

Growth of *Mycobacterium paratuberculosis* in Radiometric, Middlebrook and Egg-Based Media

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ABSTRACT

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The ability of BACTEC radiometric 7H12 broth, Middlebrook 7H10 Tween broth, Middlebrook 7H10 agar, and Herrold's egg-yolk medium to provide early detection of *Mycobacterium paratuberculosis* was evaluated. The minimum detection times in days for the various media were: 7H12, 9; 7H10 agar, 23 (plate), 28 (slant); 7H10 Tween broth; 27; and Herrold's egg-yolk medium, 43 (plate), 49 (slant). The radiometric broths provided the earliest detection of *M. paratuberculosis*, and 3625 organisms ml⁻¹ were required to produce a positive, radiometric growth-index reading. Of the non-radiometric plate and slant media evaluated, microscope examination of the translucent 7H10 agar plate resulted in the earliest detection and highest mean colony counts (387) as compared with Herrold's egg-yolk agar plate (208). Similar results were noted for 7H10 and Herrold's egg-yolk agar slants; however, accurate colony counts could not be determined because of confluent growth. All media were supplemented with 2 µg ml⁻¹ of mycobactin J and excess amounts of this supplement inhibited the growth of *M. paratuberculosis* in radiometric 7H12 media.

INTRODUCTION

Mycobacterium paratuberculosis is the causative agent of Johne's disease in ruminants. This progressive disease is usually fatal and is of considerable economic importance throughout the world (Merkal, 1970; Larsen, 1972; Kopecky, 1973; Julian, 1975; Buergelt and Duncan, 1978; Chiodini and Van Kruiningen, 1982). This organism has a strict growth requirement of the hemosiderin compound, mycobactin (Francis et al., 1953; Snow, 1954; Merkal et

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al., 1964; Thoen and Muscolplot, 1979). While histological examination of tissue is very useful for establishing mycobacterial infections in ruminants, the isolation of *M. paratuberculosis* is essential for a definitive diagnosis. Existing cultural procedures using egg-based media systems are cumbersome to prepare and require extended incubation periods ranging from 2 to 4 months (Merkal et al., 1964, 1968; Merkal and Thurston, 1966; Merkal, 1970).

Damato et al. (1987) showed that BACTEC radiometric 7H12 broth supplemented with mycobactin permitted the rapid detection of growth of *M. paratuberculosis*. Radiometric growth systems have been shown to detect growth of mycobacteria earlier than conventional bacteriologic culture systems (Morgan et al., 1983; Roberts et al., 1983). The indicator of growth for this test system is the release of $^{14}\text{CO}_2$ from metabolized labeled substrates as initially described by Cummings et al. (1975) and Middlebrook et al. (1977). Furthermore, non-egg Middlebrook media has been found to be very useful for facilitating the detection of a wide variety of mycobacteria species (Chiodini et al., 1984; Sommers and Good, 1985).

The purpose of this study was to examine the sensitivity of the radiometric test system to detect the growth of *M. paratuberculosis*; to evaluate the effect of various concentrations of the alcohol-based mycobactin J supplement on this test system; and to determine if non-radiometric Middlebrook media facilitated detection of *M. paratuberculosis* when compared with the currently used egg-based media such as Herrold's egg-yolk medium.

MATERIALS AND METHODS

Isolate preparation

Thirty-four isolates of *M. paratuberculosis* from bovine (20), caprine (7) and ovine (7) origins were used in this study. An additional isolate was also obtained from the Mycobacterial Culture Collection, TMC 807 (National Jewish Hospital and Research Center, Denver, CO). All organisms were initially cultured on Herrold's egg-yolk medium (Herrold, 1931), with and without mycobactin J (National Animal Disease Laboratory, Ames, IA) to confirm mycobactin dependence. Stock suspensions were prepared by diluting each isolate to equal the No. 0.5 McFarland Turbidity Standard using 0.2% fatty-acid-free albumin and 0.02% Tween 80 in Butterfield's Buffer ($85 \mu\text{g ml}^{-1} \text{KH}_2\text{PO}_4$ in double-distilled water, pH 7.2). Ten μl of the stock suspension, which contained approximately 10^3 organisms ml^{-1} , was then used to inoculate the various test media (Fig. 1). In addition, slides were prepared for auramine fluorescent stain (Difco, Detroit, MI) to confirm the fluorescent staining characteristics of *M. paratuberculosis* (Sommers and Good, 1985).

