Acta vet. scand. 1979, *20*, 191—199.

From the National Veterinary Institute, Oslo, Norway.

ISOLATION OF MYCOBACTERIUM PARATUBERCULOSIS FROM SHEEP AND CATTLE IN ICELAND*

By

Eggert Gunnarsson

GUNNARSSON, EGGERT: *Isolation of Mycobacterium paratuberculosis from sheep and cattle in Iceland.* Acta vet. scand. 1979, *20*, 191—199. — Culture experiments concerning the Icelandic variant of Mycobacterium paratuberculosis are described. Various decontaminating agents and culture media were employed and the colonial morphology of freshly isolated strains on different media described. The growth rate and culture requirements are compared with those of the Norwegian goat-pathogenic variant of M. paratuberculosis. For primary isolation modified Herrold's medium gave the best results. However, on all the various culture media used, the growth of the Icelandic variant was much more sporadic than that of the Norwegian goat-pathogenic variant. It is concluded that bacteriological culture is not useful for the diagnosis of Johne's disease caused by the Icelandic variant of M. paratuberculosis.

Mycobacterium paratuberculosis; Icelandic variant; cattle and sheep; cultivation.

Johne's disease was demonstrated in Iceland after an import in 1933 of 20 Karekul sheep from Germany. Five of these were later proved to have been latent carriers of the disease. Johne's disease has since become widespread in Iceland and has caused quite serious economic losses (*Pálsson* 1962). The condition is most common in sheep but also occurs in cattle, often in the form of a sub-clinical infection. It has also been demonstrated in goats and reindeer (*Sigurdarson* 1976).

The Icelandic variant of Mycobacterium paratuberculosis deviates from other known strains in its cultural characteristics.

* This work was supported by grants from the Agricultural Research Council of Norway and the Institute for Experimental Pathology, Keldur, Iceland.

So far there is only 1 single report of it being cultivated (*Taylor* 1951).

However, experience from Norway has shown that bacteriological culture is an important diagnostic aid in the control of Johne's disease (*Fodstad & Gunnarsson* 1979). The development of suitable methods for the culture of the Icelandic variant would therefore be of great significance.

MATERIALS AND METHODS

Samples

The material consisted of portions of small intestine and associated mesenteric lymph nodes from a total of 56 sheep and 10 cows. In 27 and 8 of these, respectively, Johne's disease was diagnosed on the basis of the demonstration of acid-fast bacilli on microscopy of smears and/or on histological examination. The remaining animals had shown positive or suspicious complement fixation titres.

Decontamination

Either 5 % oxalic acid or 0.1 % benzalkonium chloride was used to decontaminate the material. Pre-treatment of the sample material was otherwise as described by *Gunnarsson & Fodstad* (1979) according to the methods of *Stuart* (1965) and *Merkal* (1970).

Media

Finleyson's medium (*Taylor* 1950), modified Dubos' medium (*Stuart*) and modified Herrold's medium (*Merkal*) were used for culture purposes. All media contained 2 % mycobactin obtained by alcoholic extraction from Mycobacterium phlei (*Smith* 1953).

Incubation and examination

Inoculated tubes were incubated in a horizontal position for 48—72 h at 37°C, and then in an upright position. Weekly examination was made after 4 weeks of incubation. After 3 months' incubation, smears from all tubes, in which growth of M. paratuberculosis was suspected, were stained by Ziehl-Neelsen's method. After 8 months' incubation, any growth was graded as described by *Gunnarsson & Fodstad.*

Experimental method

Three comparative experimental series were employed.

I. Samples from 18 sheep and 4 cows, in which Johne's disease had been demonstrated by direct microscopical and/or histological examination, were treated with the decontaminants. Three tubes of each of the mentioned culture media were inoculated with material from each individual sample.

II. Samples from 36 sheep and 2 cows with positive or suspicious complement fixation titres and samples from 2 sheep and 4 cows, in which infection had been demonstrated by direct microscopy or histological examination, were pretreated with oxalic acid. Three culture tubes of the Dubos' medium were inoculated in each case.

III. Subcultures were established on Dubos', Herrold's and Finleyson's medium from 3 strains isolated primarily on Herrold's medium in experimental series I. Equally old primary and secondary cultures of the Norwegian goat-pathogenic variant of M. paratuberculosis on the different media were used for the direct comparison of growth characteristics and colonial morphology.

