Onderstepoort J. vet. Res., 49, 79–83 (1982)

THE REPRODUCIBILITY OF RESULTS IN BOVINE BRUCELLOSIS SEROLOGY AND THEIR CORRELATION WITH THE ISOLATION OF *BRUCELLA ABORTUS*

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ABSTRACT

HERR, S., ROUX, D. & PIETERSON, P. M., 1982. The reproducibility of results in bovine brucellosis serology and their correlation with the isolation of *Brucella abortus*. *Onderstepoort Journal of Veterinary Research*, 49, 79–83 (1982).

In both the complement fixation test (CFT) and the serum agglutination test (SAT) titres were reproducible for the most part within a twofold range. They seldom exceeded these limits and never a fourfold range. *Brucella abortus* was successfully isolated in 86% of serologically positive cases and evidence is presented to confirm the use of the 30 International Units/mℓ level in the CFT as being diagnostically significant. The SAT, when done in microtitration plates, is even more reproducible than when done in tubes. The incidence of infected animals aborting or calving down with negative titres was found to be low.

INTRODUCTION

In an investigation involving 25 laboratories in 18 countries, Morgan, Davidson & Herbert (1973) found up to 400-fold differences in complement fixation test (CFT) titres with the 2nd International Standard Anti-*Brucella abortus* serum. Mackinnon (personal communication, 1980), on the other hand, reported that he aimed for results within a twofold range and for the most part achieved this in his own laboratory in the CFT. Alton (personal communication, 1980) reported CFT results from regional laboratories in Australia which seldom exceeded the twofold range. The CFT has been shown to be most efficient in identifying infected animals (Alton, Maw, Rogerson & McPherson, 1975; Morgan, 1977). Because of the discrepancies in the reproducibility of results, each national laboratory ought thus to undertake an evaluation of the reproducibility of their test and to re-evaluate the correlation between serological diagnosis and the successful isolation of *Brucella abortus*.

MATERIALS AND METHODS

Sera

Sera used to study the reproducibility of CFT and serum agglutination test (SAT) results. A standard serum was inactivated for 30 min at 58 °C and stored at 4 °C in 1 mℓ quantities in the freeze-dried state. This serum, when reconstituted with 1 mℓ distilled water and titrated against the 2nd International Standard Anti-*Brucella abortus* Serum (1 000 IU/mℓ) proven to have 1 600 IU/mℓ, was used as a working standard in the CFT. During the period April–August 1980 this standard serum was used as a positive control in the "Dynatech"[1] system on 139 occasions, while from August–October 1980 it was used on 489 occasions with the "Compu-Pet"[2] system. Before use, the serum was reconstituted with 1 mℓ of distilled water and, after 6 mℓ of veronal buffer had been added, the mixture gave an effective dilution of 1/7 equivalent to 228,6 IU/mℓ. Five freeze-dried sera derived from field cases were also used on a number of occasions to test the reproducibility of test results. These sera were supplied to the laboratory unmarked, with the request that they be tested on one or more occasions and results be reported.

For the SAT the *Brucella abortus* National Standard Positive Serum (OPNS 1, 1979)[3] containing 800 IU/mℓ was reconstituted with 1 mℓ of distilled water and diluted 1/10 with phenol-saline, giving an effective 80 IU/mℓ. This was used as a positive control in the tube test 222 times from January–November 1980 and 158 times in the microtitration test during February/March 1981. The same 5 field sera as above were tested in tubes on a number of occasions in 1980.

Sera used to study the correlation between CFT titres and isolation of Brucella abortus. Sera from 56 animals were used. Of these, 35 had not previously been inoculated, and titres were detected either on routine checks or around the time of abortion or calving. There were 11 that had been inoculated as adults but these had passed a negative test 6 months later and had developed titres subsequent to this. In 8 animals the vaccination history was unknown, but adult vaccination was suspected. Two cases were vaccinated as heifers and were adult when examined.

