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# Developing a practice-based microbiological laboratory to guide mastitis treatment on dairy farms

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**Abstract**

Culture-based treatment of mastitis has been advocated to reduce expenses of treatment and discarded milk and to improve treatment results. Ideally, identification of mastitis pathogens should be performed in a laboratory accessible to dairy farms within a reasonable amount of time. Results can usually be made available to farms the following day, before the time of day that treatments are normally administered. Mastitis pathogens can be successfully identified in a practice-based laboratory using standardized methods. Examples of specific, step-by-step methods, including needed materials, are described to aid in developing and operating a mastitis laboratory. A novel method of reporting results utilizing Google Docs is described.

**Key words:** dairy, mastitis, laboratory, treatment

**Résumé**

Le traitement de la mammite par l'entremise des cultures bactériologiques a été mis de l'avant afin de réduire les dépenses reliées au traitement et à la perte de lait et pour améliorer les résultats du traitement. Idéalement, l'identification des agents pathogènes associés à la mammite devrait être faite dans un laboratoire capable de fournir aux fermes laitières des résultats dans un délai de temps raisonnable. Les résultats sont habituellement disponibles le jour suivant avant que ne débute les traitements administrés à la ferme. Les agents pathogènes associés à la mammite peuvent être identifiés avec succès dans un laboratoire de pratique vétérinaire utilisant des méthodes standardisées. Des exemples de méthodes spécifiques décrites pas à pas, incluant le matériel nécessaire, sont présentés afin d'assister au développement et à l'opération d'un laboratoire dévolu à la mammite. Une nouvelle méthode de présentation des résultats par l'intermédiaire de Google Docs est présentée.

## Introduction

Microbiological identification of mastitis pathogens has typically been performed retrospectively to identify pathogens to guide treatment of subsequent cases, or to obtain an antibiogram of pathogens.[37] More recently, culture-based treatment has been advocated to reduce drug use, reduce treatment costs or increase treatment success.[8,9,13,16,17,18,20,21,24,25,27,31,32,42,43]

On-farm culture has been successful on some farms, although its use is often limited because of questionable results or discontinuation of the practice.[42] Additionally, bi-plate or tri-plate media and the 3M Petrifilm systems available for use by on-farm laboratories are designed to identify only the most common bovine mastitis pathogens.[8,13,22] As a result, their use on farms without use of microscopic evaluation and other tests may result in misdiagnosis, particularly if the mastitis is caused by less common mastitis pathogens such as yeasts, *Arcanobacterium*, *Corynebacterium*, *Pseudomonas*, *Serratia*, *Prototheca*, and others.[42]

Delay of treatment for 24 hours to seven days is not detrimental to treatment success,[30,31] thus a 24- to 48-hour interval between first diagnosis of clinical mastitis and treatment, typically achievable in a veterinary practice-based lab, should not reduce treatment success.

While methods to identify mastitis pathogens utilizing microbiological techniques have been described,[9,10,15,22,29,35,36,37,38,39,44] few resources that offer detailed, complete, step-by-step methodology needed to properly identify mastitis pathogens are available to the veterinary practitioner desiring to design a mastitis laboratory. This article will list and explain detailed methods utilized in the authors' laboratory to assist other practitioners wishing to develop a laboratory or those wishing to refine processes in an existing laboratory. This paper is not meant to represent the only or best methods available for use in a practice laboratory, nor is it suggested that identification techniques elucidated in this paper are new or novel. Furthermore, it should

be stressed that often a diagnosis can be made without completing the entire diagnostic scheme outlined for any particular organism in this paper. In general, simpler and faster is better, but at times additional testing will be needed to correctly identify a pathogen. For this reason, a relatively thorough methodology is described.

In the authors' practice, dairy producers typically treat animals based on protocols developed by the farm veterinarian following delivery of laboratory results to the farm. Often at least 50% of dairy farm mastitis samples produce growth of a coliform bacteria or no growth at all.[8,16,19,21,25,26,33] Common treatment protocols for these cases suggest no antibiotic treatment unless the animal is ill,[8,13,16,17,18,24,25,27,32,33,37] thus antibiotic usage for mastitis is reduced. Since more than 50% of antibiotic treatments used on a typical dairy farm are for mastitis, use of culture-based treatments can substantially reduce the overall use of antibiotics.[12,28,31] In addition, more appropriate treatment protocols, either in choice of antibiotic or duration of treatment, may be used when pathogens are identified, such as extended-duration therapy for treatment of mastitis caused by *Staphylococcus aureus*. The combined practice-based laboratory and on-farm treatment protocols have been well received by clients in the authors' practice. Nearly all clinical cases of mastitis are cultured on many farms, and cows are treated based on culture results.

## Sampling

Proper sample collection is critical for obtaining correct, meaningful results. Sampling methods have been well described by others.[9,29,37]

## Timing of Sampling

Mastitis is most often detected at milking time, which is a convenient time to collect samples. Samples may also be taken from fresh cows when mastitis is suspected during colostrum collection. Samples should be refrigerated or immediately frozen if they cannot be delivered to the laboratory within 24 hours. Samples should be delivered to the laboratory under refrigeration.

## Recording Samples

When samples arrive at the laboratory, a record should be created with a chronological listing of accessions.[3,34] This log should include the farm name, test(s) requested, date completed, technician's name or initials, and an area for notation that results have been reported. A form to record test results should also be created for each sample at this time. This form will be updated as microbial identification is completed. After preliminary results are obtained (usually 18 to 24 hours after the samples arrive), a notation of "preliminary" is made on the form and results are reported to the farm. An example form is shown in Figure 1. Final results should be reported on standardized, easily readable forms which can be developed using a variety of software programs.

