J. Hyg., Camb. (1967), **65**, 165
Printed in Great Britain

The sensitivity of the biotypes of *Brucella abortus* to three antibiotics used in selective media, and the description of a new biotype

By I. D. FARRELL and L. ROBERTSON
Public Health Laboratory, Preston

(*Received* 7 *December* 1966)

Brucella abortus can be isolated on selective agars from samples of untreated cows' milk and from other contaminated sources. The selective agars in current use are modifications of the one described by Kuzdas & Morse (1953). This medium contains a combination of three antibacterial antibiotics—polymyxin B sulphate, circulin, and bacitracin; an anti-fungal antibiotic—actidione; and an aniline dye—ethyl violet, to inhibit the growth of contaminant organisms.

If the composition of the selective agars used for the isolation of *B. abortus* from 1953 onwards is studied, it will be noted that two recent additions to the collection of antibacterial and anti-fungal antibiotics incorporated in the agars are nitrofurfuryl methyl ether (Morris, 1956) and amphotericin B (Report, 1964*a*). Otherwise it appears that each successive medium has been elaborated from that described by Kuzdas & Morse and neither in the original nor in any subsequent publications has experimental evidence been given to show that the concentrations of antibiotics used were in fact the most effective for the purpose. Therefore it was decided to investigate the two selective agars used in this laboratory, viz. Mair's medium (1955) and a modification of Morris's medium (1956), in order to determine whether they contained the optimal concentrations of antibiotics necessary for the isolation of *B. abortus* from contaminated material.

During the course of the investigation, which is not yet complete, it has become apparent that there were certain similarities between the antibiotic-sensitive strains and the dye-sensitive strains of *B. abortus*.

The object of this communication is to report the sensitivity of biotypes of *B. abortus* to the three antibiotics, polymyxin B sulphate, bacitracin, and amphotericin B; and to propose that the dye-sensitive variant of biotype 4 should be considered as a new biotype.

MATERIAL AND METHODS

The following 219 strains of *Brucella* were examined during the course of the investigation:—

(*a*) Ten strains of the prototypes belonging to the WHO collection, viz. *B. abortus*, biotypes 1, 2, 3, 4, 5, 6, 7, 9; (biotype 8 was not available); *B. melitensis*, biotype 1, and *B. suis*, biotype 1.

(*b*) Two hundred and nine 'wild' strains isolated from cattle in Lancashire.

All strains were maintained on serum-dextrose agar slopes and kept at a tempera-

ture of 4° C. When required for investigations they were subcultured on 5 % blood agar and incubated in air containing 20 % carbon dioxide for 3 days at 37° C.

Typing

The biotype of each strain of *Brucella abortus* was determined on the basis of the following criteria (Wilson, 1933; Cruickshank, 1954; Report, 1964*b*):

(1) Carbon dioxide dependence of the primary isolate.

(2) Production of hydrogen sulphide.

(3) The sensitivity of the organism to the two aniline dyes—basic fuchsin and thionin at the concentrations recommended by the Expert Committee on Brucellosis.

(4) The ability of a suspension of the organism to agglutinate with *Brucella abortus* and *Brucella melitensis* monospecific sera.

(5) The sensitivity of *Brucella abortus* bacteriophage.

Antibiotics

Polymyxin B sulphate was supplied in glass vials containing 500,000 international units (i.u.) as a dry sterile powder (Burroughs Wellcome). A stock solution containing 10,000 i.u./ml. was prepared in sterile distilled water.

Amphotericin B (Fungizone) was supplied in glass vials containing 50 mg. of sterile dry powder (E. R. Squibb and Sons). A stock solution of 5000 μg./ml. was prepared in sterile distilled water.

Bacitracin was supplied as an unsterile dry powder (Calmic Ltd.). A stock solution of 8,000 i.u./ml. was prepared and sterilized by Seitz filtration.

Fresh solutions of antibiotics were prepared for each batch of ditch plates.

Technique for determining the antibiotic sensitivity of the various strains

An agar diffusion (ditch plate) technique was employed to determine the sensitivity of each strain of *B. abortus* to the antibiotics polymyxin B sulphate, amphotericin B, and bacitracin.

All glassware was washed and sterilized by autoclaving at 15 pounds per square inch for 15 min. Plastic Petri dishes sterilized by gamma-radiation were used throughout the experiments; the bottoms of these dishes have a flat surface thus giving an agar plate of uniform depth.