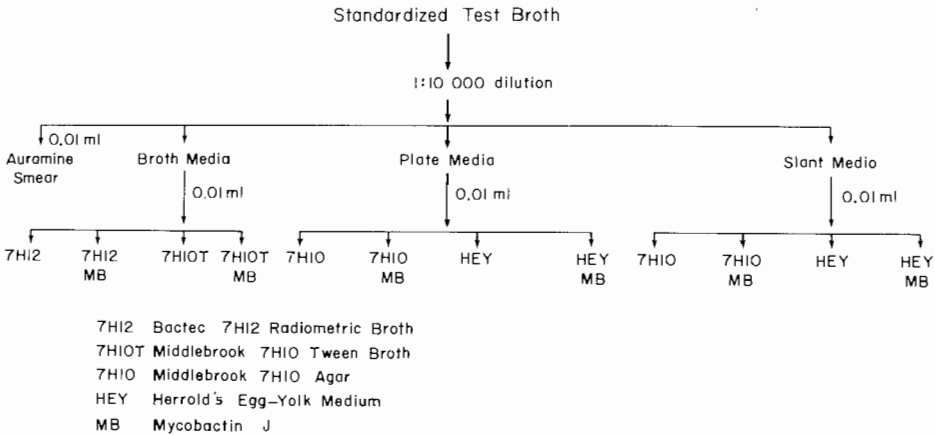


Fig. 1. Media inoculation protocol.

Growth supplement

The mycobactin J growth supplement (Merkal and McCullough, 1982) is not readily soluble in aqueous solutions. It was necessary initially to dissolve the mycobactin J in 3.7 ml of 95% ethanol, then dilute it to 70% ethanol by adding 1.3 ml of sterile double-distilled water. The resulting stock solution contained 0.4 mg ml^{-1} of mycobactin J. All media were subsequently inoculated with the stock solution to produce a final concentration of $2 \mu\text{g ml}^{-1}$ of mycobactin J.

Radiometric media

To evaluate the detection limits of the radiometric test system, the number of organisms necessary to produce a positive test was determined. A 1:10 dilution of the stock-solution containing approximately 10^2 organisms ml^{-1} was prepared and inoculated into 2 BACTEC 7H12 Middlebrook media bottles (7H12) (Johnson Laboratories, Towson, MD). One broth was supplemented with $2 \mu\text{g ml}^{-1}$ mycobactin J and the other, unsupplemented broth was used as a negative growth control. Both of the bottles were flushed with 5% CO_2 and incubated at 37°C . The gas was sampled using a BACTEC Model 301 (Johnson Laboratories, Towson, MD) that also automatically replenished the 5% CO_2 content of each bottle during the testing cycle. Sampling continued until a growth index (GI) of 8 was obtained; however, viable count determinations were performed with samples having GI readings of 2 and 8. A GI reading of 10^2 is equivalent to $0.025 \mu\text{Ci}$ of ^{14}C released as CO_2 . The GI reading of 2 was selected because it was the first point on the BACTEC 301 digital scale that could be accurately interpreted. Duplicate viable counts were performed by plating serial dilutions from the 7H12 broth using the previously described Butterfield's Buffer on mycobactin J supplemented Middlebrook, 7H10 agar.

