

PROZONES AND DELAYED REACTIONS IN THE ROSE BENGAL TEST FOR BOVINE BRUCELLOSIS

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ABSTRACT

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A prozone and a somewhat delayed reaction were found to be present in the rose bengal test (RBT) when sera, delayed in transport, gave rise to false negative results. A postulate as to the mechanisms possibly involved in this slow prozone reaction is offered.

INTRODUCTION

The incidence of a high percentage of negative rose bengal test (RBT) sera which showed a high titre in the serum agglutination (SAT) and complement fixation tests (CFT) was noted in certain batches of sera during routine testing for bovine brucellosis. As a similar phenomenon has not previously been recorded, an investigation was undertaken to establish whether in this case a prozone reaction like that described in other tests (Plackett & Alton, 1975) was present.

MATERIALS AND METHODS

Sera

During September 1979-January 1980, 9 985 sera, arriving in 286 batches of varying numbers, were received from the field for routine testing. The phenomenon of negative RBT, high titre SAT and CFT results was seen in 55 sera distributed in 14 batches. Amongst 3 807 sera comprising 65 batches and subjected to all 3 tests, the same phenomenon occurred in 14 sera from 4 batches. Whenever possible, these sera were subjected to a titration in the RBT as described below. Repeat collections of some of these batches were arranged at intervals of up to 35 days, and, subsequently, collections that were dispatched to reach the laboratory within 2 days were also tested. A further 1 038 sera were tested within 2 days of collection.

A questionnaire was compiled to establish some common denominator which would account for this phenomenon. The following aspects were inquired into: breed, age, sex, degree of inbreeding, relationship of animals involved, pregnancy status, feeding regimen, other treatments, vaccination history (brucellosis and other), methods of collection and handling of specimens, and type of farming operation. Other aspects considered for investigation were the type of immunoglobulin present and the success of isolation of *Brucella* from animals exhibiting the phenomenon.

Serology

The RBT was done in WHO haemagglutination 80-well plates (Anon., 1980). Equal volumes (0,025 ml) of serum and antigen, produced by the Veterinary Research Institute, Onderstepoort⁽²⁾, were used. Eighty sera were dispensed per plate with a micropipette⁽³⁾ and the antigen was added with a pipette dropper⁽⁴⁾. The plate was then tapped vigorously for 20 counts, placed on a rotary shaker⁽⁵⁾ for 4 min and read over an X-ray viewing box⁽⁶⁾.

All reactions from a well-defined rimming to a complete clearing of the supernatant fluid were regarded as positive.

An RBT titration was developed and carried out as follows: A 0,025 ml volume of serum was dispensed into the first 3 wells in the plate. An equal volume of serum was then dispensed into the 2nd well. Phenol-saline (0,025 ml) was then dispensed into the 3rd to 8th wells. A twofold serial dilution was done commencing at the 3rd well, transferring 0,025 ml each time and discarding 0,025 ml from the 8th well, giving effective dilutions of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ and $\frac{1}{64}$. A repeat of the single volume test in the 1st well and a double serum volume test in the 2nd well were carried out. The same antigen (0,025 ml) was then dispensed into all the wells and the test completed as described above. The results for each well were recorded as + = discernible rimming (agglutination seen around the edges of the fluid only), ++ = rimming and just discernible agglutination in the central fluid, +++ = obvious agglutination in the central fluid and ++++ = complete agglutination with clearing of the supernatant fluid. After 4 min the test was read, the plates were replaced on the rotary shaker for a further 4 min and then re-read. This process was repeated twice so that test times of 4 min, 8 min, 12 min and 16 min could thus be spaced.

Periodically, the titration was repeated after a different antigen⁽⁷⁾ was used, or different methods (clear glass plates and opaque white plastic plates) were employed. One serum was taken to another laboratory⁽⁸⁾ and the test carried out under their conditions. The pH⁽⁹⁾ of the sera was also periodically checked and compared with that of normally reacting sera.