Serum-dextrose agar was poured into 70 mm. diameter Petri dishes to a depth of about 6 mm. A strip of agar, 15 mm. wide, was removed from each plate. The central ditch thus formed was filled with serum-dextrose agar containing an appropriate amount of antibiotic solution. The range of concentrations of antibiotics against which each strain was titrated was selected so as to give a range of doubling dilutions which started at the concentration of the particular antibiotic used in the selective agars. In the case of polymyxin B sulphate seven dilutions were used, ranging from 5 to 320 i.u./ml.; for amphotericin B six dilutions were used, ranging from 10 to 320 μg/ml.; for bacitracin seven dilutions were used, ranging from 25 to 1600 i.u./ml

Diffusion of the antibiotic from the central ditch was allowed to take place

overnight at a temperature of 4° C., after which time an equilibrium state was assumed to have been reached (Ericsson, 1960). Using smooth colonies, a suspension of the organism was prepared in sterile physiological saline to give a density comparable with a Brown's opacity tube no. 2. A standard loopful of a suspension of each strain was streaked across the ditch plate at right-angles. Eight strains were tested on each ditch plate.

The performance of each plate was controlled by inoculating suspensions of the WHO prototype strains of *B. abortus*, biotype 1, and *B. abortus*, biotype 2, prepared in the same way, across each plate.

Bacitracin and amphotericin B diffuse into the medium and in higher concentrations inhibit growth some distance from the ditch. Polymyxin B sulphate, on the other hand, diffuses hardly at all even though time to reach an equilibrium state has been allowed and the only possible endpoint reading for this antibiotic is inhibition of growth over the ditch. So that the endpoint readings for the three antibiotics would be uniform, the endpoint of each titration was expressed as the lowest concentration which completely inhibited growth of the organism over the area of the ditch itself.

Each suspension was also inoculated on serum-dextrose agar to check its purity and viability. The plates were incubated in air containing 20 % carbon dioxide at a temperature of 37° C. for 3 days.

RESULTS

Table 1 shows the endpoint titrations for the eight WHO biotypes of *B. abortus*, *B. melitensis*, biotype 1, and *B. suis*, biotype 1, to the antibiotics polymyxin B sulphate, amphotericin B, and bacitracin. This shows that the WHO prototype of *B. abortus*, biotype 2, differs from all the other biotypes and from *B. melitensis* and *B. suis* in being much more sensitive to the three antibiotics polymyxin B sulphate, amphotericin B and bacitracin.

Table 1. *The sensitivity of WHO prototype strains to polymyxin B sulphate, amphotericin B and bacitracin*

Organism	Biotype	Polymyxin (i.u./ml.)	Amphotericin (μg./ml.)	Bacitracin (i.u./ml.)
Brucella abortus	1	160	⩾ 320	1600
	2	80	40	100
	3	320	⩾ 320	800
	4	320	⩾ 320	800
	5	320	⩾ 320	800
	6	320	⩾ 320	800
	7	320	⩾ 320	800
	9	160	⩾ 320	800
B. melitensis	1	⩾ 320	⩾ 320	800
B. suis	1	⩾ 320	⩾ 320	800

Table 2 is a synoptic presentation of the endpoint titrations obtained for 209 'wild' strains of the biotypes isolated from cattle in the north of Lancashire.

With the exception of strains of biotype 2 and the dye-sensitive variant of biotype 4 the strains of other biotypes were sensitive to 160 or 320 i.u./ml. poly-

Table 2. *The sensitivity of* 209 *wild strains of* Brucella abortus *to polymyxin B sulphate, amphotericin B, and bacitracin (number of strains sensitive to the different concentrations)*

	Polymyxin (i.u./ml.)							Amphotericin B (μg./ml.)							Bacitracin (i.u./ml.)						
Biotype	5	10	20	40	80	160	320	10	20	40	80	160	320	> 320	25	50	100	200	400	800	1600
Brucella abortus biotype 1, 24 strains	0	0	0	0	0	13	11	0	0	0	0	0	0	24	0	0	0	0	0	12	12
B. abortus biotype 2, 126 strains	0	0	0	2	100	24	0	1	3	92	19	1	0	10	1	6	89	28	2	0	0
B. abortus biotype 4, 11 strains	0	0	0	0	0	6	5	0	0	0	0	0	0	11	0	0	0	0	1	5	5
B. abortus, biotype dye sens. 4, 8 strains	0	0	0	0	2	6	0	0	0	8	0	0	0	0	0	0	2	6	0	0	0
B. abortus, biotype 5, 20 strains	0	0	0	0	0	19	1	0	0	0	0	0	0	20	0	0	0	0	0	20	0
B. abortus, biotype 9, 20 strains	0	0	0	0	0	20	0	0	0	0	0	0	0	20	0	0	0	0	0	20	0

320 μg./ml. is the limit of the solubility of amphotericin B in the medium.