TABLE 1

Final concentration of ethanol and mycobactin after dilution in BACTEC 7H12 broth

Supplement volume (μ)	BACTEC 7H12 (μ l)	Ethanol diluent concentrations		Mycobactin J (μ g ml ⁻¹)
		70%	95%	
10	2000	0.4%	0.5%	1.99
20	2000	0.7%	0.9%	3.95
50	2000	1.7%	2.3%	9.80
100	2000	3.3%	4.5%	19.05

Note: The concentration of mycobactin J in both the 70% and 95% ethanol solution was 400 μ g ml⁻¹.

In order to determine the effect of various 70 and 95% alcohol concentrations on the radiometric test system, the 7H12 broths were supplemented with mycobactin J in a manner which differed from that previously described. A series of 7H12 broths was inoculated with 0.01, 0.02, 0.05 and 0.1 ml of the mycobactin J supplement reconstituted with either 70 or 95% ethanol. The final concentration of ethanol and mycobactin J used in the 7H12 broth system is shown in Table 1. Each radiometric broth was then inoculated with *M. paratuberculosis* TMC 807. Duplicate radiometric broth were incubated at 37°C and examined daily for 12 days with a BACTEC 301.

Radiometric broths considered positive for *M. paratuberculosis* demonstrated increasing GI readings while the controls, which did not contain mycobactin, had GI readings less than 50, which leveled off and subsequently declined. Radiometric broths were interpreted as negative when GI readings were less than 50, leveled off and declined in a manner similar to that observed for the negative control broth. All positive radiometric broths were auramine-stained and subcultured to 7H10 agar with and without mycobactin J to confirm that the organisms require mycobactin J for growth and to determine that the fluorescent staining characteristics of the organisms were consistent with the isolates used in the study.

Conventional media

These media included Middlebrook 7H10 Tween broth (7H10 T), Middlebrook 7H10 agar (7H10) (Russel and Middlebrook, 1961). (BBL, Cockeysville, MD) and Herrold's egg-yolk medium (Herrold, 1931). All media were prepared with and without mycobactin J (final concentration 2 μ g ml⁻¹). Medium without mycobactin was used as a negative growth control. Plate media were dispensed into 100 mm petri plates in 25-ml aliquots. The agar slants were prepared by pipetting 10 ml of 7H10 or Herrold's egg'yolk medium into 20 × 150 mm screw-top tubes and laying the tubes flat until the medium solid-

