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Fluid and solid media for isolation of *Brucella abortus*

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SUMMARY

The use of a fluid-enrichment technique increased by 10–16% the recovery rate of *Brucella abortus* from milks of herds positive to a milk ring test. Three solid media for direct cultural techniques were also tested on the same milks. Combinations of these techniques produced a recovery rate of *ca.* 88% from MRT-positive-herd milks using a mixture of the centrifuged deposit and cream layer as the inoculum as against a recovery rate of *ca.* 75% using the MRT cream layer as inoculum. *B. abortus* biotypes 1–5 and 9 were isolated and growth of all the biotypes was supported by all media used.

INTRODUCTION

The serum dextrose agar of Mair (1955) may be adequate for the recovery of *B. abortus* from infected tissues, products of gestation and the like from animals, especially cattle. It is not a particularly suitable medium for the isolation of *B. abortus* from milk supplies which, almost invariably, contain a wide variety of bacteria even with the best of dairy hygiene.

In this laboratory before August 1967, Mair's medium was used to recover *B. abortus* from milk samples. Between August 1967 and January 1968, a total of 1273 milk samples were screened by the milk ring test (MRT) (Report, 1956) and the 364 MRT-positive milks were cultured both on Mair's medium and the medium of Ryan (1967). Although 171 yielded *B. abortus* on Ryan's medium, not a single isolation was obtained on Mair's medium. The biotypes recovered included types 1–5 and 9 (Sinton, 1973).

Between January and June 1971, Ryan's medium was compared with the medium of Farrell (1969) as modified by Payne. Of a total of 500 MRT-positive milks, *B. abortus* was isolated from 125 on Ryan's medium and from 209 on modified Farrell's medium. These results, with the formula of the modified medium, were reported by Brodie & Sinton (1972).

The investigations now recorded were directed towards finding a fluid medium in which milk samples might be enriched and so give an even better recovery rate. At the same time, further modications were made to Farrell's medium in attempts to improve its selectivity.

MATERIALS AND METHODS

All milk samples received through Public Health Authorities for routine cleanliness checks, including pasteurized milks which failed the tests for adequate pasteurization, were examined, if MRT-positive, by the various cultural techniques for the presence of *B. abortus*.

Enrichment broth

Various broths were compounded and tested. Inhibitory agents were added to the broths and a final formula giving maximum inhibition of contaminants with maximum yield of *B. abortus* was chosen. This broth contained the same inhibitors as used in Payne's modification of Farrell's medium with minor alterations in the concentrations of the ingredients and the further addition of cycloserine. This formula achieved greater control of contaminants such as *Pseudomonas* spp., *Alkaligenes faecalis*, *Proteus* spp., *B. cereus* and *Citrobacter freundii*. This fluid medium consisted of oxoid tryptone soya broth (CM 129) with 5% sterile horse serum added. The following substances were included to give final concentrations per ml. of

Bacitracin	20 units
Polymyxin B sulphate	5 units
Nalidixic acid	5 μg.
Vancomycin	20 μg.
Nystatin	100 units
Cyclohexamide	100 μg.
Amphotericin B	4 μg.
Cycloserine	312·5 μg.

The broth was dispensed aseptically in 5 ml. lots into universal containers and stored at 4° C. for up to 4 weeks without loss in efficiency.

Solid media.

For direct plating and plating out for colonies after broth enrichment, a number of media were tested and the following chosen.

Medium A consisted of oxoid Brucella medium (CM 169) containing 5% final concentration of sterile horse serum and all the other ingredients, except the cycloserine, used in the enrichment broth.

Medium B was the same as medium A except that cycloserine was added as in the enrichment broth.

Medium C was the same as medium A but the cycloserine content was half of that in medium B.

Medium D consisted of oxoid tryptose blood agar (CM 233) containing 5% sterile horse blood.

Media A, B and C were stored at 4° C. and, immediately before use, were dried with the lids off at 37° C. for 3 hr. Experience with these media showed *B. abortus* did not grow at all well on the surface of thinly poured plates and, in consequence,

one might fail to achieve maximum isolation. A Petri dish of 9 cm. diameter required at least 30 ml. of medium to give good growth of *B. abortus*.

