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AN IMPROVED MEDIUM FOR CULTURE OF MYCOBACTERIUM PARATUBERCULOSIS FROM BOVINE FAECES*

By

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JØRGENSEN, J. BERG: *An improved medium for culture of Mycobacterium paratuberculosis from bovine faeces.* Acta vet. scand. 1982, *23*, 325—335. — Löwenstein-Jensen medium with mycobactin, cycloheximide, penicillin, and chloramphenicol, and enriched with sodium pyruvate, was compared with an ordinary L.-J. medium with mycobactin. Faeces samples from cattle experimentally infected with M. paratuberculosis were cultured on both media. The improved medium gave 11 % more positive cultures and 90 % more colonies. Of the positive cultures 97 % showed detectable growth after 8 weeks of incubation, and the contamination rate was reduced to 0.4 %. By culture of faeces samples from naturally infected cattle the improved medium identified 23.2 % more infected animals than the basic medium, mostly due to a reduction of the contamination rate to about 3 %.

Mycobacterium paratuberculosis; culture; media; cattle.

The in vivo diagnosis of bovine paratuberculosis is based on bacteriological and immunological methods. Several studies indicate that immunological tests are lacking in both sensitivity and specificity (*Pearson & McClelland* 1962, *Merkal et al.* 1968, *Buergelt et al.* 1977) and that the diagnosis of paratuberculosis, especially in the non-clinical stages, must be based on culture from faeces. The problems in culturing M. paratuberculosis from faeces are the slow growth of the organism and the heavy contamination of the culture material. Sodium pyruvate has been found to stimulate the growth of tubercle bacteria, and *Merkal &*

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Curran (1974) have described its enhancing effect on the growth of M. paratuberculosis, but there have been no reports of its use on a larger scale. Contamination can be controlled by adding antibiotics and antimycotics to the media, and in the present study penicillin, chloramphenicol and cycloheximide were used, as proposed by *Stuart* (1965). Two media for primary culture of faeces were compared in an experiment with calves inoculated orally with M. paratuberculosis and using faeces samples sent in for routine examination. The sensitivity of faeces culture was evaluated. The results are presented and discussed in the following.

MATERIAL AND METHODS

Experimental animals

Sixteen 6-week-old calves were inoculated orally with M. paratuberculosis (strain 578/77 of bovine origin) on 10 consecutive days, with total doses ranging from 25—200 mg w/w.

Media

Two media were compared: 1) Löwenstein-Jensen (L-J) medium with 0.16 g mycobactin and 0.75 g cycloheximide (Sigma No. C-6255) per 1000 ml medium (*Stuart* 1965). This medium is designated M-medium. 2) M-medium with addition of 200,000 units of benzyl-penicillin-sodium (Leo Pharmaceutical Products, Denmark), 0.2 g chloramphenicol and 4 g sodium pyruvate (Merck, Art. 6619) per 1000 ml medium. This medium is designated Pc-medium.

Culture technique

Three g of faeces were decontaminated with 4 % sodium hydroxyde and 5 % oxalic acid as described by *Beerwerth* (1967). After the final centrifugation the sediment was suspended in 4 ml saline and 0.1 ml hereof inoculated onto 4 slants of each medium. The cultures were incubated at 37° C and read after 4 weeks and then weekly for a further 12 weeks. The media were evaluated on the basis of growth or no growth of M. paratuberculosis (qualitative evaluation), number of colonies (quantitative evaluation), speed of growth, and degree of contamination.

A faeces sample was taken from the rectum of each calf every fourth week. The total number of samples was 528.

The routine material consisted of 1413 faeces samples from bovines suspected of paratuberculosis. Each sample was decontaminated and inoculated onto 2 slants of each medium and read after 4, 8 and 12 weeks.

Sensitivity

A series of 10-fold dilutions of a culture suspension in saline was prepared starting with 1 mg/ml and ending with 10^{-8} mg/ml (w/w). One ml of each dilution was mixed with 1 g of heat-sterilized faeces and subjected to a decontamination treatment as previously described. In a parallel series, saline was substituted for sodium hydroxyde and oxalic acid in order to produce a reference for evaluation of the killing effect of base and acid on M. paratuberculosis.

RESULTS

Material from experimental animals

Qualitative evaluation. The material includes 463 faeces samples taken after inoculation (Table 1). Growth or no growth of M. paratuberculosis was recorded. Cultures were positive on both media in 306 (66.1 %) samples, negative in 91 (19.7 %). Fifty-eight samples (12.5 %) were positive on Pc-medium only, and 8 (1.7 %) on M-medium only.