Serology

Rose bengal test in WHO haemagglutination plates. The rose bengal test (RBT) was done in WHO haemagglutination 80-well plates (Anon, 1980). The antigen[4] was produced by the Veterinary Research Institute, Onderstepoort, equal volumes (0,025 mℓ) of serum and antigen being used. Eighty sera were dispensed per plate with a micropipette[5] and the antigen added with a pipette dropper.[6] The plate was tapped vigorously for 20 counts, then placed on a rotary shaker[7] for 4 min and read over an X-ray viewing box.[8] All reactions from a well-defined rimming to a complete clearing of the supernatant fluid were regarded as positive.

The SAT in tubes. The methods of Morgan, Mackinnon, Gill, Gower & Norris (1978), as modified by Herr, Bishop, Bolton & Van der Merwe (1979), were followed, except that the test was carried out in 2 mℓ volumes and that an automatic syringe with a 16 gge, 75 mm cannula was used for the serial dilutions. The reading of the test was based on the International Unitage (IU/mℓ) table with the 1 000 IU/mℓ end-point defined as 50% agglutination at a final dilution of 1/500. A four-tube test used gave final dilutions of 1/10, 1/20, 1/40 and 1/80 and a maximum reading of 212 IU/mℓ (Herr *et al.*, 1979).

The SAT in microtitration plates. Preliminary tests were carried out to determine whether the best results would be obtained in U- or V-bottomed plates. After a preliminary observation that the agglutinate tended to stick to the bottom of the wells after 16–20 h incubation,

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(2) General Diagnostics, Eastleigh, Hants. SO5 3ZQ England

(3) Veterinary Research Institute, Onderstepoort 0110

Received 27 November 1981—Editor

(4) Rose Bengal *Brucella* Antigen, Veterinary Research Institute, Onderstepoort 0110

(5) Eppendorf pipette 4700, Eppendorf Gerätebau, P.O. Box 630324, 2000 Hamburg, West Germany

(6) Cooke Engineering Company, 900 Slaters Lane, Alexandria, Virginia, 22314 USA

(7) Heidolph Type 54131 REAX 3, Heidolph-Elektro KG, D-8420 Kelheim, West Germany

(8) EAC Graphics, 180 Schoeman Str., Pretoria 0002

a shaker and centrifugation of the test at half-hour intervals were used to determine the optimum time for agglutination to occur and prevent the sticking of the agglutinate to the bottom of the wells. It was reasoned that, even had agglutination occurred, it would not immediately be seen, as the large aggregates would still have to settle out. For this reason the plates were centrifuged at 300 g for 1 min at these same half-hour intervals. From these results the following method was evolved:

Sera were dispensed (0,1 mℓ) in 96-well, U-bottomed, microtitration plates. Using the micro "Compu-Pet" multidilutor primed with phenol-saline set to pick up 0,025 mℓ and at a dilution of 1/5, 8 sera at a time were dispensed into rows 1, 5 and 9 of a clean microtitration plate, giving 0,1 mℓ of a 1/5 dilution of sera in these rows. The "Compu-Pet" was then reset to pick up 0,05 mℓ and at a dilution factor of 1/2 to deliver 0,1 mℓ. A serial dilution was then done in 4 wells to pick up 0,05 mℓ, transferring 0,1 mℓ and discarding 0,05 mℓ from the 4th well, giving serum dilutions of 1/5, 1/10, 1/20 and 1/40. Antigen[1] was used to reprime the Compu-Pet and the machine was reset at a 1/1 dilution. All the wells receiving 0,05 mℓ antigen gave final dilutions of 1/10, 1/20, 1/40 and 1/80. The plates were then placed on a shaker in an incubator for 1½ h at 37 °C. After incubation the plates were centrifuged at 300 g for 1 min and read over a black background with a diffuse light source.

The CFT. The methods employed in the CFT were those of Morgan *et al.* (1978) and of Alton (1977), as modified by Herr *et al.* (1979). The preparation of a 3% red blood cell suspension (RBC) was done spectrophotometrically and the 50% end-point spectrophotometric complement titration was used. Up until August 1980 the "Dynatech"[2] system was used for serial dilutions and dispensing, and thereafter the "Compu-Pet"[3] system was brought into use for both operations.

