

From the National Veterinary Institute, Oslo, Norway.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF MYCOBACTERIUM PARATUBERCULOSIS ISOLATED FROM GOATS IN NORWAY

By

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GUNNARSSON, E. and F. H. FODSTAD: *Cultural and biochemical characteristics of Mycobacterium paratuberculosis isolated from goats in Norway*. Acta vet. scand. 1979, 20, 122—134. — In Norway a variant of *Mycobacterium paratuberculosis* occurs which causes disease in goats but very seldom in sheep and cattle. Cultural and biochemical characteristics of this variant are investigated by comparing different pre-treatment methods and culture media for primary isolation and by subjecting a number of strains to different enzymatic and biochemical tests. Decontamination of materials with 5 % oxalic acid and 0.1 % benzalkonium chloride and culture on Dubos', Finleyson's and Herrold's medium was tested. The investigations showed that the combination oxalic acid decontamination/Dubos' medium is most suitable for isolation of the goat-pathogenic variant.

The morphology of the colonies was also most easily studied after culture on Dubos' medium from material pre-treated with oxalic acid. The biochemical tests were found to be poorly suitable for the identification of *M. paratuberculosis* and for its differentiation from other mycobacteria.

Mycobactin dependence for growth seems not to be absolute as a few goat strains produced growth on Dubos' medium without mycobactin. However, growth was in all cases far better in the presence of mycobactin.

goat; *Mycobacterium paratuberculosis*; culture; biochemistry.

Several variants of *Mycobacterium paratuberculosis* have been described, characterized inter al. by difference in growth properties, growth rate, colonial morphology and pathogenicity (Taylor 1951). Cultural characteristics are, however, difficult to determine. Growth and isolation of the organism put great demands on cultivation techniques and culture media. An incubation period of several months is usually required. Moreover, the inoculum is often grossly contaminated. Several methods and media for the cultivation of *M. paratuberculosis* have been de-

scribed (Minett 1942, Taylor 1950, Smith 1953, Stuart 1954, 1965, Larsen & Merkal 1961, Merkal & Larsen 1962, Ringdal 1963, Merkal *et al.* 1964).

It is usually necessary to add a growth factor, mycobactin, to the media in order to obtain growth of *M. paratuberculosis*. Only a few reports describe successful culture on media lacking mycobactin (Morrison 1965, Murohashi & Yoshida 1968, Nemeto 1969). Mycobactin dependence is thus an important criterion for the identification of *M. paratuberculosis*.

Cultural characteristics shown by the variant of *M. paratuberculosis* causing disease in the goat in Norway have so far not been described. The present investigations therefore aimed at comparing different pre-treatment methods and culture media for primary isolation. Colonial morphology on the various media was also studied. A number of strains were subjected to the enzymatic and biochemical tests routinely employed at the National Veterinary Institute, Oslo, for the differentiation of other mycobacteria. Corresponding tests were also carried out on 4 laboratory strains used for the production of vaccine and antigens. Finally, some strains were subjected to growth studies on media with and without the addition of mycobactin.

MATERIALS AND METHODS

Samples

The sample material consisted of the lower part of the small intestine and associated mesenteric lymph nodes from 10 infected goats.

Decontamination

Approx. 10 g of tissue material was finely ground in a mortar. Half of this was transferred to sterile 50 ml centrifuge tubes and suspended in 0.1 % benzalkonium chloride in sterilized water. After overnight storage at room temperature, the sediment was removed with a pipette, leaving the supernatant, and about 0.2 ml inoculated into each culture tube (Merkal 1970). The remaining half of the tissue material was ground in the mortar and decontaminated with 5 % oxalic acid as described by Fodstad & Gunnarsson (1979) according to the method of Stuart (1965). Prior to inoculation, direct microscopy of smears from the inoculum, stained by Ziehl-Neelsens method, was carried out.

Media

The material pre-treated with oxalic acid as well as that treated with benzalkonium chloride was inoculated onto Finleyson's medium (*Taylor* 1950), modified Dubos' medium (*Stuart* 1965) and modified Herrold's medium (*Merkal*) — 3 tubes of each medium being used in each case. All the media contained 2 % mycobactin, prepared by the alcoholic extraction of *Mycobacterium phlei* (*Smith* 1953).