Table 1. Comparison of results on Pc- and M-media — Qualitative evaluation.

Number of samples	Both media		Positive Pc-medium only	Positive M-medium only
	positive	negative		
463	306	91	58	8
	66.1 %	19.7 %	12.5 %	1.7 %

Quantitative evaluation. In 194 samples counts were made of viable units per g of faeces (Table 2). In 132 samples the number of colonies on both media was of an order permitting exact counting. The average number of colonies per g of faeces was 278 on Pc- and 147 on M-medium.

In 6 samples innumerable colonies grew on Pc-medium and an average of 600 colonies per g of faeces on M-medium. In 56 samples innumerable colonies grew on both media. For 138

Table 2. Comparison of results on Pc- and M-media — Quantitative evaluation.

Number of samples	Average number of colonies per g faeces	
	Pc-medium	M-medium
132	278	147
6	innumerable	600
56	innumerable	innumerable

samples it was possible to make exact counts on one or both media. In 119 samples the number of colonies was greater on Pc- than on M-medium, in 4 samples it was the same on both media, and in 15 samples it was greater on M- than on Pc-medium.

Speed of growth. The time required for appearance of clearly visible colonies was recorded in 208 samples (Table 3). The total number of positive samples was 199 on Pc- and 191 on M-medium. On Pc-medium 10.7 % of the samples were positive after 4 weeks' incubation, and after 7 and 8 weeks 95.0 % and 96.5 % were positive, respectively. On M-medium the first positive samples (1.6 %) appeared after 5 weeks' incubation, and after 7 and 8 weeks 38.7 % and 58.1 % were positive, respectively. The M-cultures required from 13 to 15 weeks for 95 % or more to become positive.

Table 3. Speed of growth on Pc- and M-media.

Incubation in weeks	Samples with visible growth			
	Pc-medium		M-medium	
	Number	%	Number	%
4	21	10.6		
5	126	63.3	3	1.6
6	163	81.9	23	12.0
7	189	95.0	74	38.7
8	192	96.5	111	58.1
9	193	97.0	137	71.7
10	195	98.0	157	82.2
11	197	99.0	168	88.0
12			173	90.6
13—15	198	99.5	188	98.4
16	199	100	191	100
	Total: 199 samples		Total: 191 samples	

Table 4. Degree of contamination on Pc- and M-media.

	Number of samples	Number of samples with uncontaminated tubes	Number of samples with 1 or more contaminated tubes			
			1	2	3	4
M-medium	528	390	73	29	10	26
	(100 %)	(73.9 %)	(13.8 %)	(5.5 %)	(1.9 %)	(4.9 %)
Pc-medium	528	520	8	0	0	0
	(100 %)	(98.5 %)	(1.5 %)	—	—	—

Total number of tubes per medium: 2112
M-tubes contaminated: 265 (12.5 %)
Pc-tubes contaminated: 8 (0.4 %)

Degree of contamination. The material includes all 528 samples (Table 4). On M-medium 390 samples (73.9 %) were without contaminated tubes while 73 (13.8 %), 29 (5.5 %), 10 (1.9 %) and 26 (4.9 %) samples showed, respectively, 1, 2, 3 and 4 contaminated tubes each. On Pc-medium 520 (98.5 %) of the samples were without contamination while 8 (1.5 %) showed 1 contaminated tube per sample. Of the total of 2112 tubes of each medium, 265 (12.5 %) M-tubes and 8 (0.4 %) Pc-tubes were contaminated.

Results of routine examinations

On M- and Pc-medium 31.9 % and 41.7 % of the samples were positive, respectively, and the contamination rates were 15.4 % and 2.6 %. The percentage of contaminated tubes was 20.7 and 3.1 on M- and Pc-medium, respectively. A total of 597 animals were positive on one or both media. Of these 589 (98.7 %) were positive on Pc-medium and 451 (75.5 %) were positive on M-medium (Table 5).

Sensitivity of culture

Viable unit counts showed that the original inoculum contained 16×10^9 viable units per mg. The effect of the decontamination procedure was evaluated on the portion of faeces to which 10^{-6} mg (16,000 viable units) M. paratuberculosis had been added per g. After the decontamination procedure 140 viable units were found per g. This means that each viable unit reco-

Table 5. Results of examination of 1413 faeces samples from bovines suspected of paratuberculosis.