## Materials Needed for the Typical Practice-Based Mastitis Laboratory

- Media
  - Washed bovine blood plates
  - Citrate media in tubes
  - Factor plates
  - MacConkey plates
  - Modified Hayflick plates (for *Mycoplasma*)
  - Motility media with color indicator (or Motility/Indole/Ornithase media) in tubes
  - Optional selective media, for example, potato dextrose agar for yeast and *Prototheca*
  - Modified TKT (MTKT) plates
- Candle jar (for *Mycoplasma*)
- Glass rod
- Dissecting microscope (for *Mycoplasma*)
- Filter paper
- Freezer
- Gram stain kit
- Hydrogen peroxide, 3%
- Incubator
- Microscope, slides, cover slips, and microscopy oil
- National Mastitis Council (NMC) *Laboratory Handbook on Bovine Mastitis*, 1999
- Oxidase reagent
- Potassium hydroxide
- PYR test kit
- Refrigerator
- Rabbit plasma
- *Staphylococcus aureus* cultures
- Sterile, 0.1 mL disposable inoculating loops
- Urease reagent
- Kovac's reagent (for use with MIO tubes)
- 6.5% NaCl broth with BromCresol indicator

Factor and MTKT agar are available from The Laboratory for Udder Health.[a] Other supplies may be obtained from Hardy Diagnostics[b] or other sources.

The actual list of materials used in any practice-based lab will vary. Each practitioner should choose appropriate tests for his/her own laboratory.

## Procedures for Identifying Mastitis Pathogens

A variety of techniques have been described for identifying pathogens.[9,10,15,22,29,35,36,37,38,39,44] An excellent

# Individual Culture ID Sheet
## (Lab Use Only)

| Client: | | Date samples plated: | |
|---|---|---|---|
| Date samples submitted: | | Date results reported: | |

| Cow ID | Quarter Sampled | | | | | NG | STAPH | | STREP | | | | COLIFORM | | | OTHER | Identification Tests | | | | | |
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24-Hour Reading DVM Initials: _____ 48-Hour Reading DVM Initials: _____

**Figure 1.** Example form to record milk sample information and laboratory results.

resource is the *Laboratory Handbook on Bovine Mastitis*,[15] published by the National Mastitis Council. Most organisms can be identified in a practice-based laboratory by culturing samples on selective media, and following with one or more simple laboratory tests. Reading cultures on colony morphology alone often results in misdiagnosis.[41] Reading cultures using morphology, microscopic examination, and supplemental testing may still occasionally result in an incorrect diagnosis.[14] This can occur because of variable colony morphology or variable response to supplemental testing. In addition, results of biochemical and other supplemental tests are sometimes subjective.

A practice-based laboratory designed to enable culture-based treatment of mastitis does not need to produce absolutely correct results 100% of the time. However, it is important for the diagnosis to be correct when the recommended treatment would change if the diagnosis was corrected. For example, misidentifying an *Enterobacter* species as *Escherichia coli* will likely not change the recommended treatment, but misidentifying yeast as a coagulase-negative *Staphylococcus* species will more likely change the treatment decision. Thus, a reasonable goal is to promptly produce correct results whenever possible, and to not misdiagnose organisms where the choice of treatment would be changed.

In consideration of this goal, there is no need to perform unnecessary procedures and tests to achieve a diagnosis with a reasonable level of confidence. Those considering or actively building a mastitis laboratory should understand that a diagnosis is often simple, and that the sum of the tests and procedures in this document are not needed in most cases. Two useful resources for ensuring laboratory quality are the Laboratory Proficiency Testing Program offered by Cornell University's Quality Milk Production Services,[1] and known standard

cultures which can be plated and run through the diagnostic process in the laboratory. Cultures are available from a number of sources, including Hardy Diagnostics.[b]

Standardized procedures are necessary for quality results in the mastitis laboratory. Procedures used in the authors' practice laboratory are as follows:

Milk is plated with a 0.01 mL loop on each of the following plates: washed bovine blood agar, Modified TKT agar, MacConkey agar, and Factor agar. Up to four samples can be inoculated on each plate by dividing plates into sections. Plates can typically be read 18 hours post-incubation. An attempt is made to generate preliminary results and deliver them to the farm by phone, fax, or email prior to the time of day the producer typically administers intramammary treatments. The particular time of day varies from farm to farm, therefore polling individual producers regarding daily treatment schedules is important. If samples are delivered to the laboratory on the day of collection, producers can generally administer treatments after receiving culture results the following morning.

The first step in evaluating plates is to determine upon which plates colonies are present. MTKT agar is selective for *Streptococcus* and *Enterococcus*; Factor agar selects for gram-positive organisms; and MacConkey agar selects for gram-negative organisms,[22] but there are exceptions. For example, *Pasteurella multocida*, a gram-negative organism, only grows on blood agar, while *Mannheimia haemolytica* typically grows on blood, Factor, and sometimes MacConkey agar. A variety of less common pathogens, including yeasts, fungi, and the algae *Prototheca*, will grow inconsistently on a variety of plates. Yeasts typically grow on blood, Factor, occasionally MTKT, and rarely MacConkey. *Prototheca* typically grows on all four plates, but is most recognizable on blood agar due to more rapid and robust growth. With the exceptions noted, a presumptive diagnosis can often be quickly made by examining the patterns of growth on the agar plates.

When a definitive diagnosis can be made by visual examination only, no further testing is necessary and the plates are returned to the incubator for 24 or more hours. For example, *E. coli* is often fairly easy to identify on MacConkey agar by its characteristic dry, flat, pink colonies with surrounding pink precipitate. Typically, however, performing one or more simple tests are required to confirm the identification of organisms.[9,10,15,36,37,38,39,44]

If a clear diagnosis is not reached by visual examination, a variety of simple tests can be performed in the laboratory, including gram staining. The gram stain should be performed on any colonies that cannot be definitively identified. Visual examination under high power will often lead to a diagnosis. For example, yeasts usually are easy to recognize following gram staining with a microscope, but variable colony morphology and patterns of growth on different media often make the diagnosis difficult without microscopy.