The methods of Morgan, Mackinnon, Gill, Gower & Norris (1978), as modified by Herr, Bishop, Bolton & Van der Merwe (1979), were followed for the SAT, the essential difference being 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 $\frac{1}{500}$. A four-tube test was used giving final dilutions of $\frac{1}{10}$, $\frac{1}{20}$, $\frac{1}{40}$ and $\frac{1}{80}$ and a maximum reading of 212 IU/ml, using locally prepared antigen⁽¹⁰⁾.

The methods employed in the CFT were those of Morgan *et al.* (1978) and Alton (1977), as modified by Herr *et al.* (1979). The preparation of a 3% red blood cell suspension (RBC) was done spectrophotometrically, as described, and the 50% end-point spectrophotometric complement titration was used. Until August 1980 the

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⁽³⁾ Eppendorf pipette 4700, Eppendorf Gerätebau, P.O. Box 630324, 2000 Hamburg, West Germany

⁽⁴⁾ Cooke Engineering Company, 900 Slaters Lane, Alexandria, Virginia 22314 USA

⁽⁵⁾ Heidolph Type 54131 REAX 3, Heidolph-Elektro K.G., D-8420 Kelheim, West Germany

⁽⁶⁾ EAC Graphics, 180 Schoeman St., Pretoria 0002

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⁽⁷⁾ Rose Bengal Plate Test Antigen, Central Veterinary Laboratory, Weybridge, Surrey, KT15 2NB, England

⁽⁸⁾ Allerton, R. V. L., Private Bag X9005, Pietermaritzburg 3200

⁽⁹⁾ Universalindikator pH 0-14, Merck Chemicals (Pty) Ltd, P.O. Box 1366, Johannesburg 2000

“Dynatech”⁽⁴⁾ system was used for serial dilutions and dispensing, after which the “Compu-Pet”⁽¹¹⁾ system was brought into use for both operations. The results were recorded in International Units/ml with 1 000 IU/ml giving an end-point reading of 50% haemolysis at a serum dilution of 1/220. Twofold serial dilutions were used in U-bottomed microtitration plates with the 1/2 dilution being used as an anti-complementary control and the serum dilutions extending to 1/128. This gave a maximum reading of 784 IU/ml, when locally prepared antigen⁽¹²⁾ was used.

With the CFT as the definitive test, the following criteria were accepted when the status of the animal under test was judged. In cattle that had never been vaccinated 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.

RESULTS

The phenomenon did not occur in the 1 038 sera that were tested within 2 days of collection. In the 3 807 and 9 985 sera delayed in transit for more than 48 h the overall incidence was 0,37 and 0,55%, but the distribution was not even and occurred at a much higher incidence (26–37%), and then only in a few batches.

When repeat collections of sera were examined, the phenomenon persisted for up to 32 days, when routine collection and submission were practised, but it disappeared at 35 days when the sera were submitted within 2 days (Table 1). Sera from the same animal did not always show the same reaction in repeat collections.

When RBT titrations, which included a repeat single serum volume, a double serum volume and 4 and 8 min tests, were done (Table 2), only one serum came up positive in the repeat single serum volume test. In 8 cases the RBT was positive after 4 min when double serum volumes were used, and in one case only after 8 min. After 4 min, 28% of the cases were positive in higher dilutions and a further 19% after 8 min. Eight sera were positive in single serum volumes after 8 min.

TABLE 1 The persistence of RBT negative, high titre SAT and CFT sera in certain batches delayed in transit and the disappearance of the phenomenon when tested within 2 days

Batch	No. in batch	Percentage showing phenomenon			
		1st Collection	2nd Collection		3rd Collection
		Delayed 2–5 days	Delayed 2–5 days	Delayed 4–7 days	Delayed less than 2 days
1	25	32%	28%	36%	0%
2	18	33%	33%	ND	ND
3	51	12%	12%	ND	0%

ND = Not done

TABLE 2 The incidence and patterns of prozone reactions in RBT titrations on RBT negative, high titre SAT and CFT sera compared with normally reacting sera