The blood agar plates (medium D) required only sufficient drying at 37° C. to remove excess condensation.

Inoculum

The use of gravity cream for isolation of *B. abortus* from milks was recommended by Mair (1955) whereas Ryan (1967) preferred to use the centrifuged deposit. One of the inocula used in this investigation was a compromise of both, being a mixture of the centrifuged deposit (1200 *g* for 10 min.) from 25 ml. of milk with the cream layer from the top of the same centrifuged sample (CDC). To obtain a sufficient volume for the number of inocula necessary, a channel was made through the cream layer and all but approximately 2 ml. of the skim milk was discarded. The cream layer and the deposit were thoroughly mixed in this residual 2 ml. of skim milk.

The other inocula were obtained from the cream layer of the positive MR test (RT).

Using a 30 drop/ml. pipette (calibrated with water), 5 drops of CDC or RT were used to seed each plate or each bottle of fluid medium as required. Such inocula were readily absorbed by the previously dried solid media when rubbed into the surfaces with sterile L-shaped glass spreaders.

Incubation

All inoculated plates and broth bottles with screw tops slackened were incubated at 37° C. in gas-tight containers from which the air had been evacuated and replaced with 10 % CO_2/air mixture. The optimum period of incubation was found to be 5 days, at which time the plates were removed and examined. At this time also the broths were subcultured on medium D and a further 2 days incubation at 37° C. in 10 % CO_2/air given to the subcultures before examination.

Method 1

The efficacies of broth enrichment followed by plating on medium D and direct cultures on media A and B using the CDC inoculum were tested. At the same time and with the same milk samples, the efficiencies of the broth enrichment and direct plating on medium A using RT inocula were assessed. No inoculations of RT inocula were done on medium B because of insufficiency of RT material available from each MR test performed.

The CDC inoculum was added to each of two broth bottles and to two plates each of media A and B. Only one broth and one plate of medium A were inoculated with RT.

Method 2

Method 1 was extended to include also two plates of medium C using CDC inocula.

Table 1. Brucella abortus *isolations from* 287 *MRT-positive milks: method* 1

			MRT status of samples				
			+++	++	+	±	Total
Milks examined ...			20	150	78	39	287
Milks culture-positive							
A. from CDC inocula							
	Direct plating						
Enrichment	Medium A	Medium B					
+	−	−	1	15	7	3	26
−	+	−	4	14	8	7	33
−	−	+	0	4	4	2	10
+	+	−	0	32	19	5	56
+	−	+	4	5	4	0	13
−	+	+	3	10	5	1	19
+	+	+	8	60	23	5	96
Total culture-positive			20	140	70	23	253
			(100)	(93)	(90)	(59)	(88)
B. from RT inocula							
	Direct plating						
Enrichment	Medium A						
+	−		2	24	9	2	37
−	+		6	30	24	8	68
+	+		9	71	28	5	113
Total culture-positive			17	125	61	15	218
			(85)	(83)	(78)	(38)	(76)

C. from CDC and/or RT inocula

CDC	RT	All MRT-positive milks	
+	−	37	(14·5)
+	+	216	(84·7)
−	+	2	(0·8)

Figures in parentheses are percentages.

Identification and biotyping

Following the method of Alton & Jones (1967), the biotype of each strain was determined according to (1) CO_2 dependence, (2) H_2S production on serum-dextrose agar, (3) sensitivity to basic fuchsin, thionin, methyl violet and pyronin, (4) agglutination with *B. abortus* and *B. melitensis* monospecific sera, (5) sensitivity to *B. abortus* bacteriophage, strain Tbilisi.

RESULTS

Using method 1, at least 1 of 3 broth enrichments or 1 of 5 direct culture platings might be positive either alone or in combinations. Method 2 extended method 1

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Table 2. Brucella abortus *isolations from* 147 *MRT-positive milks: method* 2