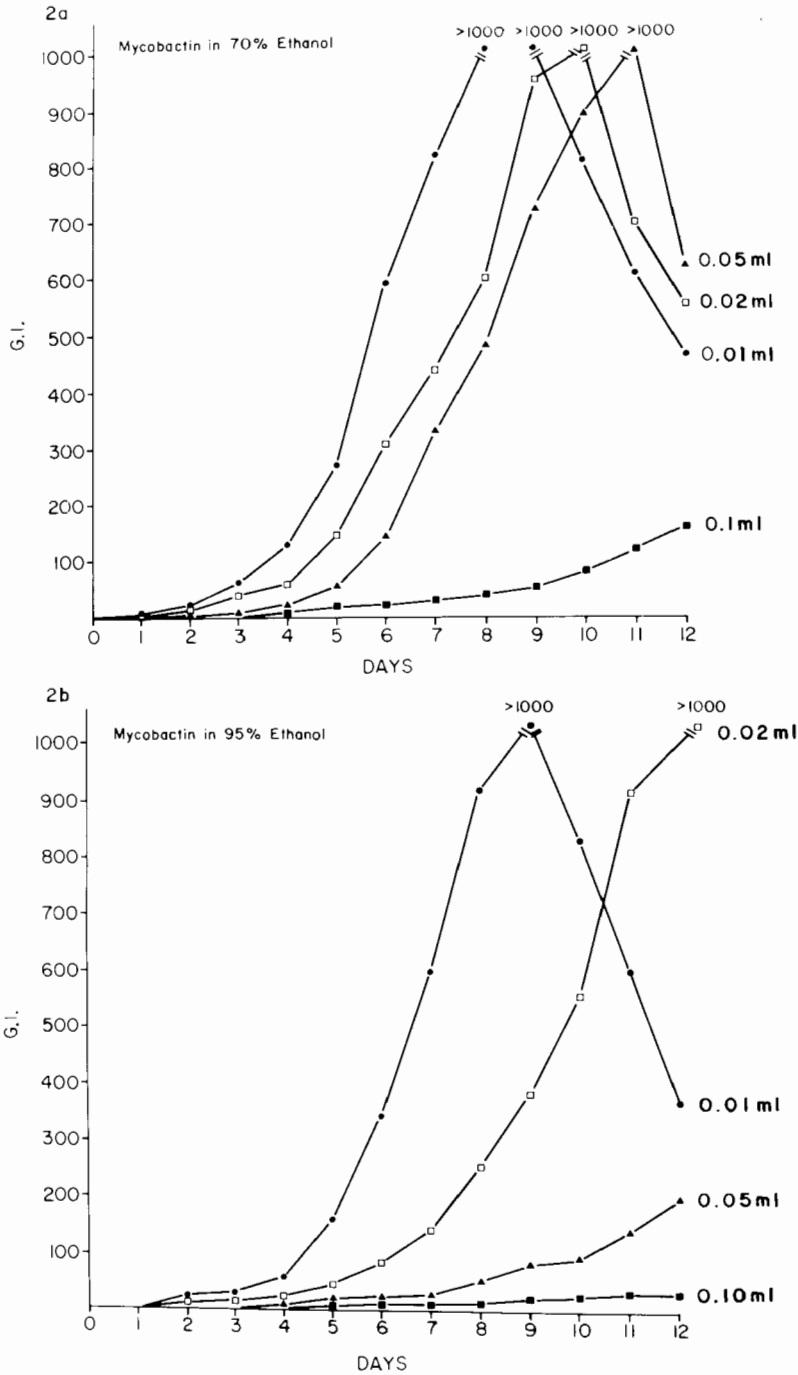


Fig. 2. The effect of increasing amounts of mycobactin supplements on the growth of *M. paratuberculosis*, using 70 and 95% ethanol as diluent. The volume of supplement added to each BACTEC broth: 0.01 ml, ●; 0.02 ml, □; 0.05 ml, ▲; 0.1 ml, ■. All control broth without mycobactin were interpreted as negative. Testing was performed in duplicate and the mean plotted.

ified. Middlebrook 7H10 T broth was dispensed into 16×125 mm screw top-tubes in 6-ml aliquots. Media were inoculated as described in Fig. 1. Plate media were subsequently streaked with a sterile bacteriologic loop to disperse bacterial growth. The 20×150 mm tubes containing the agar slants were inoculated by placing 10 μ l of inoculum directly into the agar slants. The Herrold's egg yolk and 7H10 agar plates were incubated at 37°C with 5% CO₂ (Merkal, 1970) and examined weekly. Prior to incubation, all plate media were sealed in gas-permeable 4×6-inch polyethylene bags to prevent dehydration, and all tube and bottle media were flushed with 5% CO₂ after each examination.

The conventional test media were examined macroscopically and microscopically using an AO Quebec Colony Counter, a dissecting microscope (3×, 10×, 30×), and an AO light microscope (100×) in the following manner: Herrold's egg-yolk plate and tube media (3×, 10×, 30×), Middlebrook 7H10 plate medium (3×, 10×, 30×, 100×), and Middlebrook 7H10 broth and agar tubes (10×, 30×). Herrold's egg-yolk plate medium could not be microscopically examined at 100× because it is an opaque medium.

RESULTS

Comparison of viable counts with GI readings indicated that a 7H12 radiometric broth GI reading of two had a mean bacterial plate count of 3625 ml⁻¹; with a reading of eight, the mean bacterial plate count was 37 150 ml⁻¹.