Table 3. *The biochemical and serological classification of the biotypes of* Brucella abortus *isolated from cattle in Lancashire and including* Brucella melitensis, *biotype* 1 *and* Brucella suis, *biotype* 1, *for comparison*

Species	Biotype	Phage Tb at R.T.D.	CO_2 requirement	H_2S production	Growth on dyes: Thionin	Growth on dyes: Basic fuchsin	Agglutination with monospecific sera: Abortus	Agglutination with monospecific sera: Melitensis	Remarks
Brucella abortus	1	+	+	+	−	+	+	−	Typical abortus
	2	+	+	+	−	−	+	−	Dye sensitive
	4(*a*)	+	+	+	−	+	−	+	Abortus biochem. and melitensis serological
	4(*b*)	+	+	+	−	−	−	+	As 4(*a*) but dye sensitive
	5	+	−	−	+	+	−	+	British melitensis
	9	+	−	+	+	+	−	+	Similar to British melitensis but produces H_2S
B. melitensis	1	−	−	−	+	+	−	+	
B. suis	1	−	−	+	+	−	+	−	

myxin B sulphate, were resistant to 320 μg./ml. of amphotericin B, and all but one of the strains were sensitive to 800 or 1600 i.u./ml. of bacitracin. The greater antibiotic sensitivity of biotype 2 is also revealed in Table 2. Out of 126 strains of biotype 2, 102 were more sensitive to polymyxin B sulphate, 116 were more sensitive to amphotericin B, and all of the 126 strains were more sensitive to bacitracin than strains of the other biotypes with one exception, viz. a variant of *B. abortus*, biotype 4, which is also dye sensitive (Table 3), which was found to be a little more sensitive to polymyxin B sulphate and much more sensitive to amphotericin B and bacitracin.

DISCUSSION

The genus *Brucella* is divided into three species: *B. abortus*, *B. melitensis* and *B. suis*. The system of classification depends upon the results of oxidative metabolic tests with a series of amino acids and the sensitivity of the organisms to the *B. abortus* bacteriophage.

There are nine biotypes of *B. abortus* and their classification is dependent upon the quantitative rather than the qualitative interpretation of the results of the following tests: CO_2 dependence of the primary isolate; production of H_2S; sensitivity to the two aniline dyes, basic fuchsin and thionin; agglutination reaction with monospecific abortus sera and monospecific melitensis sera (Report, 1964*b*).

The WHO method of classification of the biotypes present in the cattle population of the north of Lancashire is shown in Table 3, which also includes *B. melitensis* biotype 1, and *B, suis*, biotype 1, for comparison.

It is apparent from Table 3 that the dye-sensitive strains belong to biotype 2 and include the dye-sensitive variants of biotype 4, and that they are identified from one another by their reaction with monospecific abortus and monospecific melitensis sera.

It is evident from the results shown in Table 2 that biotypes of *B. abortus* can be divided into two classes, those that are sensitive to bacitracin and amphotericin B and those which are comparatively resistant to bacitracin and amphotericin B.

The majority of strains of biotype 2 and the dye-sensitive variants of biotype 4 were particularly sensitive to amphotericin B and bacitracin. All but two strains (132/134) were sensitive to a concentration of 200 i.u./ml. of bacitracin, and 123/134 strains were sensitive to 80 μg./ml. of amphotericin B. However, strains of the remaining biotypes were noticeably more resistant to both of these antibiotics (Table 1 and Table 2).

Ten strains of biotype 2 gave an antibiotic sensitivity pattern not seen in the other 116 strains of biotype 2—they were sensitive to concentrations of 200 i.u./ml. of bacitracin but were resistant to amphotericin B in concentrations as high as 320 μg./ml. These results would seem to form the basis of a subdivision of biotype 2, which could be of epidemiological importance, and, in fact, even when there are minor variations in antibiotic sensitivity pattern in strains of biotype 2 from different cows, we have always found that successive strains from any one individual cow have given a constant sensitivity pattern.