Sera showing this pattern (%)	Original test			Repeat tests																		
	RBT	SAT IU/ml	CFT IU/ml	4 min						8 min												
				Single volume serum	Double volume serum	Dilutions				Single volume serum	Double volume serum	Dilutions										
						1/2	1/4	1/8	1/16			1/32	1/64	1/2	1/4	1/8	1/16	1/32	1/64			
15	-	212	784	-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	212	784	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	212	784	-	-	+++	+++	+++	+++	++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++
19	-	212	784	-	-	-	-	-	-	-	-	-	-	-	+	++	++	++	++	-	-	-
6	-	212	784	-	+++	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-
13	-	212	196	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	-	27–93	30–145	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	212	784	+	-	++	++	+	-	-	-	++	-	+++	+++	+	-	-	-	-	-	-
Total 100%																						
Normally reacting sera	++	212	784	++	++	+++	+++	+++	++	+	-	++	++	+++	+++	+++	+++	+++	+++	++	+	+
	+	27–93	30–145	+	++	-	-	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	++	80	240	++	-	+++	+	-	-	-	-	+++	-	+++	+	-	-	-	-	-	-	-

- = negative
- + = discernible rimming (agglutination seen around the edges of the fluid only)
- ++ = rimming and just discernible agglutination in the central fluid
- +++ = obvious agglutination in the central fluid
- ++++ = complete agglutination in the central fluid with clearing of the supernatant

⁽¹⁰⁾ *Brucella abortus* antigen SAT, Veterinary Research Institute, Onderstepoort 0110
⁽¹¹⁾ General Diagnostics, Eastleigh, Hants., SO5 3ZQ, England
⁽¹²⁾ *Brucella abortus* complement fixation antigen, Veterinary Research Institute, Onderstepoort 0110

Whenever these titrations were repeated with the use of other RBT antigens, the results remained the same. A single serum was taken to another laboratory (Allerton) and there showed the same reaction; it became positive only in the $\frac{1}{2}$ - $\frac{1}{2}$ dilutions after 4 min and more so after 8 min. When the time for the tests was extended to 12 min, no change in the results was seen. After 16 min the test became impossible to interpret because of drying out around the edges. No pH changes could be detected in the sera that were involved. Although the phenomenon has been seen at other times, the peak incidence was between September and February, the hottest months. Using other methods of testing, e.g. glass plates and white opaque plastic, did not change the results. True negative sera, positive control sera and normally reacting positive sera did not exhibit any of these phenomena (Table 2).

DISCUSSION

The fact that the phenomenon was (a) not seen in sera tested within 2 days of collection, (b) occurred only in sera that took longer than 2 days in transit, (c) disappeared when these sera were submitted within 2 days, (d) did not always occur in the same sera but (e) was definitely batch-related, is interpreted as an indication that some unknown factors active during transport caused the occurrence. This is supported by the higher incidence of the phenomenon during the hotter months.

Although Jones (1977) reported an increased sensitivity in the RBT when double serum volumes were used—and this was seen in 9 cases in this series—it was by no means a general finding. On the contrary, a number of sera were positive in single volume and negative in double volume. This may be due to differences in the sensitivity of the antigen used.

The inclusion of the repeat single volume serum test excludes the possibility of human error as the fact that only 1 serum came up positive in this repetition of the routine test proves. Even this is not necessarily due to human error, as some sera have tested positive only once out of 4 or 5 times.

It is noteworthy that sera showing the prozone are all high titre sera and that the same result is absent from low titre sera. By no means do all high titre sera exhibit this phenomenon, however. A postulate which would fit all

these findings is as follows: in transit at temperatures around 40 °C but probably not exceeding 56 °C [at the latter temperature the SAT titre would be affected (Morse, Schneider & McNutt, 1955; Amerault, Manthei, Goode & Lambert, 1961)], together possibly with the effect of agitation and the action of enzymes produced by specific contaminants present, a denaturation of the amino acid sequence constituting the antigen binding sites on the antibody occurs and may affect one or more of these sites and some or all of the antibody molecules. This denaturation is such that at pH 3.6 (RBT) it delays or effectively blocks antigen/antibody binding but has no effect at pH 7 (SAT).

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