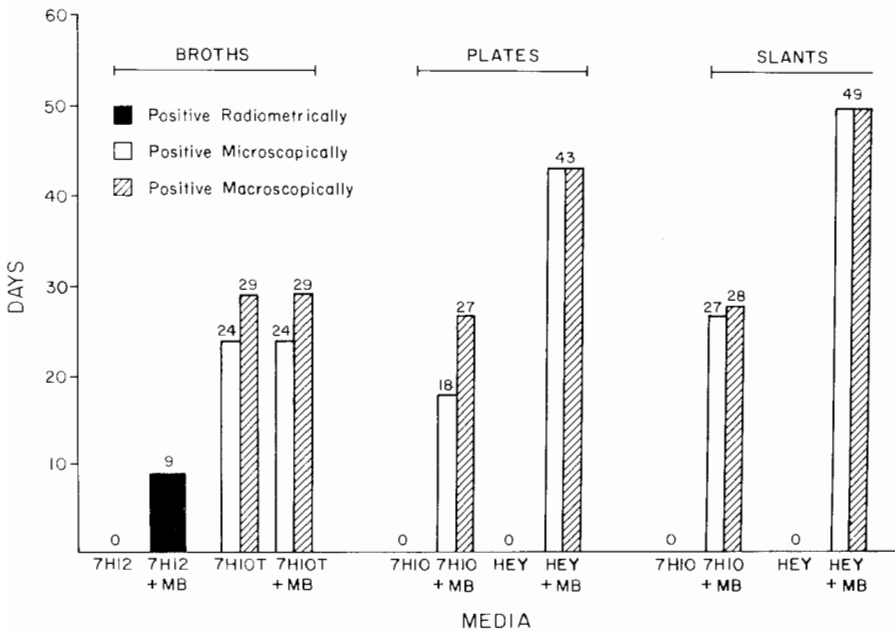


Fig. 3. Mean detection time of *M. paratuberculosis* using several media. Middlebrook 7H10 agar was examined microscopically at 3×, 10×, 30×, 100× and tubes, slants, and Herrold's egg-yolk medium at 3×, 10×, 30×. 7H12, BACTEC radiometric broth; 7H10 T, Middlebrook 7H10 Tween broth; 7H10, Middlebrook 7H10 agar, HEY, Herrold's egg-yolk medium; MB, mycobactin J.