**Simple Tests Useful for Identification of Mastitis Pathogens and Their Uses**

- **CAMP test:** this test is used to differentiate between CAMP-positive *Streptococci* (*Strep. agalactiae*, some human isolates) and CAMP-negative *Streptococci* (primarily *Strep. dysgalactiae*).
- **Catalase test:** commonly used to differentiate between staphyloccal (catalase-positive) and streptococcal (catalase-negative) organisms. It may also be used to differentiate between *Arcanobacterium pyogenes* (catalase-negative) and *Corynebacterium bovis* (catalase-positive). *Serratia* and *Proteus* are vigorous, rapid catalase reactors, while most other gram-negative mastitis pathogens are slow, weakly positive or negative reactors.[4,40]
- **Citrate test:** this test is used for differentiation between citrate-negative (*E. coli*) and citrate-positive (*Klebsiella, Enterobacter*), gram-negative organisms.
- **Coagulase test:** used to confirm coagulase-positive (*Staph. aureus*) organisms.
- **Gram stain:** commonly used to confirm the identification of yeast and *Prototheca* and to aid in identification of less common mastitis pathogens.
- **Motility media:** used to distinguish motile (*Enterobacter, E. coli*) from non-motile organisms (*Klebsiella*)
- **MIO (motility, indole, ornithase) media:** this test is used to indicate motility, production of indole, and metabolism of ornithine. It is useful for differentiation of lactose-fermenting gram-negative pathogens, including *E. coli, Enterobacter, Citrobacter, Klebsiella*, and some *Serratia* isolates.
- **Oxidase test:** this test distinguishes *Pseudomonas* (positive) from non-pigmented *Serratia* (negative).
- **Potassium hydroxide (KOH) test:** this test distinguishes between gram-negative (KOH-positive) and gram-positive (KOH-negative) organisms growing on blood agar. It is also useful to confirm that a *Mannheimia* or *Pasteurella* (gram-negative, KOH-positive) is growing on a Factor plate.
- **PYR test:** used to distinguish gram-positive, esculin-positive, PYR (pyrrolidonyl arylamidase)-positive (*Enterococcus*) organisms from PYR-negative (*Streptococcus uberis*) organisms.
- **6.5% NaCl broth (with color indicator):** used to distinguish gram-positive, esculin-positive, organisms capable of growing in 6.5% NaCl (*Enterococcus*) from those incapable of growth (*Streptococcus uberis*).
- **Urease test:** this test distinguishes urease-positive (*Proteus, Pseudomonas*) from urease-negative (*Serratia*) organisms.

Further information regarding specific tests and test procedures is available from a variety of other sources.[7,10,15,29,36,37,38,39,44]

### Testing Methods

- **CAMP test:** streak known *Staph. aureus* colonies in a straight line down the center of a blood agar plate. Streak the suspected *Strep. agalactiae* in a line perpendicular to the *Staph. aureus* so that the two lines come within 1-2mm of each other. Formation of a clear, arrowhead-shaped zone of beta-hemolysis after 24 hours of incubation is a positive reaction.
- **Catalase test:** place a drop of 3% hydrogen peroxide onto a microscope slide. Emulsify a colony into the drop of peroxide, being careful not to gouge the agar (red blood cells will create a false-positive test). Bubbling is positive; no bubbling is negative.
- **Citrate:** inoculate the tube with a few colonies. Positive isolates will turn the green media blue within 24 hours.
- **Coagulase test:** thaw a 5 mL tube of frozen rabbit plasma. Using a loop, transfer several colonies into the tube and mix thoroughly. Incubate and examine at 1, 2, 4, and 24 hours after inoculation. Semi-solid to solid gelling is a positive reaction. If no gelling has occurred at any time and the plasma is liquid at 24 hours, the test is negative.
- **Gram stain:** using a loop, mix a colony into a drop of sterile water on a glass microscope slide. Air dry and fix the slide by passing over a flame. Apply crystal violet to the slide, wait 30-60 seconds, then drain. Apply Grams Iodine to the slide, wait 30-60 seconds, then drain. Decolorize with decolorizer (75% acetone/25% isopropyl alcohol) by gently rinsing until no color is visible in the runoff. Rinse with tap water. Apply safranin and wait 60 seconds. Rinse with tap water, blot dry with bibulous paper, and examine under the microscope with oil. Note size, shape, arrangement, and color of the cells.
- **KOH test:** place a small drop of KOH onto a plastic cover slip. Use a loop to transfer several colonies into the drop. Thoroughly mix the colonies into the drop with the loop, then gently raise the loop while looking closely for any evidence of a tiny string between the drop and the loop. Stringing is a positive (gram-negative organism) reaction. A false negative may occur if there is too much KOH, too few colonies, or inadequate mixing.
- **Motility media with color indicator:** inoculate the tube with a deep stab into the media. Positive isolates will show reddish lines radiating from the stabbed area after 24 hours of incubation.
- **MIO media:** inoculate the tube with a deep stab into the media. After 24 hours of incubation, motility-positive organisms will show fuzziness radiating out from the stab line and the media may appear cloudy. Ornithase-positive organisms will keep the media purple, while ornithase-negative organisms will turn the media yellow. Indole-positive organisms will show a red line at the top of the media after adding two drops of Kovac's reagent.
- **Oxidase test:** place a circle of filter paper on top of cardboard to protect the countertop. Place a drop of oxidase reagent onto the filter paper. Pick up a colony with a loop, then drag the loop across the wet area of the filter paper. A positive reaction has occurred when the filter paper immediately turns purplish-black where it came in contact with the loop. No color change, a slight, or pink color change, or a color change occurring after ten or more seconds is negative.
- **PYR test:** moisten the paper disk slightly with distilled water. Pick two to three well isolated colonies from the blood plate and rub into a small area of the PYR disk so that there is a visible paste. Allow to react for two minutes, then add one drop of PYR reagent. A bright pink or cherry red color will appear within one minute for a positive test. No color change, or orange, salmon, or yellow color should be interpreted as a negative result.
- **6.5% NaCl broth (with color indicator):** inoculate the broth with a few colonies. Positive isolates will turn the broth blue within 24 hours.
- **Urease test:** moisten filter paper with a few drops of 10% urea agar base concentrate. Rub some culture onto the paper with a glass rod. Positive isolates will show a pink or red streak on the paper within two minutes.

