

Facilitating Identification of Lactose-Fermenting Enterobacteriaceae on MacConkey Agar

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Retrospective (904 isolates) and prospective (over 500 stock and current clinical isolates) data on common lactose-fermenting Gram-negative bacilli were used to develop a guide for interpretation of growth on MacConkey agar. Use of this guide facilitated correct conjecture on the identification of lactose-fermenting Enterobacteriaceae. This approach is useful as a teaching aid and for quality control when initial conjecture is compared with final identification and antimicrobial susceptibility testing.

INTRODUCTION

MacConkey agar (MAC), first described in 1905 (1), is a bacteriological medium which selects for Gram-negative bacilli and differentiates lactose fermenters from nonfermenters of lactose (NFL). Bile salts selectively inhibit Gram-positive organisms. When Gram-negative bacilli ferment lactose, mixed acid byproducts are formed. These acids cause a localized decrease in pH, shown by Neutral Red, producing pink-red colonies. Some lactose fermenters, such as *Escherichia coli*, also produce a zone of precipitated bile salts around the colonies (2). NFL produce no color.

MacConkey agar is a commonly used primary plating medium in many clinical microbiology laboratories. However, manual and computer literature searches showed only a few past (1, 3) and current (2, 4-6) references dealing with specific interpretation of growth on this agar. Since this medium is so common, and because it can provide timely clues as to the identification of some Gram-negative bacilli, it behooves microbiologists to be efficient in interpreting colonial growth. Initial conjecture as to the possible identification of an organism can then be compared with the final identification and antimicrobial susceptibility results as part of a quality control process. Our main goal was to develop a means for facilitating accurate preliminary grouping of some common lactose-fermenting Enterobacteriaceae, primarily for use by inexperienced laboratory technicians.

MATERIALS AND METHODS

Commercially prepared MAC plates (100-mm-diameter) were purchased from Austin Biological Labs (Austin, TX) and had the following formulation per liter: meat peptone, 5 g; casein, 5 g; lactose, 10 g; bile salts mixture, 1.5 g; sodium chloride, 5 g; Neutral Red, 0.03 g; Crystal Violet, 0.001 g; and agar 15 g. Final pH of uninoculated medium was 7.1. Quality control of the medium with known strains of *Escherichia coli* and *Staphylococcus aureus* showed that it worked properly throughout the study. When a drop of 0.1 N NaOH was placed on the agar, it produced a yellow clear area, whereas a drop of 0.1 N HCl produced a dark pink area with surrounding precipitate. Incubation of uninoculated MAC plates in a 5% CO₂ incubator resulted in a small amount of diffuse precipitation throughout the medium. The precipitate lasted at least 18-24 hr after the plate was removed from the CO₂ atmosphere and stored in room air at 23 °C.

Over 500 isolates of *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* were saved from Mueller Hinton agar plates (used for disc agar diffusion antimicrobial susceptibility testing) and stored at 4 °C in sterile tap water prior to testing. Next, they were subcultured onto MAC and observed for viability, purity, and initial appearance. On subsequent cultures, growth on MAC was observed daily for three days. To mimic the actual laboratory routine, MAC plates were incubated at 35 °C for 18-24 hr (atmosphere of room air, relative humidity 50-54%), then on an open bench top at room temperature (21-25 °C, relative humidity 40-70%) for the remaining two days.

Organism identification was confirmed with one or several of the following: citrate (C), MIO medium (motility-indole-ornithin decarboxylase), lysine iron agar (LIA) (Difco Laboratories, Detroit, MI), API 20E (Ana-

lytab Products Inc., Plainview, NY), Micro-ID, Enteric Panel (General Diagnostics, Organon Teknika Corp, Durham, NC), and Kligler Iron Agar (Gibco Laboratories, Madison, WI). Lactose broth (Difco Laboratories) was used to detect/confirm lactose fermentation.

RESULTS

This study was done in several phases: 1) retrospective analysis of initial interpretations of clinical isolates on MAC and their final identification (from actual previous cultures), 2) observation of stock and clinical isolates on MAC, and 3) development and evaluation of an interpretation guide. We concentrated our efforts on the Enterobacteriaceae most common in our laboratory and which fermented lactose more than 5% of the time (7), as noted in Table 1.

Single isolates of *E. cloacae*, *E. coli*, and *K. pneumoniae* were inoculated onto MAC and incubated at 35 °C in room atmosphere and in 5% CO₂ to determine the influence of incubation atmosphere on growth, precipitation, and colony texture. Growth and colony texture were somewhat similar, but background precipitation was greater and precipitation around *E. coli* was more diffuse under CO₂.