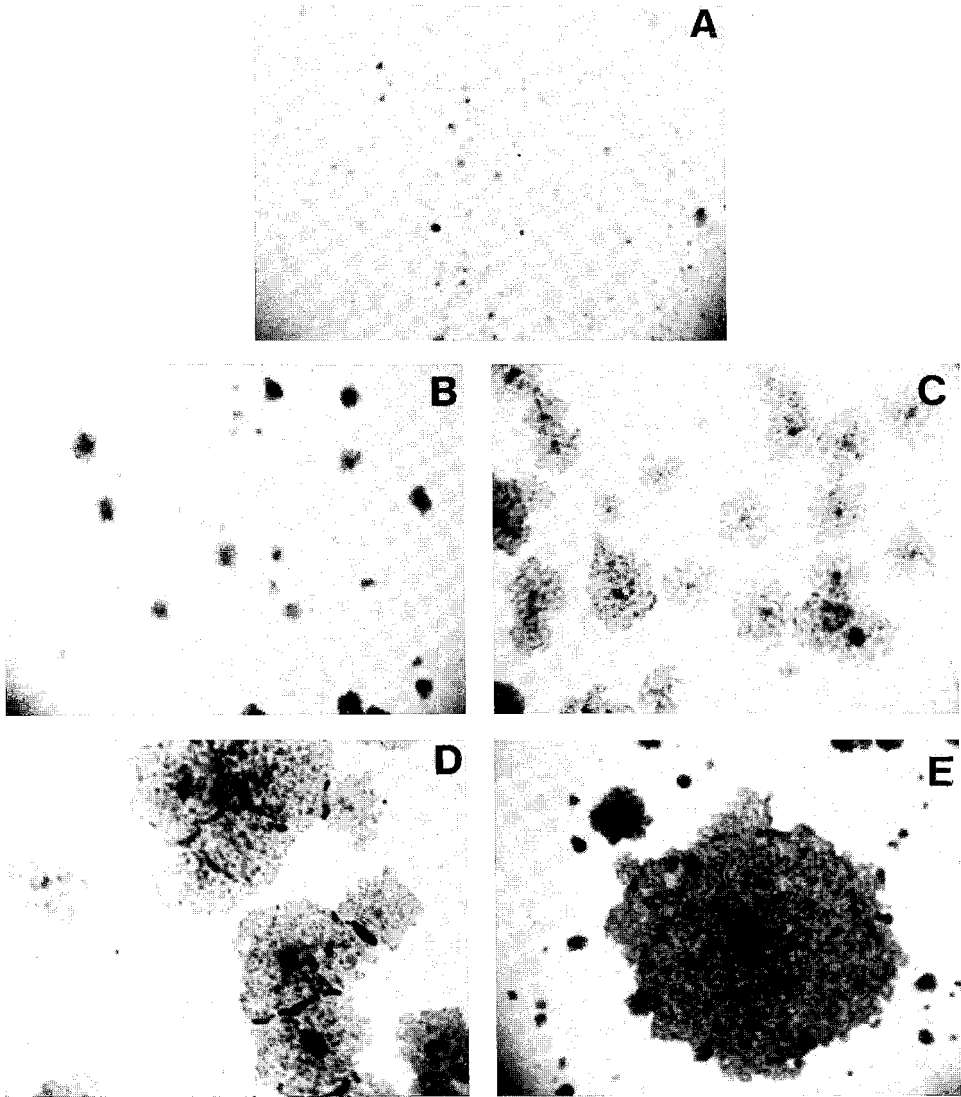


Fig. 4. Typical *M. paratuberculosis* colonial morphology ($100\times$) with translucent Middlebrook 7H10 agar containing $2\ \mu\text{g ml}^{-1}$ mycobactin J: A, 10 days; B, 14 days; C, 21 days; D, 28 days; E, 35 days.

Figure 2 illustrates the growth curves, as assessed by GI readings, observed when different volumes of mycobactin diluted in 70 or 95% ethanol were used to enrich the radiometric broths. An increase in the volume of the supplement depressed the GI reading, and the GI readings were most severely depressed when the mycobactin was reconstituted with 95% ethanol (Fig. 2b).