Interpretation of CFT titres. Using the CFT as the definitive test, we accepted the following criteria in judging the status of the animal under test. In cattle that had never been vaccinated with *B. abortus* S19 vaccine or had been vaccinated between the ages of 4 and 10 months and tested as adults (over 18 months), or where the vaccination history was unknown, a titre of 18–24 IU/mℓ was regarded as suspicious and 30 IU/mℓ or higher as positive. Where adult vaccination had occurred, 30–49 IU/mℓ was taken as suspicious and 60 IU/mℓ or higher as positive.

Isolation and typing of Brucella abortus

In a preliminary study undertaken on 10 animals, specimens were taken at slaughter from retropharyngeal, lumbar, iliac and supramammary lymph nodes, spleen, uterus or uterine content, if the animal was pregnant, and from each udder quarter. These were plated out on 5 different *Brucella* media before and after concentration by centrifugation and also after the pooling of certain of the specimens. Biological isolation and serology were performed, using guinea-pigs. These results were evaluated (Herr & Roux, 1980; 1981) and in the remaining 46 cases tissue was taken only from iliac and supramammary lymph nodes and the 4 udder quarters. The method of concentration was employed as previously described and the same 5 media were used, but biological isolation and serology in guinea-pigs were discontinued. The typing of the isolates was as described and followed the recommendations of Alton, Jones & Pietz (1975).

[1] *Brucella abortus* antigen SAT, Veterinary Research Institute, Onderstepoort 0110

[2] Cooke Engineering Company, 900 Slaters Lane, Alexandria, Virginia, 22314 USA

[3] General Diagnostics, Eastliegh, Hants., S05 3ZQ England

RESULTS

Reproducibility of CFT and SAT results

In the CFT, using the standard serum in the "Dynatech" system (Fig. 1), 136/139 (97,8%) of the results fell within the twofold range (196–392 IU/mℓ). The balance was between 145–480 IU/mℓ (within the fourfold range). With the "Compu-Pet" 480/489 (98,2%) of the results were within twofold (196–392 IU/mℓ) and the balance within fourfold. The CFT results with the test sera (Table 1) showed the greatest variance on sera numbers 4 and 5 to be 1,5- and 1,8-fold respectively, when a negative reading was arbitrarily taken as equivalent to 10 IU/mℓ. With serum 13 this figure rose to 3,5-fold, but 19/22 (86,4%) of the results were within the twofold range (120–240 IU/mℓ). Serum 14 showed the greatest variance of 3,3-fold, but 18/22 (81,8%) of the results fell within the twofold range (98–196 IU/mℓ). Serum 27 had a greatest variance of 2,4-fold, but 17/18 (94,4%) fell within twofold (49–98 IU/mℓ).

In the SAT, in which the OPNS 1 serum in tubes (Fig. 2) was used, all the results fell within the twofold range (53–106 IU/mℓ). In the microtitration plates all the results fell within a 1,5-fold range (53–80 IU/mℓ). With the test sera (Table 2) in which the same criteria for the greatest variance as in the CFT were used, sera 4, 13 and 27 showed 1-, 1,3- and 1,7-fold ranges respectively. Serum 5 had the greatest variance of 2,7-fold, but 14/15 (93,3%) of results fell within the twofold range (10–20 IU/mℓ). Serum 14 showed a 3,1-fold range, but 14/15 (93,3%) fell within 1,6-fold (67–106 IU/mℓ).

