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Isolation of *Mycobacterium paratuberculosis* from Intestinal Mucosa and Mesenteric Lymph Nodes of Goats by Use of Selective Dubos Medium

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To isolate *Mycobacterium paratuberculosis* from contaminated material, a selective medium, selective Dubos medium (SDubos), was developed by supplementing conventional Dubos medium (CDubos) with carbenicillin, polymyxin, trimethoprim, and amphotericin B. The intestine and mesenteric lymph nodes of 1,501 goats were cultured in parallel on SDubos and CDubos after decontamination with oxalic acid. The contamination rate was reduced more than 150 times by the use of SDubos. The number of positive specimens (18, or 1.2%), i.e., those which revealed growth of *M. paratuberculosis*, was too small to evaluate the number of specimens which could have been missed due to contamination on CDubos. However, the number of specimens positive on SDubos (16, or 89%) showed that the antibiotics used were not harmful to *M. paratuberculosis*.

The problem of isolating slow-growing mycobacteria from heavily contaminated material such as sputum or feces is that they are easily overgrown by other bacteria or fungi. To solve this problem, various decontaminating procedures and selective media have been developed, and these have been reviewed by Songer (12).

As far as paratuberculosis, or Johne's disease, an intestinal disease of ruminants, is concerned, the causative organism, *Mycobacterium paratuberculosis*, is isolated from feces or the intestinal mucosa and the associated mesenteric lymph nodes of infected animals. Not only is such material heavily contaminated, but the organism requires 9 to 14 weeks to grow (8). Decontamination procedures used for *M. paratuberculosis* have been reviewed by Chiodini et al. (1), but there are few reports on the use of selective media. Stuart (13) used Dubos serum-agar medium supplemented with penicillin and chloramphenicol, and Jørgensen (3) used Löwenstein-Jensen medium with the same antibiotics plus cycloheximide as an antimycotic agent.

In Norway paratuberculosis was a serious disease of goats, but it has been brought under control by means of vaccination (10). The disease is kept under continuous surveillance, and specimens of the small intestine and mesenteric lymph nodes of all slaughtered goats are examined. Occasioned by the report of Rothlauf et al. (9), who successfully used selective 7H10 medium for isolation of other mycobacteria than *M. paratuberculosis*, Dubos medium with the same antibiotics was used in the present study for isolation of *M. paratuberculosis*. To test whether these antibiotics were harmful to *M. paratuberculosis*, stock cultures from 10 goats were inoculated in parallel on Dubos medium with and without antibiotics. This preliminary investigation revealed no qualitative or quantitative differences in growth on the two media.

Media. Conventional Dubos medium (CDubos) was prepared as previously described (11, 13). Selective Dubos medium (SDubos) was CDubos with the addition of antibiotics and an antimycotic agent (amphotericin B). Final antimicrobial concentrations were 100 μg of carbenicillin (Sigma Chemical Co., St. Louis, Mo.) per ml, 200 U of polymyxin B sulfate (The Wellcome Foundation, London, England) per ml, 10 μg of amphotericin B (E. R. Squibb & Sons, London, England) per ml, and 19.5 μg of trimethoprim lactate (Norsk Medisinaldepot, Oslo, Norway) per ml. In contrast to pure trimethoprim (15 μg/ml) from Sigma, the lactate was easily soluble in water and therefore preferred. The medium, which contained 2% mycobactin prepared from *Mycobacterium phlei*, was enriched with 0.4% sodium pyruvate, which according to Jørgensen (3) enhances the growth of *M. paratuberculosis*. After cooling of the melted agar, serum and antibiotics were added, and the medium was dispensed in quantities of about 12 ml into 25-ml screw-capped cylindrical vials in a slanting position.

Specimens and processing. The specimens, which in most cases comprised parts of jejunum and ileum with associated lymph nodes, were decontaminated with 5% oxalic acid and processed as previously described (2). Each specimen was inoculated into two vials of each medium, and the vials were read after 3 months of incubation at 37°C. Acid-fast bacilli which required at least 8 weeks to grow and which grew only in the presence of mycobactin were identified as *M. paratuberculosis* (1). Specimens which revealed growth of *M. paratuberculosis* were designated as positive. Those which did not do so were designated as negative. Moreover, direct examination for acid-fast bacilli was performed by staining smears and histological sections by the Ziehl-Neelsen method.