### Procedures for Identification Using a Plate-Based Identification Flow

One method to identify organisms is to serially examine all of the plates for growth as follows:

1. Remove plates from incubator and let stand for 20 minutes. This allows the esculin reaction to take place on the MTKT plates.
2. Examine the blood agar plate.
   a. No growth – fewer than three identical colonies (one or more *Staph. aureus* or *Strep. ag.* colonies are considered positive growth) are present.
   b. *Arcanobacterium pyogenes* – white, pinpoint beta-hemolytic colonies, which typically do not appear until after 30 to 48 hours of incubation, are present. Also *A. pyogenes* grows on the Factor plate and sometimes is visible on the MTKT plate. Confirm with a

catalase test (negative) and gram stain (tiny purple rods).

c. *Bacillus* – large, flat colonies, which can be rough or moist, dry or slimy, and sometimes asterisk-shaped, are present. Sometimes beta-hemolysis is present. Consider *Bacillus* a contaminant unless no other organisms are present and there are three or more colonies. *Bacillus* also grows on the Factor plate.
d. *Corynebacterium bovis* – tiny, white or gray non-hemolytic colonies, which do not appear until after 30 to 48 hours of incubation, are present. They should also grow on Factor plate. *C. bovis* may be confused with *Prototheca*. Confirm the diagnosis with a gram stain (tiny, pleomorphic rods) and catalase test (positive).
e. *E. coli* – colonies are 3-5mm in size, gray, moist, and have a fecal odor. Less than 15% are hemolytic. They do not grow on Factor or MTKT plates. Refer to MacConkey plate to differentiate.
f. *Enterobacter* and *Citrobacter* – appear similar to *E. coli*, are non-hemolytic, and have a fecal odor. The organisms do not grow on Factor or MTKT plates. Proceed to MacConkey plate.
g. *Klebsiella* – appear similar to *E. coli*, are often mucoid, and are non-hemolytic. They do not grow on Factor or MTKT plates. Proceed to MacConkey plate.
h. *Pasteurella* and *Mannheimia* – colonies often look like spilled milk (wet, confluent, or lobed, white or grayish). Examine for growth on MacConkey and Factor plates (*Mannheimia*), Factor but not MacConkey plates (*Pasteurella* or *Mannheimia* species), or blood agar only (*Pasteurella multocida*). Confirm with a KOH test (positive) and gram stain (tiny, pink, bipolar staining rods that may look like two cocci stuck together).
i. *Proteus* – colonies are gray, slimy, and swarming with a putrid odor. Colonies may quickly cover the entire plate. *Proteus* also grows on MacConkey agar. A gram stain may show long, filament-like "swarm cells".[29] *Proteus* is urease-positive and oxidase-negative.
j. *Pseudomonas* – large, 3-4mm, flat, grayish-blue colonies are present. Beta-hemolysis is usually present. Occasionally the colonies can be rough or dry. A grape-like odor typically is noticeable. Pseudomonads are urease- and oxidase-positive.
k. *Prototheca* – small, dry, gray, flat colonies that are often difficult to notice at 24 hours, are present. *Prototheca* grows on all four types of agar, but grows fastest on blood agar. Gram stain to confirm the identity of the organism (large cells, 10 to 20 times the size of bacteria, five times the size of yeast, variable in color, variable in shape and size, often can see daughter cells pinching off of parent cells).
l. *Staphylococcus* – large, 2-5 mm, smooth, white, creamy, yellow, gold, or grayish-white colonies are present. They may or may not be hemolytic; any degree of beta-hemolysis is suggestive of *Staph. aureus* (use coagulase test to confirm). Grows on Factor, but not on MTKT plates.
m. *Streptococcus* and *Enterococcus* – small, 1-3 mm, smooth, translucent, convex colonies are present. No hemolysis, alpha- or beta-hemolysis may be present. Examine MTKT plate.
n. *Serratia* – small, 2-3 mm in diameter, white, yellow, or gray colonies are present, which may resemble *Staphylococci*. *Seratia* may be hemolytic, and may develop bright red color at room temperature.
o. Yeast – white, waxy-textured, or sometimes gray and raised colonies are present. Yeast also grow on Factor, possibly MTKT, and rarely MacConkey plates. Gram stain to confirm. They will be about five times the size of bacteria, usually purple, but variable in color, fairly consistently sized oval-shaped cells; one can often see evidence of budding; fungal hyphae may be seen.
p. Contaminated sample – if the sample is from a single quarter, and more than two distinct colony morphologies are present (not including *Bacillus*), the sample is considered contaminated. If the sample is from more than one quarter, report all organisms with more than two colonies (one or more for *Staph. aureus* and *Strep. agalactiae*) on the plate.

3. Examine the Factor plate.
   a. If no growth, proceed to the MacConkey plate.
   b. *Bacillus, Mannheimia, Pasteurella, Prototheca, Arcanobacterium,* and *Corynebacterium* – see blood plate above. *Mannheimia* and some *Pasteurella* species will grow on Factor agar; *Pasteurella multocida* will not. *Mannheimia* may grow, sometimes with pinpoint red colonies,[29] on MacConkey agar.

*Mannheimia haemolytica* will show beta-hemolysis on blood agar; most *Pasteurella* species will not.

c. Beta-hemolytic colonies – set up a coagulase test to confirm *Staph. aureus* (positive). If this test is negative, report as coagulase-negative *Staph*.

d. Non-hemolytic, or alpha-hemolytic colonies – check to see if they are growing on MTKT plate.
   i. Growth on MTKT – proceed to MTKT flow.
   ii. No growth on MTKT – two possibilities remain:
      1. Yeast – white, waxy-textured, or sometimes gray and raised colonies are typical. See blood agar above.
      2. Coagulase-negative *Staph* – colonies are typically white, yellow, or gray, and shiny.