Incubation and examination

Inoculated tubes were incubated with the surface of the medium in the horizontal position for 48—72 hrs. at 37°C and then in an upright position, weekly readings being taken after 4 weeks. After 12 weeks of incubation, growth was graded as shown in Table 1.

Enzymatic and biochemical reactions

Subcultures were made onto modified Dubos' medium from positive cultures. Good growth was obtained in most of the culture tubes after 8 weeks of incubation. Fifty goat strains were tested for hydrolysis of Tween 80 (*Wayne et al.* 1964), acid phosphatase activity (*Käppler* 1964), aryl sulphatase activity (*Wayne* 1961) and catalase activity at 68°C (*Kubica et al.* 1966). Eleven of these strains were also tested for nitrate reduction (*Virtanen* 1960) and catalase activity at 37°C (*Kubica et al.*).

Subcultures were also made onto modified Dubos' medium from cultures on fluid media of the laboratory strains 2E, 316F, Teps and Str. 18. These strains are all of bovine origin. Strains 2E and 316F come originally from the Central Veterinary Laboratory, Weybridge, England; Teps from the National Institute of Medical Research, Farm Laboratories, Mill Hill, London, England, and Str. 18 from the Animal Research Laboratory of the Bureau of Animal Industry, Auburn, Alabama, USA. They were tested, after 8 weeks incubation, for enzymatic and biochemical activity using the same test reactions as for the goat strains.

Mycobactin dependence

In some cases, parallel incubations onto Dubos' medium with and without mycobactin were made in connection with primary isolation from organ samples and when subculturing.

RESULTS

Direct microscopy of smears prepared from material treated with oxalic acid and benzalkonium chloride showed no differences in the abundance of bacteria. In the oxalic acid treated material, the bacilli were evenly spread without any background staining worth mentioning. However, in smears from material treated with benzalkonium chloride, the bacilli showed a tendency to agglomerate together with background-stained material.

The inoculum decontaminated with oxalic acid produced growth of *M. paratuberculosis* on 1 or more of the culture media as regards all 10 goats. Inoculum decontaminated with benzalkonium chloride produced growth from 3 of the goats. Some tissue particles were usually transferred together with the inoculum from the latter material. This made examination difficult, especially after a short period of incubation. Inoculation onto Dubos' medium gave much more abundant growth after decontamination with oxalic acid than with benzalkonium chloride. This is shown in Table 1. The transparent nature of this medium showed up the bacterial colonies in sharp contrast. After only 4 weeks of incubation, small dust-sized colonies could be

Table 1. Percentage distribution of culture tubes according to degree of growth.

Decontaminating agent	Culture medium	Growth after 12 weeks at 37°C					cont.
		+++	++	+	—		
5 % oxalic acid	Dubos'	80	0	10	6.7	3.3	
	Herrold's	0	6.7	60	30	3.3	
	Finleyson's	0	0	16.7	76.7	6.6	
0.1 % benzalkonium chloride	Dubos'	13.3	13.3	43.4	30	0	
	Herrold's	13.3	10	43.4	33.3	0	
	Finleyson's	0	0	10	73.3	16.7	

+++ : Dense growth of typical colonies. Very certain reading.

++ : Growth of typical colonies, though relatively sparse (less than 10 colonies/tube).

+ : Growth, but impossible to determine macroscopically. Must be verified by microscopy of smears.

— : No growth.

cont. : Contaminated tube.

observed. At that time of incubation, it was usually necessary to verify the diagnosis by microscopy. Typical colonies were usually first visible after 8—12 weeks' incubation.

Inoculation onto Herrold's medium produced the densest growth after decontamination with benzalkonium chloride. Growth intensity was about the same as on Dubos' medium using the same decontamination technique. Inoculation onto Finleyson's medium gave very poor results with both methods of decontamination. Each decontamination group consisted of 30 tubes of each medium. Table 1 shows the percentage distribution of the culture tubes according to the degree of growth after 12 weeks of incubation at 37°C. It can be seen from the table that the combination oxalic acid decontamination/Dubos' medium is the one most suitable for isolation of the Norwegian goat variant of *M. paratuberculosis*. Eighty percent of the culture tubes within this group showed dense growth (+++).

Finleyson's medium gave the poorest results with more than 70 % negative tubes, both after pre-treatment with oxalic acid and with benzalkonium chloride. This medium also produced most contaminated tubes. The other combinations of decontaminating agent/culture medium took an intermediate position in that about $\frac{1}{3}$ of the tubes were negative.

