

## Comparison of Two Commercial Formulations of the MacConkey Agar Test for Mycobacteria

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Recent evaluations of the MacConkey agar test for differentiation of rapidly growing mycobacteria have revealed that certain strains of *Mycobacterium fortuitum* and *M. chelonae* that were expected to grow on MacConkey agar failed to do so. Investigation of two formulations of MacConkey agar showed that these two species grew better on the medium when the crystal violet dye was omitted. Several possible reasons for this difficulty are discussed. It is recommended that clinical laboratories engaged in differential identification of mycobacteria utilize commercial MacConkey agar without crystal violet when testing rapidly growing species of this genus.

The differential inhibition of certain mycobacteria by MacConkey agar (2) has been one method of value in the taxonomic separation of rapidly growing acid-fast bacilli (2, 3, 5, 6, 10). *Mycobacterium fortuitum* (9) and the two subspecies of *M. chelonae* (6), commonly regarded as the potentially pathogenic fast-growing mycobacteria, usually grow on MacConkey agar, whereas the normally saprophytic species are inhibited by this medium.

A recent evaluation of clinical laboratories in Oregon (Charles E. Gardner, personal communication) revealed that those laboratories that utilized MacConkey agar without crystal violet generally observed growth of the species *M. fortuitum*, whereas those laboratories employing the same medium with the dye often failed to grow this organism.

Stanford et al. (10) reported some strains of *M. chelonae*, notably those observed in Europe, grew poorly if at all on MacConkey agar, whereas strains isolated in the United States grew well on the medium. Observations in our own laboratory revealed that a number of strains that earlier had grown on MacConkey agar were now unable to do so. Many of these strains formerly had been segregated as Rapid Grower A (3), a group closely related to *M. peregrinum*, which is a species now incorporated with *M. fortuitum* (6). Because a number of Rapid Grower A isolates were inhibited by the mixture of methylated pararosanilines referred to as methyl violet (3), and because the more purified hexamethyl pararosaniline crystal violet (7) is a component of certain formulations of MacConkey agar, we reasoned that

either the dye or the present inclusion of *M. peregrinum* in the taxon *fortuitum* (or a combination of both) might be contributing to the currently recognized failures of the once-reliable MacConkey agar test for differential identification of certain rapidly growing mycobacteria.

In an attempt to quickly reinstate the validity of the MacConkey agar test, before more time-consuming studies would hopefully reveal the true reason for its failure, the present study was undertaken to determine if MacConkey agar without crystal violet could be substituted for the dye-containing medium with a restoration of expected differential growth patterns for mycobacteria.

### MATERIALS AND METHODS

**Media.** Since we became aware of this problem, comparative studies on selected strains of rapidly growing mycobacteria have been carried out on four different lots of MacConkey agar with crystal violet (MCV) obtained from two commercial sources, as well as three lots of MacConkey agar without crystal violet (MA). Our largest comparative study, presented here, was conducted with media from only one supplier, but comparable results (with fewer strains) have been observed on all preparations of this medium. MacConkey agar (Difco) with and without crystal violet (both of which represent a modification of the original formula of MacConkey [8]), were prepared according to directions and poured into plastic petri dishes (15 by 100 mm) as described earlier (4).

**Cultures.** Sixty-six cultures of mycobacteria, obtained either from the authors' collection (Trudeau Mycobacterial Culture Collection) or from a previous international cooperative study (6), were examined. The species distribution was as follows: five strains of *M. chelonae* and 10 of *M. chelonae* subsp. *abscessus*

(6); 25 strains of *M. fortuitum* of which 13 previously were identified as *M. peregrinum*; two strains of *M. diernhoferi*; 6 of *M. flavescens*; 3 of *M. phlei*; 8 of *M. smegmatis*; and 7 strains of *M. vaccae*.

**Test procedure.** For each strain tested, one 3-mm loopful of organisms, obtained from a 7-day culture in enriched Middlebrook 7H-9 broth (Difco), was inoculated to both plates of MacConkey agar, using the petri dish turntable method (3, 4). All plates were incubated at 37 C in polyethylene bags; some few strains, which appeared to grow better at 33 C, were incubated at both temperatures. Plates were examined from 5 to 14 days, and growth was recorded as before (3). Cultures that failed to react as expected were retested to confirm the observed results. Plates of Dubos oleic-albumin agar (Difco) inoculated with each culture served as controls on colony type of test organisms.

## RESULTS AND DISCUSSION

The comparative growth of all cultures on the two preparations of MacConkey agar is summarized in Table 1.

Forty of the 66 cultures tested (25 strains of *M. fortuitum* and 15 strains of *M. chelonae*) were expected to grow on MacConkey agar. Of these, 28 strains (15 of *M. fortuitum* and 13 of *M. chelonae*) grew on both types of MacConkey agar, while five strains (three *M. fortuitum* and two *M. chelonae*) grew on neither medium. Seven strains grew only on MA. All three of the strains of *M. fortuitum* that failed to grow on either agar, and five of the seven that grew only on MA, had been segregated earlier as *M. peregrinum* (now part of the taxon *M. fortuitum*). The former taxon *M. peregrinum*, closely related to bacilli once referred to as Rapid Grower A, had been shown earlier to be inhibited by a related methyl violet dye (3); hence, we were not too surprised by these results.

