Comparison of Four Different Culture Media for Isolation and Growth of Type II and Type I/III *Mycobacterium avium* subsp. *paratuberculosis* Strains Isolated from Cattle and Goats

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Culture is considered the definitive technique for Johne’s disease diagnosis, and it is essential for later applications of certain molecular typing techniques. In this study, we have tested four solid media (Herrold’s egg yolk medium [HEYM] with sodium pyruvate and mycobactin [HEYMm-SP], HEYM with mycobactin and without sodium pyruvate [HEYMm], Middlebrook 7H11 with mycobactin [Mm], and Löwenstein-Jensen with mycobactin [LJm]) for isolation of *Mycobacterium avium* subsp. *paratuberculosis* strains in 319 tissue samples from cattle herds and goat flocks. We have shown that each of the two main groups of *M. avium* subsp. *paratuberculosis* (type II and type I/III) has different requirements for growth in the culture media studied. The recommended solid media for isolation of type I/III strains are LJm and Mm, since the combination of both media allowed the recovery of all these strains. The most widespread culture medium, HEYM, is not suitable for the isolation of this group of *M. avium* subsp. *paratuberculosis* strains. Regarding the type II strains, HEYMm-SP was the medium where more strains were isolated, but the other three media are also needed in order to recover all type II strains. The incubation period is also related to the strain type. In conclusion, because the type of strain cannot be known in advance of culture, coupled with the fact that cattle and goats can be infected with both groups of strains, we recommend the use of the four solid media and the prolongation of the incubation period to more than 6 months to detect paratuberculous herds/flocks and to determine the true prevalence of the infection.

Paratuberculosis (Johne’s disease) is a chronic inflammatory enteritis of domestic ruminants that has been recognized to be present worldwide but with important differences in prevalence (20). The etiological agent is *Mycobacterium avium* subsp. *paratuberculosis*, which is included in the *M. avium* complex (43). *M. avium* subsp. *paratuberculosis* has also been isolated from intestinal samples or other tissues of wild ruminant species, such as red deer, fallow deer, and mule elk (9, 34); a wide variety of nonruminant species, such as rabbit, hare, fox, stoat, weasel, crow, rook, jackdaw, raccoon, rat, wood mouse, badger, and wild boar (2, 3, 10, 17, 30); and human beings (4, 16, 32). This bacterium is resistant to environmental conditions and can be isolated from water (37), pastures (50), and insects (15).

Culture is considered the only definitive and critical technique for Johne’s disease diagnosis (5, 31, 49), and it is an essential step for later application of the standardized molecular typing techniques IS900 restriction fragment length polymorphism (IS900-RFLP) (35) and pulsed-field gel electrophoresis (PFGE) (42). Characterization of the strains is needed to understand the pathogenicity and epidemiology of this fastidious bacterium. Since the first in vitro isolation of *M. avium* subsp. *paratuberculosis* (44), the culture protocol and media have been improved, although there are still two major problems: (i) the long incubation periods required to obtain visible colonies (5, 45), especially when working with animals infected by extremely slow growing isolates (12, 14, 36), and (ii) the failure to isolate *M. avium* subsp. *paratuberculosis* isolates in some samples, depending on the host (23), the clinical sample (tissue or feces) (41, 46, 48, 51), and the disease stage (clinical or subclinical) (19, 25).

Several media are used for the primary isolation of *M. avium* subsp. *paratuberculosis*: (i) egg-based media like the Herrold’s egg yolk medium (21) and Löwenstein-Jensen medium (19); (ii) serum-based media such as Dubos medium (39); and (iii) synthetic media, for example, Middlebrook (11) or Watson-Reid (29) medium.

There are few studies on the efficacy of culture media for the isolation of *M. avium* subsp. *paratuberculosis*. Most of the studies regarding culture of this pathogen refer to host of origin instead of the type of *M. avium* subsp. *paratuberculosis* strain. Moreover, the design of these studies is variable with regard to the source of samples and media used, making the comparison of results difficult and hampering a definitive criterion for paratuberculosis diagnosis by culture.

*M. avium* subsp. *paratuberculosis* strains have been classified into three groups based on culture characteristics and molecular characterization by PFGE and IS900-RFLP (types I, II, and III) (7, 13, 35, 42). Type II *M. avium* subsp. *paratuberculosis* strains are slow growers that were first described in cattle and have subsequently been isolated from a wide variety of hosts and geographical locations (35, 42) and can be clustered by the PCR method described previously by Collins et al. (6).