				MRT status of samples				Total
				+++	++	+	±	
Milks examined ...				5	69	48	25	147
Milks culture-positive								
A. from CDC inocula								
	Direct plating on medium							
Enrichment	A	B	C					
+	−	−	−	0	6	2	1	9
−	+	−	−	1	4	2	5	12
−	−	+	−	0	1	2	0	3
−	−	−	+	0	2	1	0	3
+	+	−	−	0	0	1	2	3
+	−	+	−	1	1	1	0	3
+	−	−	+	0	2	0	1	3
+	+	−	+	0	14	14	2	30
+	+	+	+	1	21	14	2	38
−	+	+	−	0	0	2	0	2
−	+	−	+	1	6	5	0	12
−	+	+	+	1	4	0	1	6
Total culture-positive				5	61	44	14	124
				(100)	(88)	(92)	(56)	(84)
B. from RT inocula								
	Direct plating							
Enrichment	Medium A							
+	−			1	13	5	0	19
−	+			1	17	16	4	38
+	+			2	22	20	3	47
Total culture-positive				4	52	41	7	104
				(80)	(75)	(85)	(28)	(71)

C. From CDC and/or RT inocula

CDC	RT	All MRT-positive milks	
+	−	20	(16·1)
+	+	104	(83·9)
−	+	0	—

Figures in parentheses are percentages.

by adding to it 2 further direct culture platings, giving a total of 10 procedures likely to be positive alone or in combinations. The isolations achieved have been gathered together according to the degree of MRT positivity of the milk samples and the type of inoculum used (Tables 1, 2). From these tables it is obvious that no single method gave the full recovery of *B. abortus*.

Without broth enrichments in method 1 (Table 1), 26 (10·2 %) of the isolations made using CDC inocula would have been missed. Similarly, using the RT inocula 37 (17·1 %) would have been lost. Again with method 2 (Table 2), broth enrichment

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Table 3. *Efficacy of* B. abortus *isolations by techniques in method* 1

(Total milks positive 255.)

	No.	%
(1) Using CDC inocula:		
(*a*) Milks positive if each technique had been used alone:		
Direct culture on medium A only	204	80
Direct culture on medium B only	138	54·1
After enrichment in broth with recovery on blood agar only	191	74·9
(*b*) Milks positive within combinations of techniques:		
Direct culture on media A and B	207	81·2
After enrichment in broth and direct culture on medium A	243	95·3
After enrichment in broth and direct culture on medium B	220	86·3
After enrichment in broth and direct culture on media A and B	253	99·2
(2) Using RT inocula:		
(*a*) Milks positive if each technique had been used alone:		
Direct culture on medium A only	181	71
After enrichment in broth with recovery on blood agar	150	58·8
(*b*) Milks positive within combination of techniques:		
After enrichment in broth and direct culture on medium A	218	85·5
(*c*) Milks positive using RT inocula, negative CDC inocula	2	0·8

Table 4. *Efficacy of* B. abortus *isolations by techniques in method* 2

(Total milks positive 124.)

	No.	%
(1) Using CDC inocula:		
(*a*) Milks positive if each technique had been used alone:		
Direct culture on medium A only	103	83·1
Direct culture on medium B only	52	41·9
Direct culture on medium C only	92	74·2
After enrichment in broth and recovery on blood agar only	86	69·3
(*b*) Milks positive within combinations of techniques:		
Direct culture on media A and B	109	87·9
Direct culture on media A and C	109	87·9
Direct culture on media B and C	100	80·6
Direct culture on media A, B and C	115	92·7
After enrichment in broth and medium A	118	95·2
After enrichment in broth and medium B	97	78·2
After enrichment in broth and medium C	107	86·3
After enrichment in broth and media A and B	121	97·4
After enrichment in broth and media A and C	121	97·4
After enrichment in broth and media B and C	112	90·3
After enrichment in broth and media A, B and C	124	100
(2) Using RT inocula:		
(*a*) Milks positive if each technique had been used alone:		
Direct culture on medium A only	38	30·6
After enrichment in broth and recovery on blood agar	19	15·3
(*b*) Milks positive within combination of techniques:		
After enrichment in broth and direct culture on medium A	104	83·9

Table 5. *Numbers of* Brucella abortus *biotypes isolated by methods* 1 *and* 2

Biotype	Isolations by Method 1		Method 2	
1	161	(63)	82	(66)
2	23	(9)	14	(11)
3	2	(1)	2	(2)
4	11	(4)	2	(2)
5	45	(18)	19	(15)
9	13	(5)	5	(4)
Total	255		124	

Figures in parentheses are percentages.

only made 9 (7·3 %) of the isolations using CDC inocula and 19 (18·3 %) using RT inocula. Of the solid media used for direct cultures, medium A was superior to either medium B or C.