4. Examine the MTKT plate.
   a. *Prototheca* – see blood agar plate above.
   b. Esculin-positive colonies (darkened or black agar surrounding the colonies)
      i. *Strep. uberis* – colonies appear black or gray. The organism will not grow in 6.5% NaCl broth, and is PYR-negative.
      ii. *Enterococcus* – colonies appear black, gray, greenish-gray, or brown. Confirm with PYR test (positive) or NaCl broth (positive).
   c. Esculin-negative colonies
      i. *Strep. agalactiae* – a zone of beta- (clear) hemolysis will be present around colonies. Perform CAMP test (positive). If negative, report as *Strep. dysgalactiae*. It may be necessary to submit CAMP-positive samples to another lab, or use an API system[c] for confirmation.
      ii. *Strep. dysgalactiae* – non-hemolytic, esculin-negative colonies of variable size and color are present.
   d. Opportunities for occasional misdiagnosis using this scheme include the isolation of an occasional esculin-positive *Strep. dysgalactia*, or isolation of other streptococcal species that are less common causes of bovine mastitis.[15] More definitive diagnoses can be made with an API, or similar system.
5. Examine the MacConkey plate.
   a. *Prototheca* – see blood agar above.
   b. Lactose-fermenting colonies (pink and opaque)
      i. *E. coli* – dry, pink colonies are surrounded by precipitated bile salts (surface of the agar around the colonies turns pink). *E. coli* are citrate-negative.
      ii. *Enterobacter* – typically pink, dry colonies, but sometimes wet and mucoid colonies are present. There is no bile precipitate. Organisms are citrate-positive, motility-positive, and ornithase-positive, but indole-negative. (MIO +-+)
      iii. *Klebsiella* – mucoid, wet-looking, pink colonies with a cream-colored center. Precipitated bile salts are not present. *Klebsiella* are citrate-positive, motility-negative, and ornithase-negative. Most *Klebsiella* are indole-negative (MIO ---), but *Klebsiella oxytoca* is indole-positive (MIO -+-).
      iv. *Serratia rubidaea* – this species of *Serratia* ferments lactose, and is an unusual cause of bovine mastitis. Colonies are pink and wet-looking. Sixty percent of isolates will have red pigment and appear as bright red colonies. Pigment is more likely to be produced at room temperature. Organisms are motility-positive, indole-negative, ornithase-negative, and citrate-positive (MIO +--). *Serratia* are vigorous catalase reactors, while other lactose-fermenters are slow reactors, negative, or weakly positive.[4,40]
      v. *Citrobacter* – wet-looking colonies are present. *Citrobacter* are motility-, indole-, ornithase-, and citrate-positive (MIO +++).
      vi. *Mannheimia haemolytica* – pinpoint reddish colonies may be present due to fermentation of lactose. Look for growth on Factor plate; note "spilled milk" morphology on blood agar. Gram stain to confirm. *Mannheimia* is negative for motility, indole, and ornithase, (MIO ---), citrate-negative, and oxidase-negative or weakly positive.
      vii. Special note for diagnosis of lactose-positive organisms: often a diagnosis of *E. coli, Klebsiella*, and *Enterobacter* can be made by colony morphology alone, but the possibility exists that misdiagnosis will occasionally occur. For example, *Enterobacter* are typically dry-looking, pink colonies with no bile precipitate. However, some *Enterobacter* will be mucoid, and thus may be confused with *Klebsiella*. Non-pigmented *Serratia rubidaea* may also be confused with *Klebsiella*. *E. coli* can occasionally cre-

ate mucoid colonies. *Citrobacter* is an uncommon cause of bovine mastitis. MIO and citrate testing can definitively diagnose most lactose-fermenting organisms.

c. Non-lactose fermenting colonies (translucent, gray, tan, or yellow):
   i. *Pseudomonas* – colonies are tan, translucent, and may have a grape-like odor. Confirm with the oxidase (positive) test. If oxidase-negative, report as *Serratia*.
   ii. *Serratia* – most *Serratia* isolates are non-pigmented, and will appear very similar to *Pseudomonas*. The remainder produce a red pigment, especially if left out for 20 minutes at room temperature. Perform an oxidase test to confirm (negative). *Serratia* are vigorous catalase reactors.[4,40]
   iii. *Proteus* – swarming, slimy colonies often cover the entire plate. A putrid odor may be present. *Proteus* are vigorous catalase reactors.[4,40] *Proteus* are urease-positive, while *Serratia* are urease-negative.

d. Differentiation between gram-negative organisms, and particularly the *Enterobacteraciae*, may be difficult in some situations. Table 1 and Table 2 show typical reactions of gram-negative organisms to a variety of tests. These tables have been compiled by the authors from a variety of sources.[10,15,29,39,44] Tables are provided as an aid in diagnosis when less thorough testing does not result in a diagnosis. Alternatively, an API 20E system[c] or other similar system can be used to definitively identify pathogens in such circumstances. Typically, however, such systems are more costly than simple tests that can be run in a practice laboratory.

6. Special considerations for *Nocardia* and *Mycoplasma*
   a. *Nocardia* – this gram-positive organism is seldom seen on mastitis cultures. Infection is uncommon, and refrigeration and freezing of milk samples make isolation difficult. They are gram-positive, irregular rods with filamentous branching, have no spores, and are catalase-positive. Airborne hyphae are potentially pathogenic to humans, so plates with suspected or confirmed *Nocardia* should be handled carefully.
   b. *Mycoplasma* species require microaerophilic conditions for growth. This can be accomplished with a carbon dioxide incubator or a candle jar. Plates are examined at 24 to 48 hours after plating for growth. Samples are not considered negative unless no growth is present at seven days. Specific procedures for culture and examination are found elsewhere.[15,29] If *Mycoplasma* culture is to be performed in a practice-based laboratory, one should follow the procedures described above for other organisms, and then supplement results with *Mycoplasma* culture results, which are typically available seven days after plating.