DEVELOPMENT AND EVALUATION OF AN INTERPRETATION GUIDE

The important variables for interpretation were: age of culture, degree of colony isolation, color and texture of colony, and precipitation occurring around the colony. Several generalizations can be made regarding growth and appearance of test organisms and precipitates on MAC over a period of days: 1) colony color tended to fade to a lighter shade, 2) precipitates tended to become diffuse and occasionally disappear, and 3) color of medium tended to go from pink (on plates with lactose fermenters) to yellow. The influence of nutrient exhaustion with subsequent pH change was not studied in detail. A few strains of *E. cloacae* generated both lactose fermenters and NFL subpopulations. Table 2 defines how our isolates reacted on MAC.

A suggestion for using Table 2 is: decide if you are going to observe well isolated or clustered colonies and the age of the culture; define the color, surrounding precipitation, and texture of colony using the definitions at the bottom of Table 2; then find numbers that match your interpretation as closely as possible under the appropriate columns. For example, if you are observing isolated colonies on a 24-hr MAC plate and they appear pink to dark pink, dry, and surrounded by opaque precipitate, the appropriate code would be 1-2 for color, 1 for precipitation, and 1 for colony texture. The most appropriate possibilities would include *E. coli* and *C. freundii*, both in Group I. Since *E. coli* is more common than *C. freundii* in our laboratory, the chances are greater that the isolate is an *E. coli*. However, the order of occurrence might vary among laboratories. Also, other media/tests (e.g., sheep blood agar, spot indole test) might be available for simultaneous evaluation.

Rare mucoid strains of *E. coli*, *C. freundii*, *C. diversus*, and *E. cloacae*, and NFL (including rare NFL *E. aerogenes*) are not noted in Table 2. Several *Hafnia alvei* were originally misidentified as NFL *E. aerogenes*.

In order to verify lactose fermentation and detect slow lactose fermenters, all 'NFL' (at 24 hr of incubation) on MAC were also tested in lactose broth, with incubation at 35 °C for up to five days. Observation of gas in the inverted Durham tubes was considered a positive test for lactose fermentation. Such fermentation in lactose broth and on MAC plates was identical if the MAC plates were read carefully at 48 hr. However, slow lactose fermenters appeared as NFL by both media at 24 hr. None of our *C. diversus* isolates fermented lactose, whereas many of our slow- lactose-fermenting *E. cloacae* were originally called NFL.

DISCUSSION

MacConkey or Eosin Methylene Blue and 5% sheep blood agars are recommended as primary plating media for most routine aerobic bacteriological cultures (4). Hemolysis of sheep blood, on primary plating media, by Gram-negative bacilli is a presumptive identification aid (8). In this study, we concentrated on efficient use of growth of common lactose-fermenting Gram-negative Enterobacteriaceae as another such aid.

Although colony color and precipitation on MAC have been mentioned in other

TABLE 1. Growth of Gram-negative bacilli on MacConkey agar

Growth			No Growth
Lactose Fermentation			
Positive	Variable ^a	Negative	
In this study:			
<i>Escherichia</i> (E)	<i>Citrobacter</i> (E) ^b		
<i>Klebsiella</i> (E)	<i>Enterobacter</i> (E) ^b		
Not in this study:			
<i>Actinobacillus</i>	<i>Aeromonas</i>	<i>Alcaligenes</i>	<i>Cardiobacterium</i>
	<i>Acinetobacter</i>	<i>Chromobacterium</i>	<i>Eikenella</i>
	<i>Citrobacter</i> (E) ^c	<i>Edwardsiella</i> (E)	<i>Francisella</i>
	<i>Enterobacter</i> (E) ^c	<i>Hafnia</i> (E)	<i>Haemophilus</i>
	<i>Flavobacter</i>	<i>Morganella</i> (E)	<i>Kingella</i>
	<i>Pasteurella</i>	<i>Proteus</i> (E)	<i>Legionella</i>
	<i>Plesiomonas</i>	<i>Providencia</i> (E)	<i>Pasteurella</i>
	<i>Pseudomonas</i>	<i>Salmonella</i> (E)	
	<i>Serratia</i> (E)	<i>Shigella</i> (E)	
	<i>Vibrio</i>	<i>Yersinia</i> (E)	

^a fermentation depends on species or individual isolate

^b lactose fermenters

^c nonfermenters of lactose

(E) Enterobacteriaceae

TABLE 2. General guidelines for interpretation of growth of lactose fermenters on MacConkey agar