Correlation between CFT titres and the isolation of Brucella abortus

Since out of the 48 animals whose vaccination history was known 36 cases were classified as positive serologically and *Brucella abortus* biotype 1 was also isolated from 5 and biotype 2 from 26, 31/36 (86%) isolations were successful (Table 3). An isolation was also successful in the single suspicious reactor from this group. Another 3 animals that tested negative serologically nevertheless yielded *Brucella* organisms on culture. In the 8 cases whose vaccination history was un-

TABLE 1 Reproducibility of CFT titres (IU/mℓ) of 5 freeze-dried test sera repeated blind throughout 1980 in a single laboratory

Date tested	Serum No. 4	5	13	14	27
80/10/13	—[1]	—	196	172	86
80/10/13	—	—	344	172	86
80/10/13	—	15	196	196	86
80/10/13	—	—	344	196	86
80/10/13	—	15	196	172	86
80/10/21	15	15	240	240	86
80/10/21	—	—	196	240	86
80/10/21	—	—	196	240	98
80/10/21	—	—	240	196	120
80/10/21	18	—	240	145	72
80/10/28	—	—	145	98	49
80/10/28	—	—	145	98	49
80/10/28	—	—	196	98	49
80/10/28	—	—	172	98	72
80/10/28	15	—	172	172	49
80/8/12	—	—	98	98	49
80/8/18	—	—	120	98	86
80/8/21	—	—	120	98	49
March 1980	—	—	120	72	ND
March 1980	—	15	172	98	ND
Jan 1980	15	—	120	98	ND
Jan 1980	15	—	120	98	ND[3]
Greatest variance[2]	1,8	1,5	3,5	3,3	2,4

[1] — = negative

[2] Greatest variance = The highest titre in the series divided by the lowest titre recorded taking a negative result arbitrarily as equivalent to 10 IU/mℓ

[3] ND = Not done

TABLE 2 Reproducibility of SAT titres (IU/mℓ) of 5 freeze-dried test sera repeated blind throughout 1980 in a single laboratory

Date tested	Serum No. 4	5	13	14	27
80/10/13	—[1]	—	106	93	53
80/10/13	—	—	106	106	53
80/10/13	—	—	106	106	67
80/10/13	—	—	106	106	47
80/10/13	—	20	106	106	47
80/10/21	—	—	93	93	80
80/10/21	—	—	93	80	67
80/10/21	—	—	106	93	53
80/10/21	—	—	106	93	53
80/10/21	—	—	106	106	53
80/8/12	—	—	106	93	67
80/8/18	—	—	106	106	53
80/8/21	—	—	106	106	67
80/4/14	—	17	106	67	ND
Jan 1980	—	27	80	34	ND[3]
Greatest variance[2]	1,0	2,7	1,3	3,1	1,7

[1] — = negative

[2] Greatest variance = The highest titre in the series divided by the lowest titre recorded taking a negative result arbitrarily as equivalent to 10 IU/mℓ

[3] ND = not done

known but where adult vaccination was suspected, only 2 of the positive reactors yielded positive cultures (25%).

Previous serological tests on the uninoculated animals and on those that had been adult inoculated but subsequently tested negative (Table 3) had all proved negative in the routine bi-monthly tests applied, except for the following 3 cases. Case No. 34 gave a positive serological result on RBT, and 47 and 196 IU/mℓ in the SAT and CFT, respectively, 6 days before slaughter. Case No. 47 was positive, with 160 and 18 IU/mℓ in the 3 tests, respectively, 9 days prior to slaughter. Case No. 56 was positive to RBT but negative to SAT and CFT 27 days before slaughter and had a full term viable calf 18 days after this test. Unfortunately, this last case was not tested on the day of calving, and no other animal in the series proved negative on the day of calving or abortion and subsequently developed a titre.

DISCUSSION

Although the goal of achieving all results in both tests within the twofold range has not been achieved, it is within sight, as at present, for the most part, upwards of 90% of results are within this range and no results exceed