**Table 1.** Differentiation of gram-negative mastitis pathogens with biochemical tests.

| Organism | Oxidase | Motility | Lysine | Ornithine | Urease | Citrate | Indole | Lactose |
|---|---|---|---|---|---|---|---|---|
| *Enterobacter aerogenes* | - | + | + | + | - | + | - | + |
| *Enterobacter cloacae* | - | + | - | + | +/- | + | - | + |
| *Klebsiella* | - | - | + | - | + | + | - | + |
| *Klebsiella oxytoca* | - | - | + | - | + | + | + | + |
| *E. coli* | - | (+)most | (+)most | +/- | - | - | + | + |
| *Citrobacter* | - | (+)most | - | (+)most | +/- | (+)most | + | + |
| *Serratia marcescens* | - | + | + | + | (-)most | + | - | - |
| *Serratia rubidaea* | - | (+)most | +/- | - | - | + | - | + |
| *Pseudomonas* | + | +(top) | - | | + | +/- | | - |
| *Pasteurella* | weak+ | - | | + | - | - | + | (-)most |
| *Mannheimia* | +/- | - | | - | - | - | - | (+)most |
| *Proteus* | - | + | - | +/- | + | +/- | +/- | - |

**Table 2.** Motility/Indole/Ornithase (MIO) test interpretation.

| | Motility | Indole | Ornithase |
|---|---|---|---|
| *Enterobacter* | + | - | + |
| *Klebsiella pneumoniae* | - | - | - |
| *Klebsiella oxytoca* | - | + | - |
| *E. coli* | (+)most | + | +/- |
| *Citrobacter* | (+)most | + | (+)most |
| *Serratia marcescens* | + | - | + |
| *Serratia rubidaea* | (+)most | - | - |

## Procedures for Identification of Mastitis Pathogens Using Flow Charts

Flow charts are an alternative way to represent processes of identification of mastitis pathogens. Figures 2 through 4 are flow charts representing the decision process used to identify pathogens in the authors' laboratory. Note that *Pasteurella multocida* grows only on blood agar, and *Prototheca* typically grows on all four agar types. It should again be stressed that these flow charts are examples and are not meant to represent the best, or only available, diagnostic methods.

## Susceptibility Testing

Susceptibility testing has been advocated as a basis for therapy selection for nearly 50 years. However, the disk-diffusion method typically used has several limitations.[2,4,5,6,7,11] In the authors' opinion, routine susceptibility testing of bovine mastitis isolates is seldom indicated for formulating routine treatment decisions. Typical sensitivity patterns of mastitis pathogens using minimum inhibitory concentration data are available, and can be used to formulate treatment protocols.

## Veterinarian Involvement

Having both a veterinarian and a veterinary technician examine results and plates is advisable and serves several purposes.[3] First, it allows for another opinion. On occasion some judgment is required to formulate final results, and thus another trained individual is of value. Second, veterinarians can soon become proficient in identification procedures, and can be utilized to determine results when a technician is absent, or on weekends. Third, having the veterinarian sign-off on every report can help maintain a standard of quality in the laboratory. In the authors' laboratory, both preliminary and final results are examined by a practice-member veterinarian. The veterinarian may agree, disagree, or request further testing. The tracking form is initialed by the veterinarian both for preliminary and final results.

## Reporting Results

Cows are often treated at a particular time of day on many dairy farms. This might, for example, be during milking, or following a particular milking shift. It is often possible to provide preliminary results to the farm on the day after plating so that treatment is not delayed. Cellular phones make dairy farm personnel nearly always available, and many farms have fax machines for reporting results. Email is also very commonly used on farms; however, a farm computer or smart phone is required to receive results. Recently a commercial software program has been utilized to electronically report results.[23] A combination of reporting methods may be used, but it is advisable to find the preferred method for each farm and then use that method to consistently report results.

A relatively new method combines email with "cloud" storage. An example of such a system is Google Docs. A document can be created in word processing, spreadsheet, or presentation format from the Google Docs website. Alternatively, a document using one of these formats can be uploaded to Google Docs. Once a document is created or uploaded, the user can designate individuals with which to share the document. Sharing can mean viewing, editing, or both, depending on the designation used by the owner of the document. When an individual is allowed to share a document, an email notification is sent, which allows the person to click on a portion of the message and be forwarded to the online document for viewing. The editing function can be useful for veterinarians to add comments, such as treatment recommendations.

Google Docs is used by the authors' laboratory and has been very useful. As soon as results are available, an Excel spreadsheet containing the results is created by the laboratory technician using a report template. The document is then uploaded to Google Docs, with the farm veterinarian and the appropriate farm personnel listed as viewers. The farm veterinarian is also designated as an editor. Both the veterinarian and the producer receive email notification of completed results, allowing them to see the document with one click. The veterinarian can add a notation, or send an email notation to the producer at the time of viewing. When a notation is made, the producer will receive another email indicating a document is ready to be viewed. This system offers several advantages. First, it can be done quickly and the document is available almost instantaneously. Second, there is no need for a hard copy of the document to find its way into a veterinarian's inbox, or on a desk, or in a pile of documents somewhere in the office. Third, the document is available for viewing from virtually any internet-connected device at any time. Unlike email attachments, for example, it is not necessary to locate

the computer in which an electronic copy of a document is stored. A producer or veterinarian can access the information from a smart phone, tablet computer, or similar device while on the farm. Fourth, it is easy for the farm veterinarian to add comments. There are other similar services available, such as Windows Live, that are available for free on the internet.

### Treatment of Cows

Use of standardized treatment protocols on dairy farms has been advocated for a variety of reasons.[24,41] Following notification of mastitis culture results, farm staff can administer treatments according to existing farm-specific treatment protocols. Typically, contact

