The Use of the Thionin Blue Sensitivity Test in the Examination of *Brucella*

By W. J. B. MORGAN

Central Veterinary Laboratory, Weybridge, Surrey

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SUMMARY

A total of 171 cultures of *Brucella*, mostly isolated in this country from milk and foetal material, was examined for sensitivity to thionin blue as well as to basic fuchsin and thionin, and for agglutination in monospecific sera. Standard strains of *Brucella abortus*, *B. melitensis* and *B. suis* grew in the presence of thionin blue in the concentration studied. Three groups of *B. abortus* were found, however, which were sensitive to thionin blue. These were: (a) strains resembling the vaccine strain *B. abortus* strain 19; (b) dye sensitive strains (*B. abortus* type II Wilson), (c) a previously undescribed group. Cultures of this last group required added CO₂ for growth, grew on basic fuchsin and were virulent for guinea-pigs. The significance of these results is briefly discussed.

INTRODUCTION

McLeod (1944) reported that *Brucella abortus* strain 19 would not grow on media containing thionin blue in concentrations that would allow the growth of other strains of *B. abortus*. This observation was confirmed by Levine & Wilson (1949) and Cruickshank (1954). The latter author also pointed out that strains of *B. abortus* type II Wilson (Huddleson, 1955) did not grow on thionin blue media. These strains are known to be very sensitive to dyes and require the presence of serum or Tween 40 for growth (Huddleson, 1956; Sackman, 1957; Morgan, 1960). The purpose of the present paper is to record the results of the examination of *Brucella* cultures for sensitivity to thionin blue.

METHODS

Organisms. Most of the organisms studied were isolated at this Laboratory from foetal material or milk; the remainder had been sent in for confirmation of identity. Except for recently isolated cultures, they were all checked for dissociation by using obliquely transmitted light (Henry, 1933) and only smooth cultures were studied.

Media. Serum glucose agar was used for the propagation of cultures and as basal medium for the addition of dyes. The composition of this medium has been described (Morgan, 1960).

Basic fuchsin (supplied by Pharmaceutical Laboratories National Aniline Division, Allied Chemical and Dye Corporation, New York) was added to the melted and cooled medium just before pouring plate^o to give a concentration of 1/25,000.

Thionin (Allied Chemical and Dye Corp.) was used at a concentration of 1/50,000. Initially, three concentrations of thionin blue (British Drug Houses Ltd., London) were used, namely, 1/500,000, 1/1,000,000, 1/2,000,000. Later however only the 1/500,000 concentration was used for routine use. Stock solutions of the dyes were made and steam sterilized. All new batches were checked for activity against known strains. All plates were incubated overnight at 37° before use and no plate was used which had been stored for longer than one week in the refrigerator. The three F.A.O./W.H.O. reference strains *Brucella abortus* strain 544, *B. melitensis* strain 16M and *B. suis* strain 1330, as well as *B. abortus* strain 19 were always included in each series of tests.

All cultures were tested for the requirement of additional CO₂ (above that present in air) for growth, as soon as possible after isolation. Two slopes were inoculated with each isolate; one was incubated aerobically at 37° and the other in an atmosphere containing 10 % (v/v) added CO₂. Slopes were examined after 4 days of incubation.

For the dye sensitivity tests, cultures were incubated for 48 hr. in the CO₂ enriched atmosphere and the growth suspended in buffered saline (pH 6.8) to give a concentration of approximately 3×10^9 organisms/ml. One loopful of the suspension was streaked five times on each of the dye plates, without recharging the loop, using a quarter plate per suspension. The loop was sterilized between each plate. All the plates were incubated in the CO₂ enriched atmosphere and read after 5 days of incubation at 37°. The results for each dye were interpreted as negative or 1+ to 5+ depending on whether growth had occurred on 1 or all 5 streaks.

Serological test. Monospecific sera for *Brucella abortus* and *B. melitensis* were prepared from rabbit sera by absorption with the heterologous antigen. One loopful of the serum, diluted 1/5 in phenol saline, was placed on a slide and the culture emulsified. The suspension had not to be too concentrated, otherwise agglutination was delayed. Occasionally the results of slide agglutination tests were confirmed by the tube agglutination test with monospecific sera. Suspensions of the standard strains of *B. abortus* and *B. melitensis* were always included as controls.

RESULTS

The pattern of results obtained with the three F.A.O./W.H.O. reference strains of *Brucella*, together with *Brucella abortus* strain 19 (the vaccine strain), is shown in Table 1. *Brucella abortus* strain 19 did not grow at concentrations of 1/500,000 and 1/1,000,000 of thionin blue; the reference strains *B. abortus*, *B. melitensis* and *B. suis* did grow at these concentrations. At the 1/2,000,000 concentration of thionin blue, *B. abortus* strain 19 did grow, but in all cases gave only 2+ to 3+ values. *Brucella melitensis* and *B. suis* also showed less growth at the 1/500,000 concentration of thionin blue. A number of recently isolated *Brucella* cultures was examined, using the three concentrations of thionin blue; a concentration of 1/500,000 was eventually chosen for routine use, since this gave clear-cut results.