TABLE 1. Comparison of growth of mycobacteria on MacConkey agar with and without crystal violet<sup>a</sup>

		No crystal violet	
		Growth	No growth
Crystal violet	No growth	<i>M. fortuitum</i> (7) <i>M. smegmatis</i> (2)	<i>M. chelonae</i> (2) <i>M. fortuitum</i> (3) <i>M. diernhoferi</i> (2) <i>M. flavescens</i> (6) <i>M. phlei</i> (3) <i>M. smegmatis</i> (6) <i>M. vaccae</i> (7)
	Growth	<i>M. chelonae</i> (13) <i>M. fortuitum</i> (15)	

<sup>a</sup> The number of strains which grew, or failed to grow, on the two kinds of MacConkey agar are indicated parenthetically after each species name.

The important observation was that seven strains of *M. fortuitum* grew only on MA, and these results were significant at the 5% level by the chi-square test. Thus, for the diagnostic bacteriology laboratory, which is often asked to comment on the potential clinical significance of patient isolates, it is recommended that MA be used in place of MCV until more definitive evidence reveals the true reason for the failure of MCV in recent trials.

Such action is not without problems, however, for it can be seen that two strains of *M. smegmatis* also grew, even though poorly, on MA. The diagnostic difficulties posed by the rare isolation from man of *M. smegmatis* may be obviated by the concomitant performance of a 3-day arylsulfatase test; almost all strains of *M. fortuitum* and *M. chelonae* are positive in this test, whereas *M. smegmatis* rarely, if ever, is able to hydrolyze the substrate (5, 6, 11).

It is difficult to ascertain why some strains of *M. fortuitum* and *M. chelonae* no longer grow on MCV; however, it is interesting to draw from past experience and to speculate on certain components of the media as likely contributors to the problem. Examination of the formulas for MCV and MA reveals differences in content or composition of the following: peptones, bile salts, neutral red, agar, and final pH. Although the composition of peptones varies between the two formulations, the total quantity (2%) is the same in both media. The pH of both preparations used was within 0.1 U of the value recorded on the label, and because Chapman and Bernard (1) had shown the rapidly growing mycobacteria to grow quite well over a wide range of pH, it seemed unlikely that pH was a factor. In our experience, most rapidly growing acid-fast bacilli grow well in agar concentrations ranging from 1.2 to almost 2.0; because the amount of agar in both MacConkey preparations falls within this range, agar seemed an unlikely factor. Because MA contains a larger amount (0.5%) of bile salts than MCV (0.15%) and because *M. smegmatis* and *M. phlei* will not grow on MA but will grow in 1% deoxycholate (6), this content of bile salts seemed unimportant. Earlier we had observed (3) that all strains of mycobacteria tested grow so similarly on agar medium containing 0.01% neutral red that this dye was not considered for differential identification of mycobacteria. Because the maximum content of this dye (0.005%) present in MA was only 50% of the amount tested earlier (3), we have tended to disregard its importance as an inhibitory agent in MacConkey medium. However, the possibility that subinhibitory levels of neutral red, in combination with crystal

violet, are inhibitory is tenable and should be explored. Additionally, other combinations of the above ingredients taken together could account for the undesirable inhibitory effect observed in MCV, but we have not yet tested the multitude of combinations of media that would derive with six variables.

Our earlier observation (unpublished) that different lots of the same dyes (which varied in actual dye content) could yield different inhibitory growth patterns for mycobacteria was what prompted us to report the lot number and dye content of each product studied (3). For this reason we have preferred to take the more simplified view that the current inhibition on MCV of organisms in the *M. fortuitum*-*M. chelonae* group is due to a combination of two factors: (i) inclusion of *M. peregrinum* in the taxon *fortuitum*, and (ii) a possible change in content or composition of the crystal violet dye. Earlier studies (3) indicated that a group of organisms closely related to *M. peregrinum* (i.e., Rapid Grower A) were more easily inhibited by MCV and the methyl violet dye than was *M. fortuitum*. The present study included 12 strains, labeled *M. peregrinum*, which had been examined 5 years ago. At that time 10 of the 12 grew on MCV, whereas today only 5 of 12 grow on MCV, although 10 of 12 grow on MA. Additionally, 30 of the 40 strains of *M. fortuitum*-*M. chelonae* in the present study were also examined 5 years ago. In 1969, 28 of these 30 strains (93%) grew on MCV; today only 63% grow on MCV, although 90% grow on MA.

The sudden and unexplained variability of a single test is rarely of great concern to the taxonomist who may employ more than 100 test features for species characterization. In contrast, such test failure could have dramatic consequences in a diagnostic laboratory, which normally performs only a few selected but highly reliable differential tests (5, 10, 11). For this reason, the diagnostic laboratory should utilize at least two definitive tests to identify each clinically significant *Mycobacterium*, in the hope that the failure of one test (as was the case here with MacConkey agar) could be detected by another reliable test (in this instance the 3-day arylsulfatase activity).

In conclusion, for reasons that still remain nebulous, certain recent batches of MacConkey agar with crystal violet have been found to yield

spurious results when used for differential identification of mycobacteria. Until such time as more definitive data may establish reasons for this failure, it has been shown that MacConkey agar without crystal violet will give almost the same mycobacterial growth inhibition patterns as diagnostic laboratories have come to expect of the rapidly growing acid-fast bacilli. Thus, the change to a dye-free medium would serve at least as a temporary stop-gap measure that could renew our faith in this test. For this reason it is recommended that MacConkey agar without crystal violet be used to screen rapidly growing mycobacteria, and that appropriate controls (known positive and negative reactors) be included with each test run.

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