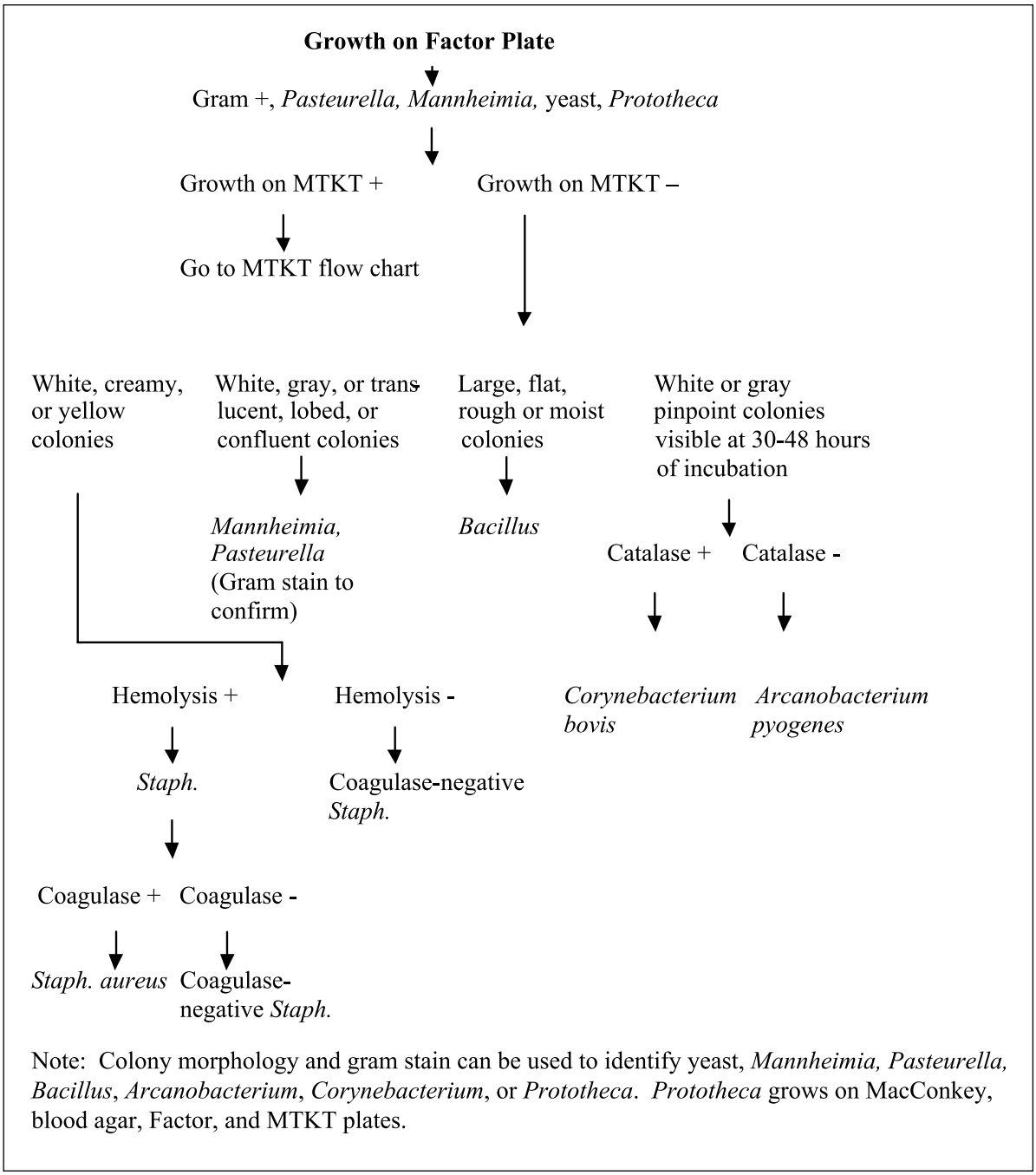


**Figure 2.** Flow chart used to identify pathogens on Factor agar plates.

with the farm veterinarian is not needed. On farms with employees, employees will usually be able to administer the designated treatments without contacting the farm owner or manager. Treatment protocols need to specify the pathogen isolated for this system to work properly. Protocols should identify the drug to use, the duration and frequency of treatment, and the proper milk and meat withholding periods. Protocols may be updated based on the perceived treatment response, product preference, cost, availability, ease of administration, and other factors. It is the veterinarian's responsibility to develop specific treatment protocols for any farm. A discussion of specific treatments is beyond the scope of this paper.

## Other Procedures

The techniques developed and materials available in the mastitis laboratory can be utilized for other testing. Cultures can be performed on cows with suspected subclinical or chronic mastitis by using the procedures outlined above. Whole-herd culture for contagious pathogens can be performed in a practice-based laboratory. Bulk-tank milk culture and differentiation, and culture of bedding, towels, calf milk, and colostrum all are relatively easy to perform once the laboratory materials and procedures are in place.

On-farm pasteurizers can be monitored by culturing pre- and post-pasteurized milk, using techniques very similar to culturing of bulk-tank milk.

## Conclusions

A practice-based mastitis laboratory can provide valuable service to dairy farms by providing pathogen identification in a timely manner. Isolation and identification methods are not difficult to learn or perform. By using available technologies it is possible to report results quickly and efficiently, allowing dairy producers to treat mastitis cases without excessive delay. This paper describes methods used in the authors' laboratory, in both text and flow chart form, and is meant to