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To our knowledge, the *M. avium* subsp. *paratuberculosis* isolates from Crohn’s disease patients characterized so far belong to type II (35). Types I and III, extremely slow growing strains, were originally described in sheep (7), although lately, there have been a few reports of their isolation in cattle and goats (12, 13, 52). Types I and III are difficult to culture and subculture, sometimes failing to give PFGE and IS900-RFLP profiles. These types can be differentiated from the type II strains by the PCR method described by Collins et al. (6); however, they cannot be distinguished from each other, and they are therefore referred to as type I/III *M. avium* subsp. *paratuberculosis* strains (12).

These differences in the epidemiology, pathogenesis, and growth rate have not been studied in detail in the context of growth requirements for the isolation of this bacterium. Therefore, the objectives of this study were (i) to test several solid culture media available for the isolation of *M. avium* subsp. *paratuberculosis* strains from bovine and caprine samples to recommend an appropriate solid medium or combination of solid media for the isolation of the different types of *M. avium* subsp. *paratuberculosis* strains and (ii) to study the differential requirements for isolation depending on the *M. avium* subsp. *paratuberculosis* type infecting the animal, regardless of the host.

### MATERIALS AND METHODS

**Samples.** The present study has been performed using a total of 319 tissue samples (pool of ileocecal valve, intestine, and mesenteric lymph node) from cattle (*n* = 122) and goats (*n* = 197). The samples received in the laboratory for diagnosis were obtained from animals that tested positive by the ELISA Parachek test (C.S.L. Ltd.). The samples were obtained from different areas of Spain (Madrid, Castilla-La Mancha, Castilla y León [central, central south, and central north, respectively], and the Canary Islands), and the total number of farms analyzed was 36 (8 cattle herds and 28 goat flocks).

**Bacteriological protocol and culture media.** The samples were homogenized and decontaminated with hexadecylpyridinium chloride as described previously (18). After the protocol was followed, the pellet was resuspended in 0.5 ml of sterile distilled water, and 100 μl was inoculated onto one slope of four specific media supplemented with 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO) and Selectatabs (code MS 24; MAST Laboratories Ltd., Merseyside, United Kingdom) to a final concentration of 200,000 U/liter of polymyxin B, 100 mg/liter of carbenicillin, 10 mg/liter of amphotericin B, and 10 mg/liter trimethoprim. The four media used in the study were Herrold’s egg yolk medium with mycobactin and sodium pyruvate (HEYMm-SP), Herrold’s egg yolk medium with mycobactin and without sodium pyruvate (HEYMm), Middlebrook 7H11 medium with mycobactin (Mm), and Löwenstein-Jensen medium with mycobactin (LJm) (Biomedics, S.L., Madrid, Spain).

Two incubations were performed, at 37°C and checked for growth every 15 days. All suspected colonies, visualized with the naked eye, were stained for acid-alcohol fastness by the Ziehl-Neelsen technique and subcultured in HEYM with and without mycobactin to determine their mycobactin dependence. Bacterial growth was recovered from the tubes, suspended in sterile purified water, heated inactivated, and subjected to a PCR analysis for identification aimed at the insertion sequence IS900 (22, 28) and the sequence 157 (38).

**Classification of *M. avium* subsp. *paratuberculosis* into type II and type I/III strains by a PCR-based test.** All the *M. avium* subsp. *paratuberculosis* isolates from cattle and goats were analyzed by PCR to discriminate between type II and type I/III *M. avium* subsp. *paratuberculosis* strains as described previously by Collins et al. (6).

Statistical calculations were performed by a cross-tabulation analysis using SPSS software package, version 11.0 (SPSS Inc., Illinois).

### RESULTS

**M. avium subsp. paratuberculosis strains.** One hundred sixty-three *M. avium* subsp. *paratuberculosis* isolates (89 from cattle and 74 from goats) were isolated from the 319 tissue samples analyzed by culture (72.9% and 37.6% from the cattle and goats samples, respectively). These strains were obtained from 26 farms (7 cattle herds and 19 goat flocks) from different geographical areas of the country.