Degree of isolation	Single, well-isolated colonies									Cluster of 5-10 colonies to heavy confluent growth								
	24			48			72			24			48			72		
Incubation, hr:	CO	PP	TX	CO	PP	TX	CO	PP	TX	CO	PP	TX	CO	PP	TX	CO	PP	TX
Organism ^(a)	CO	PP	TX	CO	PP	TX	CO	PP	TX	CO	PP	TX	CO	PP	TX	CO	PP	TX
GROUP I																		
<i>E. coli</i> (A)	1-2	1	1	1-2*	1	1	1-3	1	1	1-2	1	1	1-2*	1	1	1-3	1	1
<i>C. freundii</i> (E)	1-2*	1*	1	1-3	1-2*	1	1-3	1-3	1	1-2*	1	1	1-3	1	1	1-3	1	1
GROUP II																		
<i>K. pneumoniae</i> (B)	2-4	2-3*	3	2-4	3	3	2-4	3	3	2-4	2	3	2-4	2	3	2-4	2	3
<i>E. aerogenes</i> (C)	2-3*	3	2-3	3-4	3	2-3	3-4	3	2-3	2-3*	2-3	2-3	3-4	2-3	2-3	3-4	2-3	2-3
<i>K. oxytoca</i> (D)	2-4	2-3*	2-3*	2-4	3	3	2-4	3	3	2-4	2-3	3	2-4	2-3	3	2-4	2-3	3
<i>E. cloacae</i> ^(b) (E)	2*	3*	2	2-4*	2-3	2	3-4	2-3	2-3	2*	1-2*	2	2-4*	1-3	1-3	3-4	1-3	2-3
<i>E. agglomerans</i> (F)	2-4*	2-3*	2-3	3-4	3	2-3	3-4	3	2-3	2-4*	2-3	2-3	3-4	2-3*	2-3	3-4	3	2-3
GROUP III																		
<i>E. cloacae</i> ^(c) (E)	5*	3	2	3-4*	3	2	3-5	3	2	5*	3	2	4-5	3	2	4-5	3	2

Categories:

CO: Color of colony (1 = dark pink, 2 = pink, 3 = light pink, 4 = off-white/opaque/pink-barely-detectable, 5 = colorless/transparent); viewed from top of colony, not bottom of plate.

PP: Precipitation in agar around colony (1 = opaque, 2 = translucent, 3 = none).

TX: Colony texture (1 = dry, 2 = moist, 3 = mucoid); viewed from top of colony (e.g., moist includes any texture between the typical dryness exhibited by *E. coli* and mucoid shown by *K. pneumoniae*).

^a Order of occurrence at our hospital (A = most common, F = least common)

^b Lactose fermenters

^c Initially appear as nonfermenters of lactose, but ferment lactose slowly

* Can change after 24-48 hr

3 Bold numbers indicate most common response, if apparent.

helpful reports (1-6), factors such as degree of colony isolation, age of culture, and colony texture of MAC have not been emphasized. These factors are important in interpretation of growth on MAC. Age of culture is especially important in laboratories where assignments may vary from day to day (i.e., a person has to read plates several days old, on cultures that were originally read by another person). Colony texture may appear drier in atmospheres with low relative humidities (i.e., less than 40%).

The most appropriate use of the information in this report is for teaching and

quality control purposes. When initial conjecture of organism identification (based on appearance of colonies on available media) is compared with final identification (based on biochemical or other reliable methods) and antimicrobial susceptibility test results, an accurate result should occur more than 90-95% of the time. This last sentence may seem facetious; however, even reliable identification methods used on the same isolate can yield ambiguous results, especially for organisms such as *E. agglomerans* (9). Indeed, others have reported difficulties differentiating among some isolates of *C. diversus*, *C. freundii*, *E. cloacae*, and *E. agglomerans* using established methods (10).

There will be exceptions to the guidelines suggested in this report. For instance, medium (manufacturer, constituents, batch), individual isolate (mutants, misidentifications) and environment (incubation temperature, atmosphere, and humidity) could influence appearance of isolates on MAC. Precipitation occurs around *Serratia marcescens* colonies (a nonfermenter of lactose) on rare batches of Oxoid MacConkey agar (11). MAC plates should be incubated in room atmosphere (versus CO₂) because, as has been noted, CO₂ produces an acid environment that can increase nonspecific precipitation and therefore confuse interpretive efforts (2, 5).

The guide will not be helpful for rare mucoid strains of *E. coli*, *C. freundii*, *C. diversus*, and *E. cloacae*, which may be confused with *Klebsiella* spp., rare strains of lactose-fermenting *Serratia* spp., red-pigmented *Serratia* spp., or NFL. However, appearance of some NFL has been mentioned elsewhere (5). Our results were based on pure cultures, but actual clinical cultures will be mixed in most cases. Therefore, it is important to avoid mixing interpretations of different organisms.

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