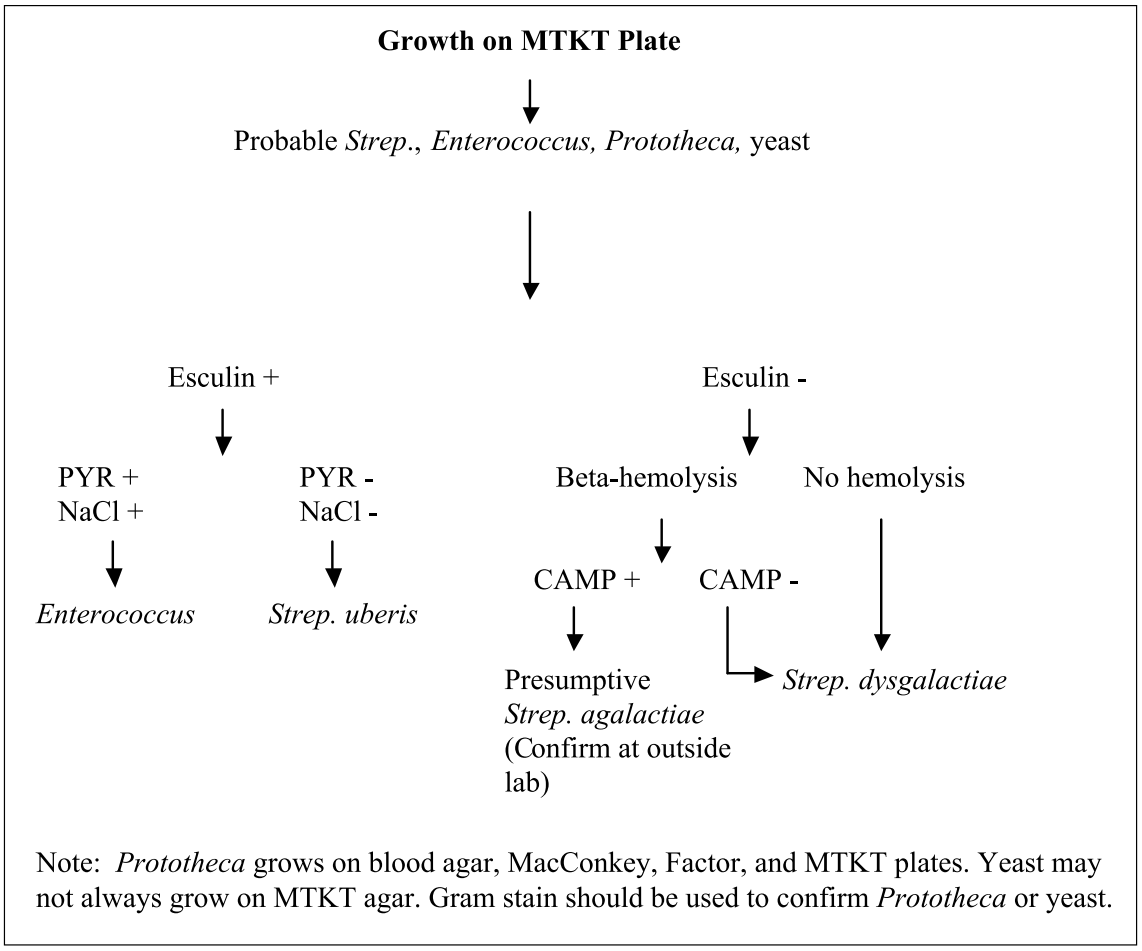


**Figure 3.** Flow chart used to identify pathogens on MTKT agar plates.

provide an example of a relatively complete "how-to" guide that other practitioners might find useful when developing a mastitis laboratory. The methods described are not meant to represent the only, or necessarily, best methods available.

### Endnotes

[a]The Laboratory for Udder Health, MVDL, University of Minnesota, St. Paul, MN, mastlab@umn.edu

[b]Hardy Diagnostics, Santa Maria, CA, www.HardyDiagnostics.com

[c]bioMérieux, Inc, Durham, NC

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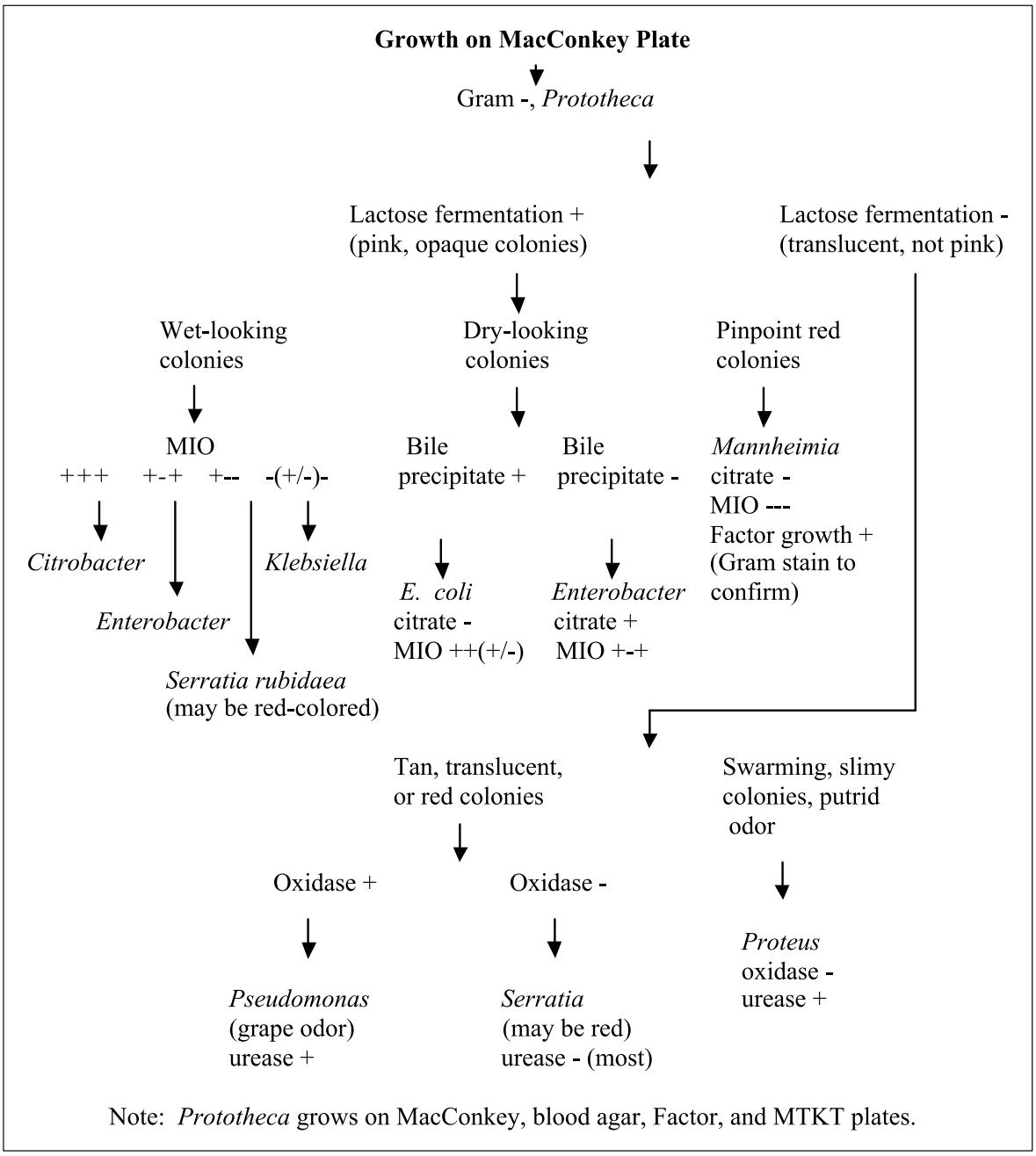


**Figure 4.** Flow chart used to identify pathogens on MacConkey agar plates.

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