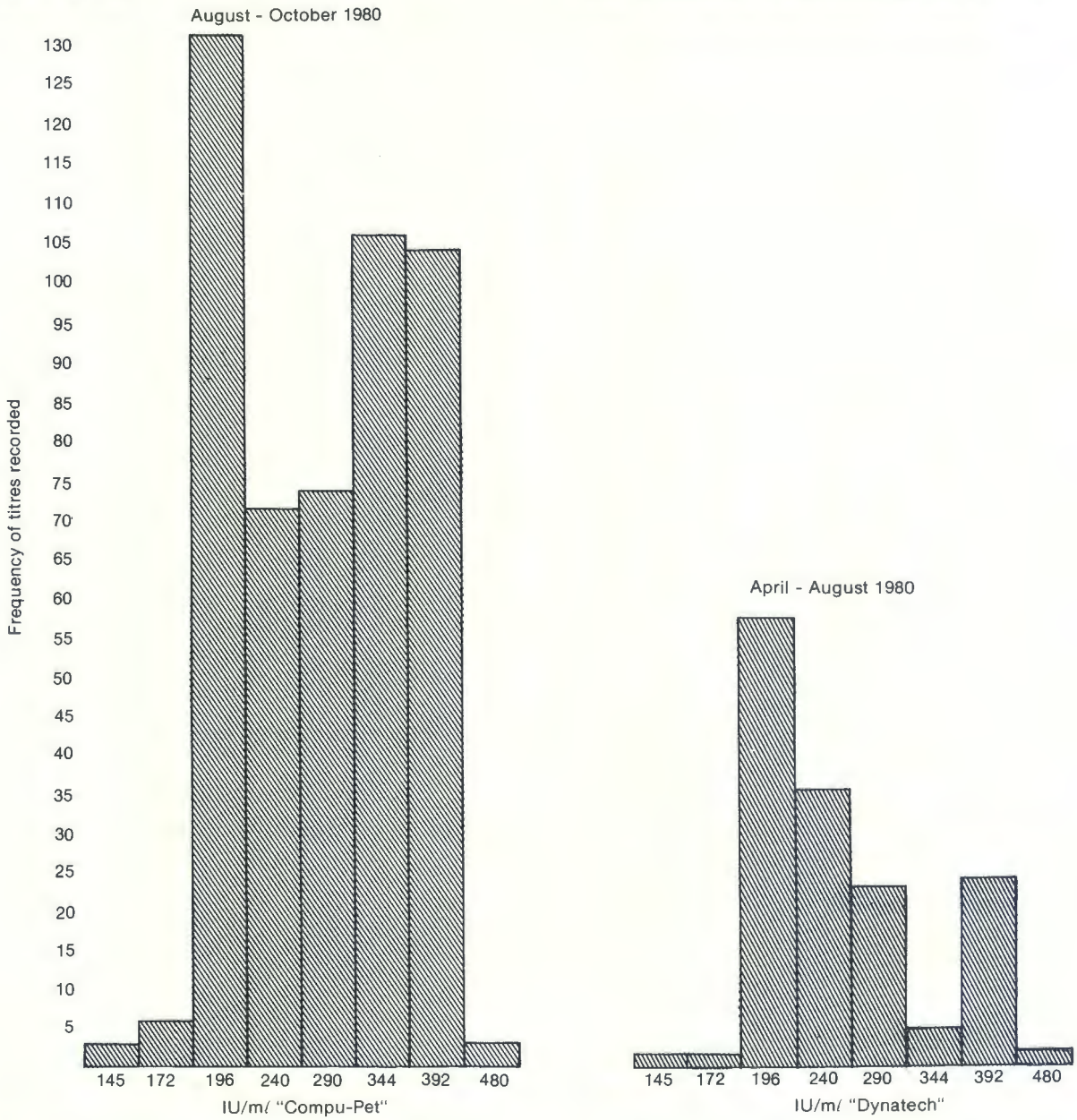

FIG. 1. Reproducibility of CFT results with a single Standard Serum in the "Dynatech" and "Compu-Pet" systems.