RESULTS

Series I

After 6 weeks' incubation, small, almost dust-like colonies were apparent on the surface of the medium in some of the culture tubes. However, the growth was not distinctive enough in any of the tubes to allow a bacteriological diagnosis to be made macroscopically. Continued incubation produced little change in growth. This led, in several cases, to other microbes becoming dominant and giving overgrowth. When smears from tubes, in which growth was suspected, were examined microscopically after 12 weeks' incubation, acid-fast bacilli were demonstrated in 1 or more of the tubes inoculated with material from 19 of the 22 samples. Samples from 2 sheep and 1 cow did not produce growth. Table 1 shows the number of samples which produced growth on the different media in the 2 decontamination groups.

Culture tubes from 12 of these samples, i.e. 36 tubes of each medium in each decontamination group, were incubated for more than 12 weeks. After 8 months' incubation, macroscopically

Table 1. Results of the bacteriological culture from 22 infected Icelandic samples.

Decontaminating agent	Culture medium	Positive samples after 12 weeks' incubation at 37°C	
		number	%
5 % oxalic acid	Dubos'	9	40.9
	Herrold's	12	54.5
	Finleyson's	3	13.6
0.1 % benzalkonium chloride	Dubos'	1	4.5
	Herrold's	7	31.8
	Finleyson's	2	9.0

visible growth was apparent in the oxalic acid group in 7, 2 and 1 culture tubes with, respectively, Herrold's, Dubos' and Finleyson's medium. As regards the benzalkonium chloride group macroscopically visible growth was present in 5 tubes with Herrold's medium.

A thin film of growth was produced in many of the culture tubes. Macroscopically, it was impossible to ascertain whether this was growth of M. paratuberculosis. If acid-fast bacilli were found in smears from these tubes, they were classified as positive. Tubes showing overgrowth of other microbes, or in which the medium had become completely liquified, were classified as "contaminated". Results are shown in Table 2 as the degree of growth, expressed in percentage, in the different culture tubes after 8 months' incubation at 37°C.

Series II

The culture tubes inoculated with organ material from 35 sheep and 2 cattle with positive or suspicious complement fixation titres did not produce visible growth of M. paratuberculosis. Nor did samples from infected animals give rise to visible growth after 3 months' incubation. However, by microscopic examination of smears from the infected animals, acid-fast bacilli in samples from 1 sheep and 1 cow were demonstrated.

Series III

Inoculation of 3 strains primarily isolated on Herrold's medium did not give visible growth after 12 weeks' incubation on any medium employed. After 8 months' incubation there was

Table 2. Percentage distribution of culture tubes according to degree of growth.

Decontaminating agent	Culture medium	Growth after 36 weeks at 37°C				
		+++	++	+	—	cont.
5 % oxalic acid	Dubos'	0	5.6	25.0	50.0	19.4
	Herrold's	0	19.5	47.2	25.0	8.3
	Finleyson's	0	2.8	13.9	19.4	63.9
0.1 % benzalkonium chloride	Dubos'	0	0	8.3	88.9	2.8
	Herrold's	0	14.0	19.4	50.0	16.6
	Finleyson's	0	0	2.8	22.2	75.0

+++: Dense growth of typical colonies. Very certain reading.
++: Growth of typical colonies, though few in number (< 10/tube).
+: Slight growth, microscopic examination of the culture necessary.
—: Negative.
cont.: Contaminated (overgrowth or completely liquified medium).

still no visible growth in the tubes containing Finleyson's medium. However, macroscopically apparent colonies were produced from all strains in tubes containing Dubos' and Herrold's medium. Growth on Herrold's medium was far denser than on Dubos' medium. Compared with secondary growth produced by the Norwegian goat-pathogenic variant on Dubos' medium, growth produced by the Icelandic variant was very poor (Fig. 2).

Colonial morphology

Due to slow and uncertain growth, it was not possible to study colonial morphology until after a period of 8 months' incubation. Growth most often was manifested as a blanket of very small colonies. It was difficult to determine whether these were M. paratuberculosis without preparing smears for acid-fast staining. Where it was possible to distingish individual colonies, these varied in size from pin-point up to 2 mm in diameter (Fig. 1). On Herrold's medium colonies were unpigmented, convex, smooth surfaced, almost mucoid and with an irregular edge. On Dubos' medium they were generally smaller, drier and more rough. Compared with the Norwegian goat-pathogenic variant of M. paratuberculosis, the Icelandic variant grew very poorly on Dubos' medium. On Herrold's medium, the difference was not so marked though the Norwegian goat strain did show denser

Figure 1. Comparison of primary growth of the Icelandic and Norwegian variant of Mycobacterium paratuberculosis after 8 months' incubation at 37°C. Tubes 1 and 2 from left contain cultures of the Icelandic variant on Dubos' and Herrold's medium, respectively, while tubes 3 and 4 show growth of the Norwegian variant on the same media. The arrows indicate individual colonies.

growth. On the other hand colonial morphology was quite similar. Because of insufficient growth, colonial morphology on Finleyson's medium could not be described.