Samples	Medium				No. of samples with			
	M		Pc		Pc pos.*	Pc pos.	Pc neg.*	Pc cont.*
	No.	%	No.	%	M neg.	M cont.	M pos.	M pos.
positive	451	31.9	589	41.7	23	115	7	1
negative	745	52.7	787	55.7				
contaminated	217	15.4	37	2.6				

Number of tubes per medium:	2826	
M-tubes contaminated:	586	(20.7 %)
Pc-tubes contaminated:	87	(3.1 %)

* pos. = positive, neg. = negative, cont. = contaminated.

vered corresponds to 16,000/140 = 114 viable units per g of the inoculum, which is, by definition, the sensitivity of the test. In the faeces not treated with base and acid 220 viable units were found per g, corresponding to a sensitivity of 72 viable units per g. Thus, in the decontamination procedure about 99 % of the bacteria were lost, of which 0.5 % were killed by the treatment with base and acid.

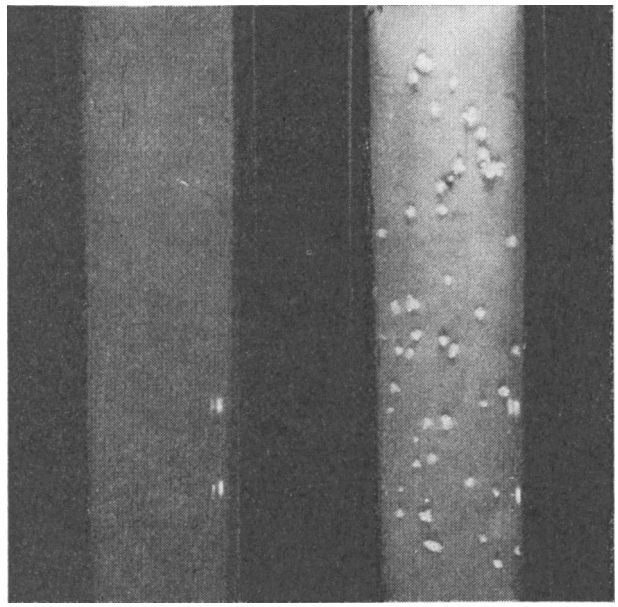

Figure 1. Colonies of M. paratuberculosis on Löwenstein-Jensen medium with (right) and without (left) sodium pyruvate. Incubation period 12 weeks.

Colony morphology

On L-J medium with sodium pyruvate M. paratuberculosis changes from dysgonic to almost eugonic growth. In the early phase (4—6 weeks), the colonies are flat and smooth, though with a slightly raised center, which later on grows to form an irregular papilla. In this phase the morphology is very similar to that of M. avium. At 5 weeks colonies on Pc-medium are about the size of colonies on M-medium at 12—16 weeks (Fig. 1).

DISCUSSION

The diagnosis of clinically manifest bovine paratuberculosis presents no great problems. Both the sensitivity and the predictive value of the complement-fixation test are ab. 90 % (*Ringdal* 1960, *Pearson* 1962, *Reinders* 1963, *Jørgensen* 1972, 1981), while the sensitivity of microscopy of faeces and rectal mucosa biopsies is about 50 % (*Jørgensen* 1980, 1981). The sensitivity of faeces culture at this stage of the disease is not known exactly, but according to unpublished investigations by the present author, which showed viable unit counts from 1.3×10^5 to 5.9×10^6 per g of microscopically positive faeces samples, it would seem to be nearly 100 %. The present investigations have shown that a positive culture can be expected if the faeces contains about 100 bacteria per g. This confirms the results of *Merkal* (1973). The diagnosis of clinical paratuberculosis can thus be based on a combination of serological and bacteriological methods.