**Classification of *M. avium* subsp. *paratuberculosis* into type II and type I/III strains by PCR tests.** Fifty out of the 89 *M. avium* subsp. *paratuberculosis* isolates from cattle belonged to the type II *M. avium* subsp. *paratuberculosis* strains (56.2%), and the rest of the isolates (*n* = 39) were type I/III *M. avium* subsp. *paratuberculosis* strains (43.8%). Regarding the *M. avium* subsp. *paratuberculosis* isolates from goats, 75.7% belonged to the type II strains (*n* = 56), and 24.3% belonged to the type I/III strains (*n* = 18).

**Culture.** An animal was considered to be infected with *M. avium* subsp. *paratuberculosis* if *M. avium* subsp. *paratuberculosis* was isolated on any single medium. Differences in culture medium performance were detected depending on the type of *M. avium* subsp. *paratuberculosis* strain. Eighty-six percent of *M. avium* subsp. *paratuberculosis* type I/III strains grew exclusively in one culture medium, whereas 62.3% of type II *M. avium* subsp. *paratuberculosis* strains grew in more than one culture medium (Table 1). Growth of type I/III strains in LJm and that of type II strains in HEYMm-SP were statistically significant (*P* = 0.001 and *P* = 0.000, respectively; 95% confidence interval). The results of recovery of *M. avium* subsp. *paratuberculosis* strains in different culture media are shown in Fig. 1.

(i) *M. avium* subsp. *paratuberculosis* isolates from cattle. (a) Type I/III strains. A total of 64.1 and 25.6% of the type I/III *M. avium* subsp. *paratuberculosis* isolates grew exclusively in LJm and Mm, respectively, and 10.3% grew in both media (Table 1). The incorporation of these two media allowed the detection

### TABLE 1. Isolation of type I/III and type II *M. avium* subsp. *paratuberculosis* strains from cattle and goats in four solid media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cattle Type I/III</th>
<th>Cattle Type II</th>
<th>Goats Type I/III</th>
<th>Goats Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall % isolation</td>
<td>% Isolation exclusively for medium</td>
<td>Overall % isolation</td>
<td>% Isolation exclusively for medium</td>
</tr>
<tr>
<td>LJm</td>
<td>74.4</td>
<td>64.1</td>
<td>44.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Mm</td>
<td>35.9</td>
<td>25.6</td>
<td>66.0</td>
<td>8.0</td>
</tr>
<tr>
<td>HEYMm-SP</td>
<td>0.0</td>
<td>0.0</td>
<td>80.0</td>
<td>18.0</td>
</tr>
<tr>
<td>HEYMm</td>
<td>0.0</td>
<td>0.0</td>
<td>44.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*P* 0.000, respectively; 95% confidence interval.
of all *M. avium* subsp. *paratuberculosis* isolates obtained from cattle in this study (Fig. 1A).

(b) **Type II strains.** The type II *M. avium* subsp. *paratuberculosis* strains grew on the four solid media tested. The combination of HEYMm-SP and Mm was the most efficient, with isolation of 96% of *M. avium* subsp. *paratuberculosis* strains (Fig. 1A).

(ii) **M. avium** subsp. *paratuberculosis* isolates from goats. (a) **Type I/III.** As described above for cattle, the type I/III *M. avium* subsp. *paratuberculosis* isolates grew mostly in LJm and Mm (Table 1).

(b) **Type II.** The type II *M. avium* subsp. *paratuberculosis* isolates can grow in the four solid media employed. The best combination of two media was HEYMm-SP and LJm, since isolation was accomplished in 91.1% of strains (Fig. 1B).

### TABLE 2. Incubation period of *M. avium* subsp. *paratuberculosis* strains belonging to type I/III or type II

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Incubation period in mo (t)</th>
<th>No. of type I/III <em>M. avium</em> subsp. <em>paratuberculosis</em> strains (%)</th>
<th>No. of type II <em>M. avium</em> subsp. <em>paratuberculosis</em> strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t ≤ 3</td>
<td>11 (28.2)</td>
<td>35 (70)</td>
<td></td>
</tr>
<tr>
<td>3 &lt; t ≤ 5</td>
<td>10 (25.6)</td>
<td>11 (22)</td>
<td></td>
</tr>
<tr>
<td>t ≥ 5</td>
<td>18 (46.2)</td>
<td>4 (8)</td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t ≤ 3</td>
<td>5 (27.8)</td>
<td>34 (60.7)</td>
<td></td>
</tr>
<tr>
<td>3 &lt; t ≤ 5</td>
<td>7 (38.9)</td>
<td>10 (17.9)</td>
<td></td>
</tr>
<tr>
<td>t ≥ 5</td>
<td>6 (33.3)</td>
<td>12 (21.4)</td>
<td></td>
</tr>
</tbody>
</table>
Incubation period and growth characteristics. Approximately 70% of the type I/III \textit{M. avium} subsp. \textit{paratuberculosis} strains took more than 3 months to grow (Table 2), 14 (50\%) of which took up to 7 months to yield visible colonies in the solid culture media. However, most of the type II \textit{M. avium} subsp. \textit{paratuberculosis} strains (70\% and 60.7\% in cattle and goats, respectively) grew in less than 3 months (Table 2).