TABLE 3 Correlation between serological titres and the successful isolation of *Brucella abortus*

Case No.	Cow No.	Vaccination history	Pregnancy status (months)	Pre-slaughter serological results			Serological diagnosis	Successful isolation and biotype	Tissue used in isolation attempts
				RBT	SAT IU/mℓ	CFT IU/mℓ			
1	1141	AISNT[1]	NR[2]	—[3]	—	—	—	—	RLISSUU[4]
2	1164	,,	,,	+[5]	20	21	—	—	,,
3	1216	,,	,,	+	212	784	+	1[15]	,,
4	1269	,,	,,	+	20	—	—	1	,,
5	2026	,,	C[14]	+	212	98	+	1	,,
6	2636	,,	NR	+	20	98	+	—	,,
7	2675	,,	,,	+	23	—	—	—	,,
8	7893	,,	,,	—	—	—	—	—	,,
9	9341	,,	,,	+	212	784	+	1	,,
10	9542	,,	,,	+	212	784	+	1	,,
11	4225	HUAVS[6]	,,	+	80	98	+	—	ISU[7]
12	4229	,,	,,	+	80	43	+(S)[8]	—	,,
13	4230	,,	,,	+	34	36	+(S)	—	,,
14	4227	,,	,,	+	40	49	+(S)	—	,,
15	4232	,,	,,	+	67	72	+	—	,,
16	165	,,	8[17]	+	212	784	+	1	,,
17	241	,,	NP[9]	+	212	784	+	1	,,
18	143	,,	5	+	212	784	+	—	,,
19	281	NV[10]	NR	+	186	172	+	2[16]	,,
20	2004	,,	,,	+	212	784	+	—	,,
21	806	,,	,,	+	212	784	+	2	,,
22	2575	,,	,,	+	212	49	+	—	,,
23	1147	,,	,,	+	47	98	+	—	,,
24	427	,,	,,	+	212	196	+	2	,,
25	2319	,,	,,	+	47	24	S[11]	2	,,
26	1382	,,	,,	+	212	784	+	2	,,
27	1390	,,	A[12]	+	186	784	+	2	,,
28	1119	,,	A	+	212	784	+	2	,,
29	1273	,,	A	+	212	784	+	2	,,
30	1689	,,	A	+	212	172	+	2	,,
31	9228	,,	A	+	80	86	+	2	,,
32	459	,,	A	+	212	784	+	2	,,
33	8349	,,	2	+	40	—	—	—	,,
34	2573	,,	NR	+	—	784	+	2	,,
35	1190	,,	3	—	—	—	—	—	,,
36	1280	,,	2	+	212	784	+	2	,,
37	892	,,	NP	+	212	784	+	2	,,
38	1133	,,	7	+	212	784	+	2	,,
39	2007	,,	5	—	—	—	—	2	,,
40	9019	,,	NP	—	—	—	—	2	,,
41	206	,,	2	+	212	290	+	2	,,
42	1556	,,	6	+	212	784	+	2	,,
43	944	,,	NP	+	212	784	+	2	,,
44	9522	,,	2	+	212	784	+	2	,,
45	1184	,,	4	+	212	196	+	2	,,
46	4645	,,	6	+	212	784	+	2	,,
47	2462	,,	2	+	212	49	+	2	,,
48	1714	,,	6	+	27	98	+	2	,,
49	4075	IAH[13]	NP	+	23	—	—	—	,,
50	3461	AISNT	NP	+	212	784	+	1	,,
51	125	NV	8	+	80	86	+	2	,,
52	4745	IAH	NP	+	40	—	—	—	,,
53	9313	NV	5	+	20	98	+	—	,,
54	2508	,,	5	+	212	688	+	2	,,
55	1398	,,	A	+	20	43	+	2	,,
56	9421	,,	C[14]	+	212	344	+	2	,,

[1] AISNT = Adult inoculated with a subsequent negative serological test
[2] NR = Not recorded
[3] — = Negative
[4] RLISSUU = Retropharyngeal, lumber, iliac and supramammal lymph nodes, spleen, udder (4 quarters), uterus or uterne content, if pregnant
[6] HUAVS = History unknown. (Adult vaccination suspected)
[7] ISU = Iliac and supramammary lymph nodes plus udder (4 quarters)
[8] (S) = Suspicious reaction if adult vaccinatoin was practised
[9] NP = Not pregnant
[10] NV = Never vaccinated
[11] S = Suspicious
[12] A = Aborted
[13] IAH = Inoculated as heifer
[14] C = Calved normally
[15] 1 = Biotype 1 isolated
[16] 2 = Biotype 2 isolated
[17] Months pregnant

