ORBM 02/03), Behringwerke AG, Marburg, West Germany

goat herds where *Coxiella burnetii* had been identified on morphological grounds from placental impression smears following abortions. Sera from cattle herds in which no such problem was suspected were also tested. The antigen was used at a 1/10 dilution, as recommended by the manufacturer. A reaction of +/5 (Schafer, 1983) has been shown to be significant, and we considered 30 SAU/mℓ equivalent to a +/8 reaction or higher as positive.

Equine piroplasmosis test

Both antigen and control sera were kindly supplied by the Diagnostic Reagents Section of the National Veterinary Services Laboratory (NVSL), Ames, Iowa, USA. Positive reactions in both the *Babesia equi* and *B. caballi* CFT were taken at antibody titres of 18 SAU/mℓ (++/4) or higher to conform to the +/5 reaction regarded as positive in the USA (Brown-NVSL, personal communication, 1982).

RESULTS

Brucellosis test

The test in 480/489 (98,2 %) of cases where the control standard serum (240 IU/mℓ) was used gave antibody titres of 196–392 IU/mℓ. The balance (1,8 %) showed antibody titres of 172 IU/mℓ (5 cases) and 145 and 480 IU/mℓ respectively (2 cases each). From the 31 cows with titres of 24 IU/mℓ or higher, *Brucella abortus* biotype 2 was successfully isolated in 27 (87 %) cases (Herr *et al.*, 1982).

Dourine test

Out of 19 batches of antigen produced, a single batch was found to have a titre lower than 1/100 and was discarded. Two other batches were anti-complementary and were also discarded. All the other batches recorded titres of 1/200–1/300 and at this optimum dilution gave readings of 172–240 SAU/mℓ with the standard serum. In the animals which were regarded as positive (titres of 120 SAU/mℓ or higher) and where the clinical signs were reported, approximately half showed swelling of the genitalia, a few were reported to be in poor condition and the balance showed no signs of dourine. Nervous symptoms were not reported in any of these cases. The animals with suspicious antibody titres (18–98 SAU/mℓ) were retested, and half of these had returned to negative status within 1–2 months. The rest showed titres in this same range when retested. None of these animals were reported to have any clinical manifestations of the disease. The use of the standard serum gave results essentially similar to those reported for the standard brucellosis serum (i.e. within two- to fourfold of the predetermined antibody concentration of 240 SAU/mℓ).

The results from the 116 serum samples from the field which were tested in parallel agreed in positive, negative and suspicious categories in both tests.

Johne's disease

The positive serological reactions came from clinically affected animals. The reproducibility of results with the standard serum were similar to that reported for brucellosis. In the tests done on sera from herds not suspected of harbouring the disease, the results were always negative. In 2 negative herds, a few transient reactions of 15–30 SAU/mℓ were experienced when samples were taken shortly after the animals had been tested for tuberculosis with bovine and avian tuberculin.

Experimental attempts to reproduce this phenomenon have been unsuccessful to date (Herr & Williamson, unpublished data, 1985).

Wherever positive serological results were checked bacteriologically, acid-fast bacilli were seen and/or *Mycobacterium paratuberculosis* was isolated. Typical clumps of acid-fast bacilli were easily demonstrated in faecal material. Isolation of the organism from faecal material was not successful. Impression smears from the intestinal mucosa and adjacent mesenteric lymph nodes regularly showed acid-fast bacilli. The culture of the organism from intestinal mucosa and/or mesenteric lymph node material was successful in 80 % of the cases attempted.

Q fever test

In the herds suspected of being infected, between 5 % and 10 % reactors were seen with titres of 30 SAU/mℓ or higher. In the herds where no suspicion of the disease existed, tests were invariably negative (titres below 15 SAU/mℓ).

Equine piroplasmosis test

In our test system, the negative antigen and negative serum consistently gave negative results. The *Babesia equi* antiserum with a reported titre of 4+/10 gave us readings of 30–60 SAU/mℓ, representing reactions of +/8–+/16. The *B. equi* antiserum with a titre of 4+/80 gave reactions of 196–392 SAU/mℓ, representing 4+/32–4+/64 titres. *B. caballi* antiserum assessed by NVSL, Ames, as having a titre of 4+/10 reacted in our test between 30–72 SAU/mℓ, which represents +/8–2+/16 titres. The *B. caballi* serum with a titre of 2+/320 consistently gave readings of 784 SAU/mℓ or higher, representing titres in excess of 4+/128. This latter serum, however, was also consistently anti-complementary in our test. This anti-complementary activity did not extend beyond the 1/8 dilution.

DISCUSSION

In the bovine brucellosis CFT, using standardized antigens and an International Standard serum, as much as four-hundredfold differences in antibody titre have been reported by various laboratories (Morgan *et al.*, 1973). Within laboratories some authors have been satisfied with a fourfold reproducibility (Thomson, Mumford, Campbell, Griffiths & Clapham, 1976). The finding in our bovine brucellosis CFT that the reproducibility was within the twofold range in more than 98 % of cases and never exceeded the fourfold range, is an indication that the test technique was reliable. Throughout the other tests, this same range of reproducibility was maintained, an indication that so far as this aspect is concerned the technique used was equally reliable.

Furthermore, in brucellosis serology it was shown that 87 % of serologically reactive animals were infected (Herr *et al.*, 1982). The fact that successful isolation was achieved in 87 % of these cases compares favourably with reported 90 % successful isolations after experimental infection (Plommet, 1977). This type of examination was not possible in the other tests. However, in the test for Johne's disease, the results were in agreement wherever isolation and serology were attempted on the same animal. The phenomenon of false positive Johne's disease reactions is not uncommon (Gorrie, 1959; Wilks *et al.*, 1981), but this phenomenon has not previously been associated with the use of bovine and avian tuberculin. Nevertheless it would be advisable to test for

Johne's desease before testing with tuberculin. In the case of Q fever, we have the circumstantial evidence that positive serology was only seen in animals on those properties where the organism had been demonstrated. This is in agreement with the findings of Schaal & Schafer (1984). The fact that spot checks on sera from cattle herds not experiencing problems invariably proved negative warrants further investigation. The agreement between clinical signs observed and positive serology was also good for dourine as many animals are known to harbour *T. equiperdum* without showing clinical signs (Blood, Henderson & Radostits, 1979; Barrowman, 1976).

Because of the availability of International Standard sera, the titres recorded in the brucellosis CFT are known to be comparable with those of laboratories in other countries. To date, it has been possible to check only the international correlation of titres in our other tests in the case of equine piroplasmosis. In this test, the findings from our techniques were well within twofold of the reported titres emanating from the laboratory of the NVSL at Ames.

In conclusion, therefore, this work shows that it may be worth while for laboratories employing the CFT in a number of disease conditions to investigate whether the variety of techniques used for the different tests is really necessary.

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