Minnesota Easy Culture System II

Quality Milk Production Services
NYS Animal Health Diagnostic Laboratory
34 Cornell Drive
Canton, NY 13617
The Quality Milk Production Services (QMPS) and the University of Minnesota Udder Health Laboratory have reached an agreement whereby QMPS will distribute supplies, and provide technical support for the Easy Culture II System in the Northeast. This system consists of culture plates (Bi-plates and Triplates) and a manual that explains their use and interpretation.

This service is intended to help you perform some relatively simple microbiological analysis procedures at your farm or veterinary clinic. It is not intended to replace a high quality diagnostic laboratory or identify all organisms that may be present in the sample.

The Bi-plate system is intended to identify a quarter as infected with Gram-positive or Gram-negative organisms, but some presumptive identification may be possible.

The Tri-plate system is intended to give more complete identification of organisms causing the problem by including an additional media. Both systems require an incubator and sterile, cotton tipped swabs and are designed to be used on quarter milk samples only. Optional materials needed to further identify microorganisms include:

- Microscope and slides
- Hydrogen Peroxide
- Flat toothpicks
- Rabbit (coagulase) plasma

Culture results can provide you with valuable decision making information. Identifying infections early will facilitate treatment decisions and allow management changes that will have the greatest impact resulting in fewer new infections. Generally, reducing and/or preventing new infections will depend on appropriate milking procedures, cow (dry and milking) comfort and housing, heifer rearing and appropriate dry cow management. It is recommended that you and your veterinarian review all culture results and decide what mastitis management strategy is appropriate for your herd.

Easy Culture II plates and manuals, as well as other laboratory supplies can be ordered through any QMPS laboratory. Quality Milk personnel are also available to provide technical support for any of the materials they distribute. This includes: training in their use, confirmation and interpretation of culture results and a Laboratory Proficiency Testing program.

<table>
<thead>
<tr>
<th>Northern Laboratory</th>
<th>Central Laboratory</th>
<th>Western Laboratory</th>
<th>Eastern Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 Cornell Drive</td>
<td>22 Thornwood Drive</td>
<td>4530 Millennium Drive</td>
<td>111 Schenectady Ave</td>
</tr>
<tr>
<td>SUNY Canton</td>
<td>Park View Tech Center</td>
<td>Geneseo, NY 14454</td>
<td>B4 Wheeler Hall-SUNY</td>
</tr>
<tr>
<td>Canton, NY 13617</td>
<td>Ithaca, NY 14850</td>
<td>585-243-1780</td>
<td>Cobleskill, NY 12043</td>
</tr>
<tr>
<td>315-379-3930</td>
<td>607-255-8202</td>
<td></td>
<td>518-235-3681</td>
</tr>
</tbody>
</table>
This laboratory manual is intended to help you perform some relatively simple microbiological analysis procedures on milk samples. It is intended to aid in the easy identification of the most common bacteria found in milk samples. It is not intended to replace a high quality diagnostic laboratory or identify all organisms that may be present in the sample.

It is a good idea to record the types of bacteria identified on your dairy. This will help you and your veterinarian decide the proper course of prevention and treatment.

If you are going to sample several cows looking for contagious agents *Staphylococcus aureus* (*Staph. aureus*) or *Streptococcus agalactiae* (*Strep. ag.*) or need antibiotic sensitivities performed it is recommended that you send the milk samples to the Laboratory for Udder Health for analysis. It is recommended that every 3-4 months several milk samples from mastitic cows be obtained, frozen and shipped to the Laboratory for Udder Health for bacteria isolation and antibiotic sensitivity analysis. This should be done sooner if your animals fail to respond to antibiotic therapy.