Mean detection times for each of the media tested are shown in Fig. 3. Except

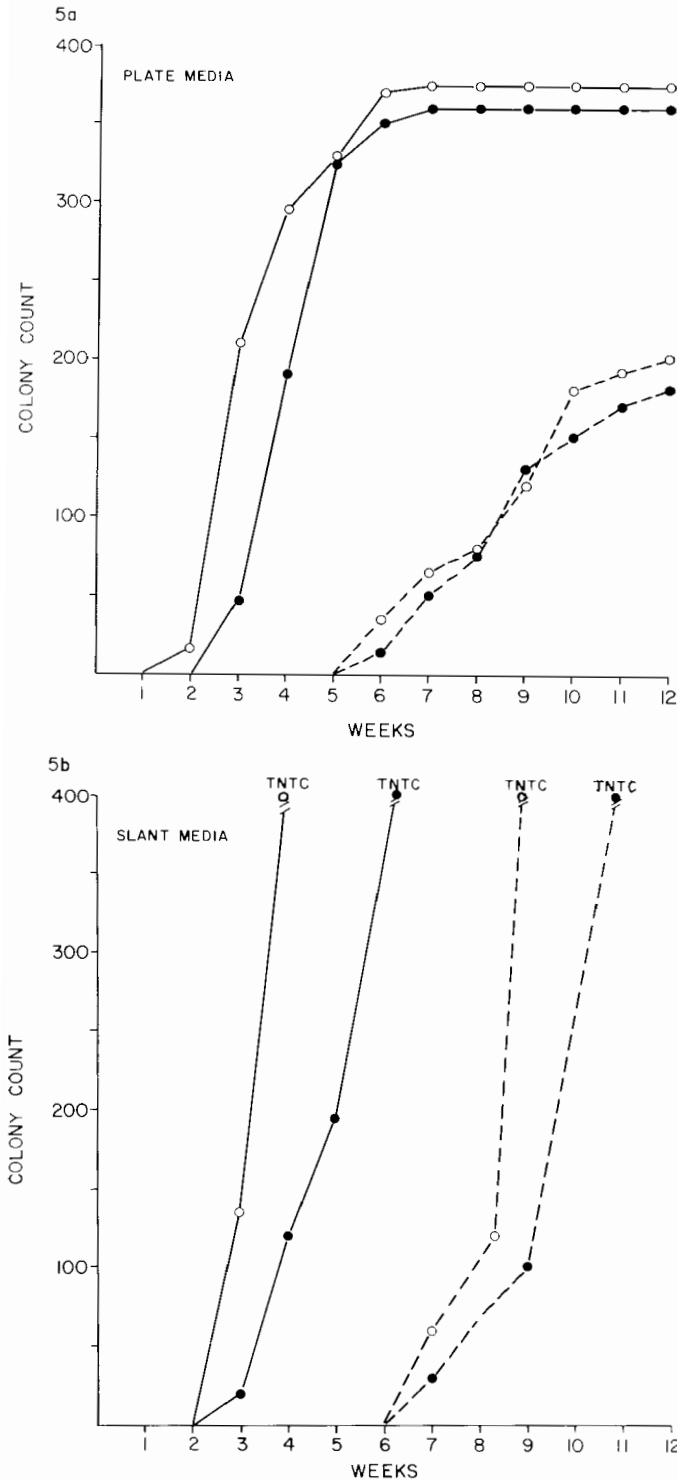


Fig. 5. Mean colony count for *M. paratuberculosis* grown on various media. 7H10 (microscopic), ○—○; 7H10 (macroscopic), ●—●; Herrold's egg-yolk (microscopic) ○--○; Herrold's egg-yolk (macroscopic), ●--●; TNTC, too numerous to count accurately. All control media without mycobactin were interpreted as negative.

for the 7H10 T negative growth control broths, all other controls without mycobactin did not support the growth of *M. paratuberculosis*. The 7H12 radiometric media with mycobactin J had the earliest mean detection time (9 days). Figure 4 illustrates the typical colony morphology of *M. paratuberculosis* on 7H10 agar supplemented with mycobactin J.

The colony count data for the various solid media are found in Fig. 5. All negative control media without mycobactin supplementation had little or no growth present. Any residual growth present on the negative growth control media was less than 10 colonies per plate. The colonies were less than 0.5 mm in diameter and exhibited atypical morphology. These findings were interesting as negative when compared with the large number of colonies noted on the mycobactin-supplemented media. Of the agar media evaluated, the 7H10 plate and slant media supplemented with mycobactin provided the earliest detection, at 23 and 28 days, respectively, as well as the highest bacterial plate counts.

Microscopic examination of the 7H10 agar plates was accomplished by viewing the surface of the agar from the reverse side. The 7H10 slant media were more difficult to read microscopically than the 7H10 plate media. The 7H10 T broths had a mean macroscopic and microscopic ($10\times$, $30\times$) detection time of 29 and 24 days, respectively. The 7H10 T broths were unique in that all the *M. paratuberculosis* isolates tested were able to grow actively in these media in the absence of mycobactin J. Auramine stain and subculture results from these broths confirmed the fact that the organisms growing in the broths were *M. paratuberculosis* and that the isolates still demonstrated their mycobactin dependence when subcultured on solid media with and without mycobactin.

Herrold's egg-yolk slant and plate media required the longest incubation prior to detecting *M. paratuberculosis* growth and had lower plate counts.