Colonial morphology

Colonial morphology varied between media. It was most easily studied after culture on Dubos' medium from material pre-treated with oxalic acid. After 2—3 months' incubation small, irregular greyish-white colonies were normally seen. After continued incubation, the size of the colonies varied somewhat according to growth density, not only in different tubes, but also in one and the same tube. Fig. 1 shows growth on Dubos' medium after 4 months' incubation.

In dense cultures, colonies were still small after a long period of cultivation. When growth was more moderate, colony size increased and morphology was easier to study. The largest colonies had a diameter of about 2 mm. Sometimes colonies fused to form confluent masses of about 0.5 cm in diameter. Individual colonies were irregular in shape, with a convex or flat, uneven surface and greasy appearance. They were only loosely attached to the surface of the medium, but adhered quite

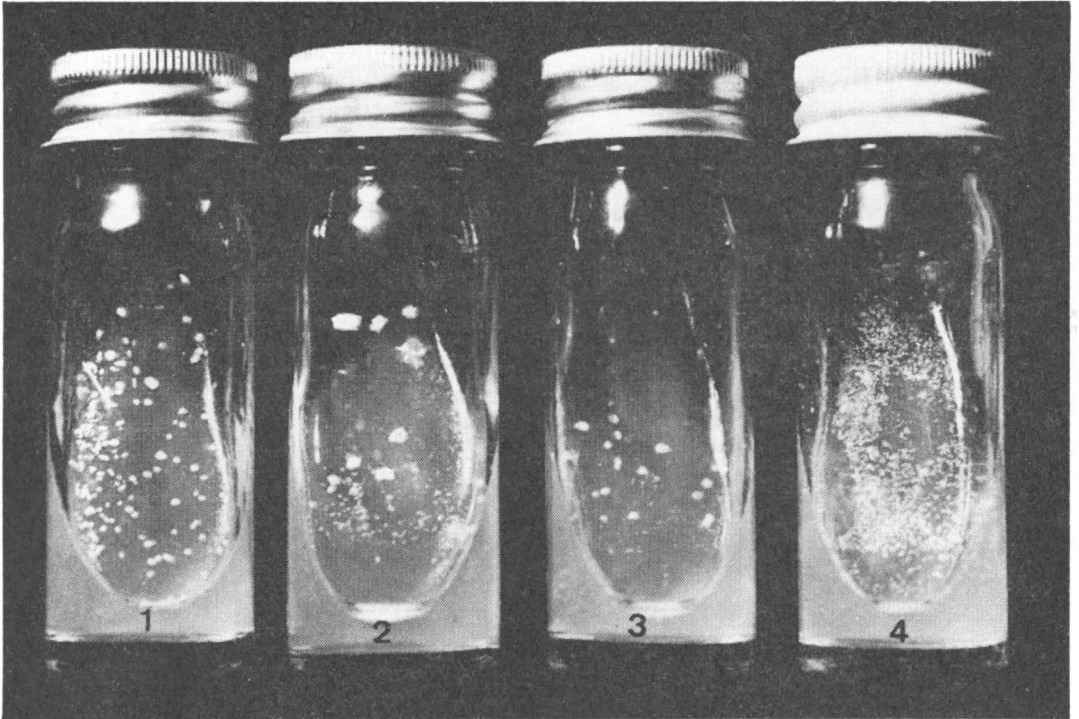


Figure 1. Growth of the Norwegian goat pathogenic variant of *Mycobacterium paratuberculosis* on Dubos' medium with 2 % mycobactin after 4 months' incubation at 37°C. Colony size varies according to density of growth. Dense growth of small colonies is seen in tube 4, while in the other tubes, growth is more moderate and the colonies larger. In some places colonies form large confluent masses.

strongly to each other making it somewhat difficult to transfer individual colonies with a loop onto a slide for the preparation of smears.

In older cultures, large colonies showed a tendency to crater formation and were greyishly discoloured centrally. Very occasionally, growth was so dense that a "blanket" was formed, making the identification of individual colonies impossible. Colonies were smaller on Herrold's medium than on Dubos' medium, and were more mucoid with a smoother surface. On Finleyson's medium, they were also much smaller and had a tendency to be somewhat yellow in colour.