During an 8-month period, April through November 1983, specimens from 1,501 goats originating from 370 herds were cultured in parallel on CDubos and SDubos, and the results are shown in Table 1.

On CDubos 156 specimens (10.4%) were contaminated, and of these, 50 (3.3%) were completely spoiled due to contamination of both vials. On SDubos only one vial of one specimen (0.07%) was contaminated, a finding which shows that the contamination rate was more than 150 times greater on CDubos than on SDubos (156 versus 1). The number of specimens negative on SDubos and contaminated on CDubos was 153, whereas no vials were negative on CDubos but contaminated on SDubos. The contamination was, in most cases, due to gram-positive spore-forming rods.

As paratuberculosis is under control in Norway at present, there were altogether only 18 positive specimens. Of these, 14 were positive on both media, 2 were positive on SDubos

TABLE 1. Comparison of culture results of 1,501 specimens[a]

CDubos	SDubos		
	Contaminated	Negative	Positive
Contaminated	1	153	2[b]
Negative	0	1,345	2[b]
Positive	0	2	14

[a] Number of vials per medium, 3,002; number of CDubos vials contaminated, 201 (6.7%); number of SDubos vials contaminated; 1 (0.03%).
[b] These two specimens were partly contaminated but negative on CDubos.

and partly contaminated but negative on CDubos, and 2 were positive on CDubos and negative on SDubos. No specimens were positive on CDubos and contaminated on SDubos. There was no quantitative difference in growth of *M. paratuberculosis* on CDubos and SDubos. Of the 18 specimens which were positive by culture, 13 (72%) were negative by direct microscopy and histology.

The findings that the contamination rate was reduced more than 150 times by use of SDubos and, moreover, that 3.3% of the specimens were completely spoiled when only CDubos was used leave no doubt that SDubos is a satisfactory selective medium. In comparison, McClatchy et al. (5) found that the contamination rate was reduced about 85% when they examined sputum samples from humans for mycobacteria. As to the kind of contamination, in the present study this was mostly due to gram-positive spore-forming rods, whereas McClatchy et al. found mostly fungi, and Matajack et al. (4) found gram-positive rods.

A greater proportion of positive specimens would have been desirable to demonstrate the usefulness of SDubos. According to several authors (3, 4, 7, 9), a certain proportion of mycobacteria is killed by decontamination with acid or base. Moreover, Mitchison et al. (7) and McClatchy et al. (5), who used the same antibiotics as in the present study, found that some species of nontuberculous mycobacteria were inhibited by these antibiotics. The small number of positive specimens, 18 (1.5%), in the present study, might therefore indicate that *M. paratuberculosis* could also be inhibited by these antibiotics. However, the findings that 14 (78%) of the specimens were positive on both media and, moreover, that 13 (72%) of the 18 positive specimens were positive by culture but negative by direct microscopy and histology reveal a low but true incidence of infection which, as already mentioned, is due to the fact that the disease has been brought under control by vaccination. The antibiotics used are therefore evidently, as earlier mentioned, not harmful to *M. paratuberculosis*.

Two of the specimens were positive on SDubos and negative on CDubos, and conversely, two specimens were positive on CDubos and negative on SDubos. This indicates a chance distribution and demonstrates, as mentioned by Matajack et al. (4), the necessity of using multiple cultures per specimen. Moreover, some specimens which were positive either on SDubos or CDubos were positive only in one vial, a finding which demonstrates the necessity of using more than one vial of each medium per specimen.

Various authors adhere to the use of different basic media for the isolation of *M. paratuberculosis*. Some workers (3, 6) prefer egg media, while others (2, 11, 13) prefer serum-agar media. As Dubos serum-agar medium is transparent and thus allows a better differentiation between *M. paratuberculosis* and contaminants than egg media, this medium was the choice in the present study.

As for decontamination, Merkal et al. (6) found the use of oxalic acid deleterious to *M. paratuberculosis* and proceeded therefore first to benzalkonium chloride and then to hexadecylpyridinium chloride. Decontamination in the present study was based on the previous investigations by Fodstad and Gunnarsson (2), who used oxalic acid and successfully detected 92% of infected goats by culture but only 54% by histology and 47% by direct microscopy.

Although selective media may be useful, several authors (4, 5, 7) maintain that heavily contaminated specimens ought to be decontaminated and, moreover, that selective media should be used in combination with conventional media. I agree with these authors, and based on the results obtained, the contamination procedure and media described in the present study are therefore still being used and will continue to be used in the future.

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