The amphotericin B resistant strains of biotype 2 may be mutants selected

through laboratory adaptation, but this seems unlikely as amphotericin B is not employed in any of the media used in this laboratory for the isolation of *B. abortus*, and also it is most unlikely that the animals would have come into contact with this antibiotic in nature. It may be that these variants of biotype 2 have arisen purely from natural mutation of the genes controlling the characteristics of the organism. It is of interest at this point to mention that Cruickshank (1954) described the subdivision of biotype 2 on the basis of pyronin sensitivity. It is hoped to obtain a larger collection of these variants and to compare their antibiotic and dye sensitivities.

The WHO prototype of biotype 4 and the 'wild' strains tested were sensitive to concentrations of 1600 i.u./ml. of bacitracin and were resistant to concentrations of amphotericin B as high as 320 μg./ml. On the other hand, the dye-sensitive variants of biotype 4 were sensitive to concentrations of 200 i.u./ml. of bacitracin and 40 μg./ml. of amphotericin B; they are as sensitive to these antibiotics as most strains of biotype 2. These dye-sensitive variants of biotype 4 are unusual in that their antibiotic sensitivity is completely different from that of the WHO prototype of biotype 4.

In this laboratory selection of resistant mutants from agars containing the two aniline dyes, basic fuchsin and thionin, and investigation of any change in the antibiotic sensitivity of the mutant, has shown that the dye sensitivity of a brucella strain can be altered without changing its antibiotic sensitivity. These results would seem to indicate that antibiotic sensitivity and the dye sensitivity of brucella strains are not genetically related properties.

The only difference between biotype 1 and biotype 2 is that biotype 2 is dye-sensitive, and this one property has been sufficient for them to be recognized as two distinct biotypes.

The dye-sensitive variants of biotype 4 are different from the WHO prototype of biotype 4 in both their antibiotic sensitivity and dye sensitivity—two genetically unrelated characteristics. In the light of this evidence it is suggested that dye-sensitive variants of biotype 4 should be reclassified. These variants seem more closely related to biotype 2 than to biotype 4; but as the classification of *B. abortus* into its nine biotypes is more a matter of degree than of positive and negative characteristics it is further suggested that this variant be raised to the status of a biotype and should be called biotype 10.

SUMMARY

An investigation into the antibiotic sensitivity of *B. abortus* biotypes to polymyxin B sulphate, amphotericin B, and bacitracin has shown that the dye-sensitive strains are also antibiotic-sensitive; and preliminary experiments suggest that these two properties are not genetically related.

The results suggest that biotype 2 can be subdivided on the basis of antibiotic sensitivity pattern, and that this could be of epidemiological significance.

Investigation into the antibiotic sensitivity pattern of the dye-sensitive variant *B. abortus*, biotype 4, leads us to believe that this strain should be reclassified as a separate biotype and provisionally called biotype 10.

We wish to thank the Medical Officers of Health and their Public Health Inspectors for their co-operation in obtaining the samples of milk from which the Brucella organisms were isolated. We should like to record our appreciation of the technical assistance of Mr F. D. Hargreaves.

REFERENCES

CRUICKSHANK, J. C. (1954). Observations on brucella species based on the examination of 800 strains. *J. Hyg., Camb.* **52**, 105.

ERICSSON, H. (1960). Laboratory Methods: determination of bacterial sensitivity. *Scand. J. clin. Lab. Invest.* (*Suppl.*) **60**, 9.

KUZDAS, C. D. & MORSE, E. V. (1953). A selective medium for the isolation of brucellae from contaminated materials. *J. Bact.* **66**, 502.

MAIR, N. S. (1955). A selective medium for the isolation of *Brucella abortus* from herd samples of milk. *Mon. Bull. Minist. Hlth* **14**, 184.

MORRIS, E. J. (1956). A selective medium for *Brucella* spp. *J. gen. Microbiol.* **15**, 629.

REPORT (1964*a*). Brucellosis in the British Dairy Herd. *Animal Disease Survey* no. 4, H.M.S.O.

REPORT (1964*b*). Joint FAO/WHO Expert Committee on Brucellosis. *Tech. Rep. Ser. Wld Hlth Org.* no. 289, Annex 2, p. 50.

WILSON, G. S. (1933). The classification of the brucella group: a systematic study. *J. Hyg., Camb.* **33**, 516.