Enzymatic and biochemical reactions

Table 2 shows the results of the tests carried out. It can be seen that *M. paratuberculosis* is not very biochemically active. The laboratory strains tested, with the exception of Teps, differed from the goat variant in that they lacked catalase activity at 68°C at pH 7. Str. 18 and 1 goat strain differed from the others in showing a positive nitrate reduction.

Table 2. Biochemical activity shown by strains of *Mycobacterium paratuberculosis*.

Strain	Nitrate reduc- tion	Hydro- lysis of Tween 80	Acid phos- phatase	Aryl sulphatase	Catalase 37°C	Catalase 68°C
A. Laboratory strains						
2 E	—	—	—	—	—	—
316 F	—	—	—	—	—	—
Teps	—	—	—	—	2 mm	+
Str. 18	+	—	—	—	—	—
B. Goat strains						
38 str.	0	—	—	—	0	+
1 „	0	—	(+)	—	0	+
1 „	—	—	(+)	—	10 mm	+
7 „	(+)	—	—	—	„	+
1 „	(+)	—	+	—	„	+
1 „	(+)	—	—	—	„	—
1 „	+	—	—	—	„	—

— : Negative.

(+): Doubtful.

+ : Positive.

0 : Not examined.

mm : Hight of foam in the test tube.

Mycobactin dependence

Inoculation of material from 6 infected samples onto Dubos' medium without mycobactin produced growth from only 1 sample after more than 4 months' incubation at 37°C. Growth was, however, very sparse, with small unpigmented, dry, irregular colonies. They consisted of short acid-fast bacilli. Subcultures onto Løwenstein's, Petragnani's, Stonebrink's and Dubos' medium without mycobactin did not produce growth. On

Dubos' medium containing mycobactin, there was abundant growth of typical colonies of *M. paratuberculosis*. Macroscopical and histological examination of the sample from which this isolation was made showed changes definitely associated with Johne's disease.

Subcultures were made onto Dubos' medium with and without mycobactin from 8 primary bacterial cultures, 1 tube with mycobactin and 2 tubes without for each culture. After 12 weeks of incubation there was dense growth in the culture tubes containing mycobactin while the tubes without mycobactin were negative. However, control readings taken about 5 months later revealed growth of small, white, irregular, dry colonies also in 6 cultures lacking mycobactin. One of the cultures produced growth in both of the tubes lacking mycobactin, while the remaining 5 produced growth in only 1. Fig. 2 shows secondary growth produced by subcultures after 35 weeks of incubation.



Figure 2. Subcultures of the Norwegian goat pathogenic variant of *Mycobacterium paratuberculosis* on Dubos' medium after approx. 8 months' incubation at 37°C. Tubes 1 and 4 contain mycobactin, while tubes 2 and 3 do not. The arrows indicate individual colonies.

DISCUSSION

Low initial pH seems to stimulate the growth of *M. paratuberculosis* when cultured on synthetic media (*Morrison 1965*). This may be the case also with cultures on serum agar medium. The good results obtained when oxalic acid decontamination was combined with cultivation on Dubos' medium, as compared with other combinations of decontaminating agents and culture media, suggest that this is indeed so.

Investigations indicate that benzalkonium chloride is a very suitable decontaminating agent when attempting to isolate mycobacteria from grossly contaminated material, and that combination with egg media is advantageous in that egg yolk neutralizes benzalkonium chloride (*Merkal 1970*). Egg media are moreover known to be good substrates for most mycobacteria (*Merkal*). It is therefore interesting to note that the results obtained with serum agar medium in combination with benzalkonium chloride were just as good as those obtained with Herrold's egg medium.

Some authors have reported good results when using serum agar for the isolation of bovine and ovine strains of *M. paratuberculosis* (*Smith 1953, Stuart 1965*). However, most authors recommend the use of egg media for this purpose (*Minett 1942, Taylor 1950, Merkal*). The differing conclusions so far reached by various authors concerning media and culture techniques for the isolation of *M. paratuberculosis* are probably due to differences in growth capabilities and growth requirements shown by the different variants of this organism. Other factors may also be involved. For example, exacting demands are put on culture techniques and culture media. At the authors' laboratory, decontamination with oxalic acid combined with culture on Dubos' medium has been employed for about 10 years for the isolation of *M. paratuberculosis* from the goat, with very good results (*Fodstad & Gunnarsson 1979*).