DISCUSSION

The present investigation seems to confirm previous reports regarding the growth characteristics of the Icelandic variant of M. paratuberculosis (*Taylor* 1951). None of the decontaminating methods or culture media tried produced satisfactory results as regards the primary isolation or sub-culture of this variant. Pre-treatment with oxalic acid gave far better results than with benzalkonium chloride. Furthermore, growth was denser on Herrold's medium than on Dubos' or Finleyson's medium

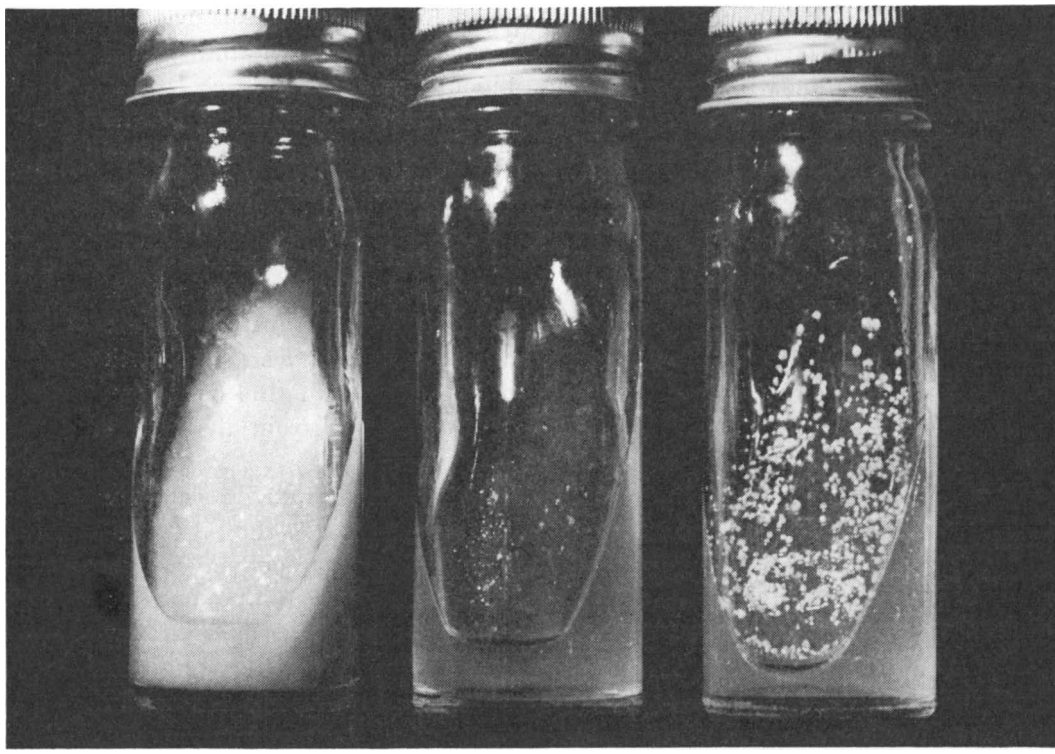

Figure 2. Secondary cultures of Mycobacterium paratuberculosis after 8 months' incubation at 37°C. Tubes 1 and 2 from left show secondary growth of the Icelandic variant on Herrold's and Dubos' medium, respectively. For comparison, secondary growth of the Norwegian goat-pathogenic variant on Dubos' medium is shown in tube 3.

(Tables 1 and 2). However, even when the combination oxalic acid/Herrold's medium was used, growth was still poor. Bacteriological culture seems therefore scarcely appropriate for diagnostic purposes in Iceland. No differences could be registered, on the basis of the material examined, between the growth characteristics of strains isolated from cattle and sheep.

The decontaminating techniques and culture media used have all been employed in cultivating other variants of M. paratuberculosis. *Taylor* (1950, 1951) obtained satisfactory results using oxalic acid and Finleyson's medium when cultivating bovine and ovine strains, inter al. the variant occurring in sheep in Iceland. However, results obtained in the present investigation using Finleyson's medium were very poor. *Smith* (1953), and later *Stuart* (1965), modified Dubos' medium (*Dubos & Middlebrook*

1947), and found it to be well suited for the culture of M. paratuberculosis. Investigations in Norway have shown that decontamination with oxalic acid and inoculation into modified Dubos' medium is the most suitable method for the isolation of the Norwegian goat-pathogenic variant (*Gunnarsson & Fodstad* 1979). In contrast, it has been concluded in the USA that a combination of benzalkonium chloride decontamination/Herrold's medium is the best method for isolation of M. paratuberculosis (*Merkal et al.* 1964). Diverging opinions as to the most appropriate methods for the isolation of M. paratuberculosis are probably due to the different growth characteristics and growth requirements shown by the different variants of this organism. The Icelandic variant seems to differ clearly from the others in its poor growth capabilities.