In the sub-clinical stages of the disease the sensitivity and specificity of the immunological methods is too low. According to *Buergelt et al.* (1977) the lymphocyte-transformation test is the most sensitive of the immunological tests, but the specificity is still too low. In these stages of the disease faeces culture seems to be the only reliable diagnostic method, but that method is not widely used, because it is laborious and time-consuming, requiring incubation for usually 12 weeks or more, and because cultures are often contaminated. In the present work decontamination with oxalic acid and sodium hydroxyde (*Beerwerth* 1967) was used. This method has been widely used for decontamination of environmental specimens. In a comparative trial (author's unpublished results) Beerwerth's method proved clearly superior to decontamination with 10 % sulphuric acid for 10 min, both with respect to number of contaminated tubes and to

number of colonies. *Ringdal* (1963) found about 20 % contaminated tubes after decontamination with 10 % sulphuric acid for 15 or 30 min, but her medium (Löwenstein-Jensen medium with 5 % heatkilled M. bovis) contained no antibiotics or antimycotics. Several authors recommend oxalic acid as decontaminant for culture of M. paratuberculosis (*Taylor* 1950, *Karpinski* 1972, *Gunnarsson & Fodstad* 1979). *Merkal et al.* (1964) compared 4 decontamination procedures and 2 media, and found that the best combination was decontamination with benzalkonium chloride and culture on modified Herrold's medium.

Antibiotics-containing media for culture of tubercle bacteria have been used for a number of years (*Mitchison et al.* 1971, *Rothlauf et al.* 1981, and others; see review paper by *Songer* 1981). For culture of M. paratuberculosis addition of 50 units of penicillin and 100 µg chloramphenicol per ml medium was proposed by *Smith* (1953). *Stuart* (1965) used 100 units of penicillin and 50 µg chloramphenicol. In the present investigation 200 units of penicillin and 200 µg chloramphenicol per ml medium were used. At these levels of concentration there is less contamination than at lower levels, but there is also a slight depression of the growth of M. paratuberculosis during the first 8 weeks of incubation. After 12 weeks the number and size of colonies is the same as on medium without antibiotics. The addition of sodium pyruvate compensates for the depressing effect (*Jørgensen* unpublished results). In the present work, the addition of 0.75 mg cycloheximide was effective in preventing fungal growth. However, *Merkal & Richards* (1972) recommend the use of amphotericin B at a concentration of about 50 µg per ml medium.

The choice of medium has been the subject of several investigations. Some authors (*Smith* 1953, *Stuart* 1965, *Karpinski* 1972, *Gunnarsson & Fodstad* 1979) prefer serum-agar media, which have the advantage of being transparent and allowing the observation of very small colonies. Other authors recommend egg media (*Taylor* 1950, *Merkal et al.* 1964, *Gunnarsson* 1979) and *Taylor* (1951) recommends media with 50 % egg yolk or more. *Nemoto et al.* (1965) found a serum-agar medium and an egg-yolk medium to be of equal value. In a comparative study with different media, a modification of Stuart's serum-agar medium (1964) was found superior to other media, including egg-yolk media (*Merkal & Curran* 1974). In the present study, only Löwenstein-Jensen medium was used as basic medium.

Merkal & Curran described the enhancing effect on the growth of M. paratuberculosis of sodium pyruvate in a concentration of 4.1 g per l medium. In the present study this observation was utilized in cultural examination of a large number of faeces samples, and it was found that cultures were found positive on pyruvate medium 2 to 5 weeks earlier than on medium without pyruvate. In addition, pyruvate medium has the advantage that colonies grow very big, so that even a single colony cannot be overlooked. In routine work the described combined medium has been found to secure a very low contamination rate, and it is superior to the basic medium in identifying infected animasl.

The nosographic sensitivity of faeces culture is not known exactly, but judging by the results of *Buergelt et al.* (1977) it would seem to be about 40 %. To increase the sensitivity should be an object of future work. The present work indicates that the loss of bacteria during the culturing procedure is not so much due to bactericidal effect of the decontaminant, but rather to loss during centrifugation, which should therefore be avoided, if possible.

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SAMMENDRAG

Et forbedret substrat til dyrkning af Mycobacterium paratuberculosis fra kvæggødning.

Löwenstein-Jensen substrat med mykobaktin, cycloheximide, penicillin, chloramphenicol og pyrodruesyre blev sammenlignet med et ordinært L.-J. substrat med mykobaktin. Gødningsprøver fra kalve oralt inficeret med M. paratuberculosis blev dyrket på begge substrater. På det forbedrede substrat fandtes 11 % flere positive kulturer. På 97 % af de positive kulturer fandtes tydelig kolonidannelse efter 8 ugers inkubation, og procenten af forurenede kulturer blev reduceret til 0,4. Ved dyrkning af gødningsprøver fra naturligt inficerede kreaturer blev diagnosticeret 23,2 % flere inficerede dyr på det forbedrede end på det ordinære substrat. Det bedre resultat skyldes hovedsageligt en reduktion af forurenede kulturer til ca. 3 %.

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