The Norwegian goat variant of *M. paratuberculosis* seems to differ from the 3 types of variants stated by *Taylor (1951)* to cause Johne's disease in sheep and goats. It differs from the Scottish sheep variant by being impigmented and from the Icelandic variant inter al. by much better growth capabilities (*Taylor 1951, Gunnarsson 1979*). Growth rate and colonial morphology are very reminiscent of that described for the classical bovine variant (*Smith, Merkal & Curran 1974*). It differs, how-

ever, from the latter in its low pathogenicity to cattle and sheep as well as having other growth requirements.

The present investigation shows that *M. paratuberculosis* is biochemically not very active. This is in accordance with the few investigations which have been carried out previously (*Merkal & Thurston* 1966, *Nemeto* 1969, *Thorell & Valette* 1976). The results show that biochemical tests are poorly suited for the identification of *M. paratuberculosis* and for its differentiation from other mycobacteria.

One of the most important characteristics of *M. paratuberculosis* is that growth normally only occurs on media containing mycobactin, both on primary isolation and subcultures (*Merkal & Curran*). The present investigations showed that mycobactin dependence is not absolute as a few goat strains produced growth in Dubos' medium without mycobactin. There can be little doubt that the strains growing without the addition of mycobactin should be classified as *M. paratuberculosis*, as they were isolated as the only acid-fast organisms from goats showing histological and macroscopical changes typical for Johne's disease. Diagnostically, this did not cause particular problems.

Mycobactin is assumed to be an iron-containing sideramine (*Zähner et al.* 1962). A splitting off of the iron component satisfies the iron requirements of most mycobacteria, though *M. paratuberculosis* does not as a rule produce enough mycobactin to cover this requirement. Some strains grow on synthetic media not containing mycobactin probably because they are able to produce endogenous mycobactin. *Morrison* reached the conclusion that the need of mycobactin varied according to the composition of the medium. In his experiments with so-called mycobactin dependent and independent strains on synthetic glucose-glycerine medium, he found that growth was indeed stimulated by the addition of mycobactin. However, his strains also grew in the absence of such addition. He considered that this was due to growth stimulating substances being formed during autoclave treatment of the culture medium, as well as to a certain growth stimulating effect of low initial pH. *Morrison's* medium (Modified Watson-Reid's medium) also contains ferriammonium citrate. Other workers have shown that this substance can replace mycobactin (*Merkal & Curran*), a circumstance which may explain *Morrison's* results.

Modified Dubos' medium contains some of the same ingredients as Watson-Reid's, but neither ferriammonium citrate nor glucose. The present investigations show that the former medium is well suited for the cultivation of the Norwegian goat pathogenic variant of *M. paratuberculosis*. This indicates that also growth factors other than those mentioned, are of significance for the culture of this bacterium. The growth of some strains of this organism on Dubos' medium without mycobactin supports this suggestion.

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SAMMENDRAG

Kulturelle og biokjemiske egenskaper hos Mycobacterium paratuberculosis isolert fra geiter i Norge.

I Norge forekommer en variant av *Mycobacterium paratuberculosis* som gir sjukdom hos geiter, men bare i unntakstilfeller hos sau og storfe. Kulturelle og biokjemiske egenskaper hos denne variant er undersøkt ved å sammenligne ulike forbehandlingsmetoder av materialet og kulturmedier for primær isolering samt ved å utføre en rekke biokjemiske og enzymatiske reaksjoner på de isolerte stammer. Materialet ble dekontaminert med 5 % oksal-syre og 0.1 % benzalkonium-

klorid og utsed foretatt på Dubos', Finleyson's og Herrold's medium. Undersøkelsene viste at kombinasjonen oksal-syre dekontaminering/Dubos' medium gir de beste resultater ved isolering av den geitepatogene variant av *M. paratuberculosis*.

Koloni-morfologien lot seg også lettest studere på Dubos' medium inokulert med materiale som var forbehandlet med oksal-syre. Biokjemiske test-reaksjoner ble ikke funnet anvendelig ved typing av *M. paratuberculosis*.

Noen få geite-patogene stammer ga vekst på Dubos' medium uten tilsetning av mykobaktin. Veksten av disse stammer var imidlertid langt bedre på Dubos' medium med mykobaktin.

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