DISCUSSION

Of the media studied, the BACTEC radiometric 7H12 broth had the earliest detection time with clinical isolates (9 days). Plate-count studies to determine the sensitivity of the radiometric system indicated that the mean number of organisms to produce a GI of two was 3625 ml^{-1} . The GI reading of two was chosen because it was the first observation point that could be accurately determined. The GI reading of eight ($37\ 150\text{ ml}^{-1}$) provided an additional accurate observation point to evaluate growth. Increasing volumes of the recommended working solution supplement of mycobactin (Merkal et al., 1968; Merkal, 1970, 1983) had an inhibitory effect on the growth of *M. paratuberculosis*. Although the higher concentration of mycobactin cannot be eliminated as a possible growth inhibitor, the known toxicity of ethanol on the growth of mycobacteria was evident when comparing the growth of this organism in 7H12 media supplemented with mycobactin J, diluted in 70 and 95% ethanol (Table 1, Fig. 2). The toxic effects of ethanol were probably enhanced by the small (2

ml) volume of 7H12 broth medium and the ability of the very sensitive radiometric test system to detect small changes in metabolic activity. It should be noted that the negative radiometric growth controls were interpreted as negative because GI readings never exceeded 50 and were 0 within 2 weeks. This residual metabolism was probably caused by the exhaustion of residual iron ions found in the test system.

Semi-quantitative turbidimetric procedures using McFarland nephelometer standards (Sommers and Good, 1985) were used to evaluate the growth of *M. paratuberculosis* in 7H10 T broth. These procedures were found to be satisfactory for determining the growth of pure cultures of this organism, but would not be feasible with actual clinical specimens because of the high background turbidity of fecal and tissue specimens subsequent to processing. Furthermore, precise growth determination in broth cultures would necessitate the use of spectrophotometric methods, which are time consuming and inconvenient, when attempting to process large numbers of clinical specimens. However, 7H10 T broth was found to be an excellent medium for the cultivation of pure cultures of this organism. The observation that 7H10 T broth without mycobactin supplementation supported the growth of *M. paratuberculosis* was probably as a result of the presence of Tween 80. This compound, acting as a dispersant or surfactant, probably enabled *M. paratuberculosis* to acquire essential iron directly from the ferric ammonium citrate ($40 \mu\text{g ml}^{-1}$) present in the medium (Macham et al., 1975).

Of the agar-based media, 7H10 plate medium gave the earliest mean detection time, because of the ease with which microscopic colonies could be detected on this translucent medium (Fig. 4).

Herrold's egg-yolk medium had the longest detection time, and plate counts were approximately one-half those noted for 7H10 media. Although this may be attributed to differences in media composition, the lower colony counts may also have been influenced by the surface irregularities and the opaque character of the Herrold's egg-yolk medium, factors found to obscure early growth. Continued incubation of these plates could have resulted in high colony counts, but additional incubation would further reduce the usefulness of this medium when compared with the other systems studied. Herrold's egg-yolk medium was also more difficult to prepare because of the use of large numbers of fresh eggs and, even with good quality control, contamination rates were higher than that noted from Middlebrook media.

The limited growth noted on the negative growth controls for the Middlebrook and Herrold's agar test systems never exceeded 10 colonies per plate and demonstrated atypical morphology when compared with the growth seen in Fig. 4. This growth was most likely a result of the scavenging of iron from other dying cells and the consumption of small amounts of residual iron found in the test system

This study provided data concerning the minimum number of organisms

required to produce a positive radiometric culture and demonstrated that using the correct supplement volume/alcohol concentration was essential for optimum test system performance. Moreover, 7H10 T broth was shown to be a good growth medium for this organism. Microscopic examination of the translucent Middlebrook 7H10 agar detected growth of *M. paratuberculosis* in approximately half the time required by the more commonly used Herrold's egg-yolk medium, thereby documenting the potential usefulness of this medium for the isolation and cultivation of *M. paratuberculosis*.

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