The type of growth was also distinctive for each solid medium. The colonies in LJm are very small, usually in a high number distributed throughout the entire surface of the medium, and difficult to be observed macroscopically compared with the colonies in the other culture media. In Mm, HEYMM-SP, and HEYMM, the colonies are rougher, larger, and more easily observed macroscopically.

**DISCUSSION**

In this study, we have tested four solid media (LJm, Mm, HEYMM, and HEYMM-SP) described in the literature for isolation of \textit{M. avium} subsp. \textit{paratuberculosis} from domestic ruminants. We have shown that \textit{M. avium} subsp. \textit{paratuberculosis} strains form a heterogeneous group with regard to the culture characteristics and that each of the two large groups of \textit{M. avium} subsp. \textit{paratuberculosis} as classified by PCR (6) have different requirements to grow in the culture media studied.

From our results, the two recommended solid media for isolation of type I/III \textit{M. avium} subsp. \textit{paratuberculosis} strains from cattle and goats are LJm and Mm. HEYM, the most widespread medium used in diagnostic laboratories, is not suitable for the isolation of this group of \textit{M. avium} subsp. \textit{paratuberculosis} strains. In this study, only one isolate of caprine origin yielded a positive result in this medium.

Regarding the type II strains, which are relatively easier to isolate, HEYMM-SP was the medium in which more strains were isolated; however, the second-best culture medium varied depending on the animal host from which it was isolated (Mm in cattle [66\%] and LJm in goats [44.6\%]).

In some laboratories, isolation of \textit{M. avium} subsp. \textit{paratuberculosis} is carried out using different media according to the species of origin of the samples. For bovine samples, Adúriz et al. (1) and Nielsen et al. (33) recommended the use of HEYM as the primary medium for detection of \textit{M. avium} subsp. \textit{paratuberculosis}. This could be certain only for type II \textit{M. avium} subsp. \textit{paratuberculosis} strains, but as described previously, cattle can be infected with type I/III \textit{M. avium} subsp. \textit{paratuberculosis} strains (12, 52), and therefore, incorporation of media suitable for both groups of \textit{M. avium} subsp. \textit{paratuberculosis} strains is necessary.

Regarding ovine samples, Adúriz et al. (1) recommended LJ without sodium pyruvate and with mycobactin J. In our experience, for testing of samples from cattle and goats infected with type I/III, which are the most frequent in sheep (24, 52), LJm without sodium pyruvate has also been the most efficient medium. However, LJm should be used in combination with Mm, as 25.6 and 33.3\% of type I/III isolates from cattle and goats, respectively, grew only in this medium.

The incorporation of sodium pyruvate into the media enhances the growth of most \textit{M. avium} subsp. \textit{paratuberculosis} strains and reduces the incubation period (26, 27, 47). In this study, we used just one medium to evaluate the effect of sodium pyruvate (HEYMM-SP versus HEYMM). In the case of type II \textit{M. avium} subsp. \textit{paratuberculosis} strains, the addition of sodium pyruvate to the HEYMM increased the recovery of isolates by 36 and 30.3\% in cattle and goats, respectively. We cannot evaluate the effect of sodium pyruvate in type I/III strains because the HEYM did not support growth of these strains.