In Table 2 are given the results of the examination of 171 cultures of *Brucella*, together with their source, and it is apparent that there were three groups of *Brucella abortus* which did not grow on thionin blue at concentrations which allowed the growth of the majority of the strains. Of these, the largest group (15.2 % of

Table 1. *The results of examining standard strains of Brucella*

Strain	Growth on serum agar plus					Agglutination in monospecific abortus/ melitensis sera	
	CO ₂ require- ment	Basic fuchsin 1/25,000	Thionin 1/50,000	Thionin blue			
				1/ 500,000	1/ 1,000,000		1/ 2,000,000
<i>B. abortus</i> strain 544 (reference strain)	+	5+	—	5+	5+	+	
<i>B. abortus</i> strain 19 (vaccine strain)	—	5+	—	—	2+	+	
<i>B. melitensis</i> 16M (reference strain)	—	5+	5+	3+	5+	—	
<i>B. suis</i> 1330 (reference strain)	—	—	5+	2+	5+	+	

Table 2. *The results of examining 171 Brucella isolates*

Organism	CO ₂ requirement	Growth on serum glucose agar plus			Slide agglutination with monospecific abortus/melitensis sera	No. of strains tested	Source distribution of strains
		Basic fuchsin (1/25,000)	Thionin (1/50,000)	Thionin blue (1/500,000)			
Typical <i>B. abortus</i>	+	5+	—	5+	+	118	51 foetal, 67 milk
Aerobic <i>B. abortus</i>	—	5+	—	5+	+	2	2 milk
<i>B. abortus</i> type II (Wilson)	+	—	—	—	+	26	19 milk, 7 foetal
<i>B. abortus</i> Vaccine strain 19	—	5+	—	—	+	8	3 foetal, 5 milk
<i>B. abortus</i> Thionine blue sensitive	+	5+	—	—	+	7	5 foetal, 2 milk
<i>B. abortus/melitensis</i>	+	5+	—	5+	—	2	2 foetal
<i>B. abortus</i> thionin-resistant	—	5+	5+	5+	+	1	1 foetal (Holland)
<i>B. melitensis</i>	—	5+	5+	3+	—	7	2 foetal, 5 milk

all cultures studied) belonged to the type II (Wilson). Such strains are known to be sensitive to most dyes. A second group, comprising 4.7 % of all strains examined, behaved biochemically exactly like the vaccine strain 19 *Brucella abortus*; these were recovered both from foetal material and from milk.

The third group (4.1 % of total isolates examined) consisted of strains that did not grow in presence of thionin blue but differed from *Brucella abortus* strain 19 in requiring additional CO₂ for growth; but they did grow in presence of basic fuchsin, thus differing from *B. abortus* type II (Wilson). Such strains were also recovered from milk and foetal material. Most of the strains (69 %) were typical of *B. abortus* in all their reactions, but two aerobic strains were also recovered. The other cultures consisted of *B. melitensis* (4.1 %) and *B. abortus/melitensis* (2.1 %). One strain of thionin-resistant *B. abortus* was received (from Holland).

Cultures obtained from guinea-pigs and the milk of cows experimentally infected with *Brucella abortus* strain 19, and with the thionin blue sensitive strains of *B. abortus* in all cases behaved exactly like the strains used for inoculation, thus confirming their stability *in vivo*.

At the 1/500,000 concentration of thionin blue, a few colonies were observed with strain 19 and with some of the CO₂ requiring thionin blue sensitive strains. After subculture on serum agar these resistant colonies gave profuse growth (5+ readings), indistinguishable from those of the standard *Brucella abortus* 544 on thionin blue at a dilution of 1/500,000.

DISCUSSION

Widespread vaccination of cattle with *Brucella abortus* strain 19 is carried out in this and other countries, and many workers have drawn attention to the need for a simple and reliable method for the differentiation of the vaccine strain from other strains of *B. abortus*. However, no single character can be singled out as characteristic of the vaccine strain only, and it is obvious that the thionin blue sensitivity test is not pathognomonic for strain 19. In addition to *B. abortus* strain 19 and *B. abortus* type II (Wilson), a third group has now been found, also sensitive to thionin blue. Cultures of this group differ from strain 19 in that they need added CO₂ for growth. Preliminary experiments at this laboratory on the virulence of cultures of the thionin blue sensitive group of *B. abortus* have shown that these are as virulent as the standard 544 strain. Virulence tests were based on the duration of bacteraemia in guinea-pigs (Cruickshank, 1957).

Since *Brucella abortus* strain 19 does not appear in the milk of cows after subcutaneous inoculation (Taylor & McDiarmid, 1949) the origin of strains recovered both from milk and foetuses, and indistinguishable from strain 19, is a matter for conjecture. Some of these strains, together with strain 19, have been examined for virulence in guinea-pigs, and all have been of equally low virulence. The only conclusion appears to be that either strain 19 can appear in the milk and foetal material in some cases, or that strains occur in the field that are indistinguishable from strain 19, by our present methods.

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