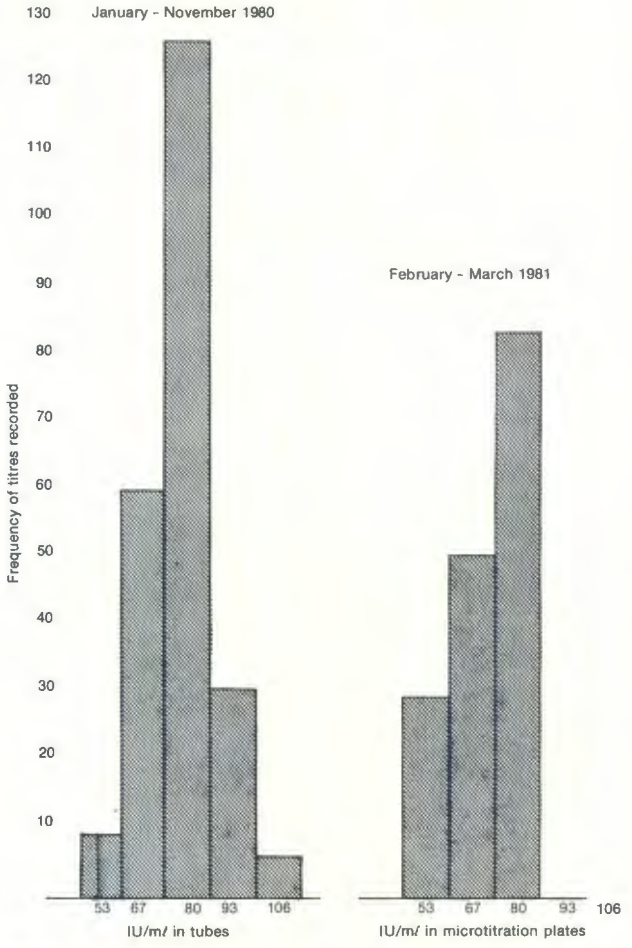

FIG. 2. Reproducibility of SAT results with the National Standard Serum in tubes and in microtitration plates.

the fourfold range. This compares well with other results reported with the CFT (Thomson, Mumford, Campbell, Griffiths & Clapham, 1976; MacKinnon personal communication, 1980; Alton, personal communication, 1980). One of the prime prerequisites for this level of reproducibility is stability of staff. Over the period under review there had been only 1 staff change working with the SAT and none with the CFT, and this consistency of staff is regarded as an important reason for the good results obtained to date.

Although the phenomenon of an infected animal aborting or calving and developing a positive titre only 1–2 weeks later is well documented (Cunningham, 1977; Thomson, 1950), the fact that not a single such case occurred in this series seems to indicate that the incidence of such a phenomenon is low.

It also becomes evident from the CFT titres recorded in cases where a positive isolation was achieved that there is justification for setting the positive limit at 30 IU/mℓ in the CFT for unvaccinated animals. Infected animals with titres of this magnitude are seen in Case No. 25, 47 and 55 (Table 3). The difficulty of distinguishing adult vaccinates from infected animals becomes evident when the above results are compared with those of Case No. 12, 13 and 14. It must be emphasized that this refers to the use of the S19 vaccine containing approximately 4×10^{10} viable organisms per dose.

The sucessful isolation of *B. abortus* from 2 animals (Case No. 39 and 40) which were negative serologically, highlights the limitations of serological tests in a disease with an incubation period as long as that found in brucellosis and is a fact to be borne in mind when isolation techniques are used to evaluate serological tests (Alton, 1977; Cordes & Carter, 1979).

ACKNOWLEDGEMENTS

The author wishes to thank Mrs E. A. van der Merwe and Miss J. C. Peens for their technical assistance.

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