Tables 3 and 4 set forth the successes of the procedures if these had been used alone and also the successes within the various combinations of the techniques. The results again bear out the superiority of using direct culture on medium A along with broth enrichment over the use of medium B (Table 3) or medium C (Table 4) with broth enrichment.

Medium B was the same as medium A except that it contained the full amount of cycloserine as present in the enrichment broth. Instead of increasing the isolation rate of *B. abortus*, this medium proved to be unexpectedly inhibitory. Medium C with half the cycloserine content was superior to medium B. If medium C had been the only medium used 74·2 % of the total *B. abortus* isolations would have been made as against 41·9 % with medium B (Table 4).

B. abortus biotypes 1–5 and 9 were isolated during these investigations and all of them were able to grow on all of the media used. The biotypes and the numbers of each isolated using methods 1 and 2 have been set forth in Table 5.

DISCUSSION

Huddleson (1920) described selective agar media containing bacteriostatic dyes for the isolation of *B. abortus*. Since then a number of solid media have been compounded each improving on the other by the addition of serum, dextrose, antimicrobial agents including antibiotics (Mair, 1955; Ryan, 1967; Farrell, 1969). Farrell & Robertson (1972), comparing Farrell's medium with that of Ryan, found that the former gave slightly higher isolation rates. Of 516 herd samples which were MRT-positive, *B. abortus* was isolated from 65 (12·6 %) against 59 (11·4 %) with Ryan's medium. Brodie & Sinton (1972) examined 500 herd samples which were MRT-positive using Ryan's medium and the modified Farrell's medium. Of the 500 samples, 25 % yielded *B. abortus* on the medium of Ryan whereas 41·8 % were positive on the modified Farrell's medium.

In the present investigation, 287 MRT-positive herd samples were examined by

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method 1 and *B. abortus* was recovered from 255, which was an isolation rate of 88·8 %. Of this total, 253 (88·1 %) were with CDC inocula and 218 (76·1 %) with the RT inocula (Table 1). The superiority of CDC inocula over RT inocula was again shown in method 2 where, of 147 MRT-positive milk samples, 124 (84·3%) were culture positive using the former as against only 104 (70·7 %) isolations using the latter (Table 2).

The tables show medium B to be the least efficient of the solid media used for direct culture. Medium C, while less efficient than medium A, was found to be a useful additional solid medium for isolation of *B. abortus* from grossly contaminated herd samples.

The introduction of the fluid-enrichment technique accounted for 26 (10·3 %) of the 253 isolations made using CDC inocula and for 37 (16·9 %) of the 218 isolations made using RT inocula with method 1 (Table 1). Without the broth enrichments, these isolations would not have been made.

Method 2 using CDC inocula credited 9 (7·2%) of the total 124 isolations to broth enrichment only (Table 2). This was a lower percentage than that obtained with method 1. However, if the three isolations made with both broth and medium C together were to be credited to the broth only, then these would become 12 which is 9·7 % of the 124 isolations, thus giving a good comparison with the 10·3 % obtained by broth enrichment only in method 1.

Only broth enrichment and direct plating on medium A were used with RT inocula in method 2 and here 19 (18·3 %) isolations from the total 104 were credited to the broth technique only (Table 2). This again gave a good comparison with the 16·9 % similarly obtained with method 1.

The use of the fluid-enrichment technique enhanced the isolation rate by some 10–18 % according to the type of inoculum employed. The best of the solid media was medium A which, if used alone, would have given 80 % of the total isolations made with method 1 (Table 3) and 83 % with method 2 (Table 4).

The use of CDC inocula and the technique of seeding 2 enrichment broths in addition to the direct seeding of 2 plates of medium A gave 95·3 % of the isolations with method 1 and 95·2 % with method 2. The addition of the direct seeding of two plates of medium C raised the percentage to 97·4 (Table 4). Medium B, although the least satisfactory of the solid media, did make its own small contribution. The need to inoculate more than one plate even of the same medium to obtain a higher isolation rate had already been indicated by previous investigations (Farrell & Robertson, 1972; Brodie & Sinton, 1972).

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