Whittington et al. (49) recommended the BACTEC 12B radiometric medium and Middlebrook 7H10 and 7H11 agars for bacteriological culture from sheep. The sensitivity of detection of \textit{M. avium} subsp. \textit{paratuberculosis} in solid media was slightly lower than that obtained using modified BACTEC 12B radiometric medium. In that study, those authors also concluded that both egg yolk and mycobactin J were essential additives for the growth of ovine strains of \textit{M. avium} subsp. \textit{paratuberculosis} in both liquid and solid media. In our study, type I/III \textit{M. avium} subsp. \textit{paratuberculosis} strains grew better in LJm than in the Mm, HEYMM, and HEYMM-SP. One of the main differences between the four media is the presence and concentration of egg yolk, pointing out that type I/III strains grow better in medium with egg yolk, but it is not essential, since 66\% and 28.6\% of type I/III \textit{M. avium} subsp. \textit{paratuberculosis} strains isolated from cattle and goats, respectively, grew in a medium without egg yolk (Mm).

The requirements for isolation of \textit{M. avium} subsp. \textit{paratuberculosis} strains by culture are directly related to the type of \textit{M. avium} subsp. \textit{paratuberculosis} strain and not to the host (animal species), as has previously been accepted (8, 14, 36). This fact has to be taken into account when a protocol for the isolation of \textit{M. avium} subsp. \textit{paratuberculosis} is designed, since the use of inappropriate media affects the detection rate and therefore leads to false-negative results. The limited use of Löwenstein-Jensen medium for culture may be a reason for the limited findings of \textit{M. avium} subsp. \textit{paratuberculosis} type I/III other than in sheep. The use of this medium allowed the detection of type I/III \textit{M. avium} subsp. \textit{paratuberculosis} strains in 28.6 and 47.4\% of the \textit{paratuberculosis}-infected cattle herds and goat flocks. The extended use may help to identify a broader host range for these strains.

Another factor that has to be taken into account is the incubation period. In general, the incubation period when working with solid media is 3 months for bovine samples (5), although when working with small ruminants, the incubation period increases up to 6 months (8). In this study, we have described type I/III \textit{M. avium} subsp. \textit{paratuberculosis} isolates in cattle that took more than 3 months to grow (71.8\%), and a large proportion (42.8\%) of these isolates took more than 6 months to grow. This is another isolation feature that depends on the strain type and not the host origin of the sample. Therefore, we recommend increasing the incubation period up to 8 months for all animal species the first time a farm is sampled to avoid false-negative results due to a relatively short incubation period.

Even though diagnosis of paratuberculosis by conventional culture is tedious and requires a long incubation period, it is still considered the “gold standard” technique. It is important to optimize the culture protocol to detect all the infected animals regardless of the type of the strain infecting the animal. We have shown that the growth requirements for the two main groups of \textit{M. avium} subsp. \textit{paratuberculosis} classified by
PCR (6) are different: type I/III strains grow better in LJm, and type II strains grow better in HEYMm-SP. This fact is important for some laboratories that use only one of these culture media for primary isolation of M. avium subsp. paratuberculosis and, therefore, false-negative results are obtained, and infections with types I/III are not detected. Because the type of strain cannot be known in advance, we recommend the use of at least the solid media LJm, MM, and HEYM-SP as well as extending the incubation period up to 8 months. The combination of these three media allowed the recovery of the 100% of type I/III strains and 98 and 94.6% of type II M. avium subsp. paratuberculosis strains in cattle and goats, respectively. In order to recover 100% type II strains, we also recommend the incorporation of HEYMm. The use of the appropriate media is essential for the detection of paratuberculosis in herds or flocks to determine the true prevalence of the infection in a farm and to establish the need to apply control programs.

Moreover, the overall sensitivities of cultures in this study were 72.9 and 37.6% in cattle and goats, respectively, and are in agreement with data from previous studies (14, 40, 48). This difference in the isolation may indicate the difficulty of the isolation of a certain group of strains because of several factors that must be studied, such as very limited bacterial burden in the sample, conditions of preservation, increased sensitivity to the decontamination protocol, specific nutritional requirements, and increased sensitivity to the inhibitory effect of antibiotics in the media. For this reason, the use of several culture media would also be appropriate in attempts to recover M. avium subsp. paratuberculosis isolates from samples from Crohn’s disease patients.

Further research is needed to understand the growth requirements of the different M. avium subsp. paratuberculosis strains from different sources and environments. The progress in the knowledge of the culture of these bacteria, coupled with molecular diagnosis, could be essential in the disclosure of several intestinal disorders in domestic and wild animals, including Crohn’s disease in humans. This knowledge would help to better understand the epidemiological relevance of the presence of M. avium subsp. paratuberculosis in the environment.

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