Investigations in Norway show that bacteriological culture of visceral material is the most certain method in the post-mortem diagnosis of Johne's disease in the goat (*Fodstad & Gunnarsson* 1979). This method has not been used in diagnostic work in Iceland. It is laborious and time-consuming and, in view of the large number of samples examined for Johne's disease each year in Iceland, would hardly seem appropriate for use in routine diagnosis. However, bacteriological culture often would be desirable, for example, deeling with samples from animals showing suspicious or positive complement fixation titres, but without demonstrable histological changes or acid-fast bacilli in smears. In some instances, reactions of this type are due to sensitization by other cross-reacting mycobacteria. A further explanation may be that the samples are from animals in such an early stage of the disease that the infection has not had sufficient time to establish itself in the tissues. In such cases, it is often possible to reveal infection by means of bacteriological culture. However, the present investigation shows that none of the culture methods tried gave a satisfactory result as regards Johne's disease caused by the Icelandic variant of M. paratuberculosis.

ACKNOWLEDGEMENT

The author wants to thank Sigurdur Sigurdarson, Head of Department, Institute for Experimental Pathology, Keldur, Iceland, for collecting the samples.

REFERENCES

Dubos, R. J. & Middlebrook: Media for tubercle bacilli. Amer. Rev. Tuberc. 1947, *56,* 334—345.

Fodstad, F. H. & E. Gunnarsson: Post-mortem examination in the diagnosis of Johne's disease in goats. Acta vet. scand. 1979, *20,* 157—167.

Gunnarsson, E. & F. H. Fodstad: Cultural and biochemical characteristics of Mycobacterium paratuberculosis isolated from goats in Norway. Acta vet. scand. 1979, *20,* 122—134.

Merkal, R. S.: Diagnostic methods for detection of paratuberculosis (Johne's disease). Proc. 74th Ann. Meet. U.S. Anim. Health Ass. 1970, 620—623.

Merkal, R. S., K. E. Kobecky, A. B. Larsen & J. R. Thurston: Improvements in the techniques for primary cultivation of Mycobacterium paratuberculosis. Amer. J. vet. Res. 1964, *25,* 1290—1293.

Pálsson, P. A.: Paratuberculosis in Icelandic sheep and its control by vaccination. Bull. Off. int. Epiz. 1962, *58,* 65—79.

Sigurdarson, S.: Garnaveiki i nautgripum, saudfé og geitum. (Paratuberculosis in cattle, sheep and goat). Handbók bænda 1976, Reykjavik.

Smith, H. W.: Modifications of Dubos' media for the cultivation of Mycobacterium johnei. J. Path. Bact. 1953, *66,* 135—381.

Stuart, P.: Vaccination against Johne's disease in cattle exposed to experimental infection. Brit. vet. J. 1965, *121,* 289—318.

Taylor, A. W.: Observation of the isolation of Mycobacterium johnei in primary culture. J. Path. Bact. 1950, *62,* 647—650.

Taylor, A. W.: Varieties of Mycobacterium johnei isolated from sheep. J. Path. Bact. 1951, *63,* 333—336.

SAMMENDRAG

Isolering av Mycobacterium paratuberculosis fra sau og storfe på Island.

Dyrkningsforsøk med den islandske varianten av Mycobacterium paratuberculosis beskrives. Forskjellige dekontamineringsmidler og kulturmedier ble brukt i forsøk på å isolere denne varianten. Vekstevne og veksthastighet hos den islandske og den norske geitepatogene varianten av M. paratuberculosis ble sammenlignet. For primær isolering ga oxalsyredekontaminering og utsæd på modifisert Herrold's kulturmedium best resultater. Imidlertid var veksten av den islandske varianten langt dårligere enn av den norske varianten også på dette medium. Det konkluderes med at bakteriologisk dyrking er lite egnet for diagnostisering av paratuberkulose forårsaket av den islandske varianten av M. paratuberculosis.

(Received September 27, 1978).

Reprints may be requested from: E. Gunnarsson, the National Veterinary Institute, P.O.Box 8156, Dep., Oslo 1, Norway.