**Interpretation of Bacteriology Results on an Individual Quarter Sample:**

Normally a quarter is not infected with more than one organism at the same time. It is possible to pick up organisms from the teat skin or teat canal in addition to those actually infecting in the quarter. Isolation of contagious organisms such as *Streptococcus agalactiae* or *Staphylococcus aureus* indicates a high probability there is infection of the gland. Environmental organisms such as *Streptococcus species* (*uberis, dysgalactiae*), *Enterococcus species*, coliforms (*E.coli, Klebsiella, Enterobacter*), *Staphylococcus species* (coagulase negative *Staph. species*), *Pseudomonas species*, *Cornyebacterium species*, and yeasts are as likely to have come from the skin or teat canal as from the gland. Isolation of these organisms depends, to some degree, on the sampling technique. However, these organisms can infect the gland and produce clinical or subclinical mastitis. An increased somatic cell count is an indicator the quarter is actually infected. In addition, the presence of clinical mastitis and the absence of contagious organisms indicate that a quarter may be infected with an environmental pathogen.
How to obtain a proper milk sample:

Avoid sampling in windy or dusty conditions. It is preferable that the sample is collected while wearing gloves. Prior to sample collection, clean and dry the udder and teats well. Next scrub the teat end of the teat to be sampled with alcohol. Discard one-half squirt before sampling. Place one to two squirts in a clean tube. Replace the top and either swab some on a plate immediately (see Day one: plating procedure below) or place immediately in a refrigerator.

Areas of Interest when Reading Plates:

There are generally two major areas of interest when reading bacteria culture plates. The first is bacteria identification. Knowing which bacteria is found in the sample will help you decide which antibiotics are appropriate to use on that cow, should you so desire. The second area of interest is colony forming units or how many bacteria are present. When there are more than one colony present, it is likely the cow is infected with two or more bacteria (if the sample is of high quality).

The Bi-plate system is intended to identify a quarter as infected with a Gram-positive or Gram-negative organism only. It will also identify a *Staphylococcus aureus* infected quarter. (See picture after text section)

The Tri-plate system is intended to give more complete identification of the organisms causing the problem. (See picture after text section)

NOTE: These plates are intended for use in culturing infected quarters only.

Media

The following media are commonly used in the microbiological analysis of milk samples: Factor, Modified TKT (MTKT) and MacConkey. Never use outdated media. These plates should have been tested prior to using to ensure proper reaction and growth of appropriate organisms. **Factor plates** are used to identify Gram-positive bacteria (staph, strep, bacillus, corynebacteria, arcanobacteria (actinomyces), and yeasts). **MTKT plates** select for *Streptococcus species* only. **MacConkey plates** select for Gram-negative
bacteria (E. coli, Klebsiella species, Enterobacter species, Proteus species, Serratia species, Pseudomonas species, etc.).

**Materials (for Bi-plate system only)**
- Sterile cotton tipped swabs
- Incubator

**Materials (for Tri-plate system only)**
- Sterile cotton tipped swabs (required)
- Hydrogen peroxide (optional)
- Flat toothpicks (optional)
- Rabbit plasma (optional)
- Microscope slides (optional)
- Incubator

**Day 1: Plating Procedure:**

**Sample mixing:**
If the sample cannot be plated immediately (within 10-15 minutes) refrigerate or freeze the sample. This is critical to obtaining quality results. Thaw the frozen milk samples in the refrigerator. Make sure the lid is properly sealed. Once the milk sample is thawed, mix each sample vial or tube well by inverting the milk samples approximately 15 times.

**Sample plating:**
Avoid touching the plate or cotton end of the sterile swab as this will result in contamination, which will give inaccurate findings. (Open the swab package such that the cotton ends remain covered and only a small portion of the stick is exposed.) Place a sterile cotton swab end in the milk sample, rolling the non-cotton end of the swab between index finger and thumb, for approximately 8 to 10 seconds, or until swab becomes completely saturated. Try to avoid plating clumped milk. If this happens be sure to note where it was so as not to confuse it with bacteria growth.

**Bi-plates**
Swab each half of the Bi-plate with the swab. Re-dip the swab in the milk sample between halves. **NOTE: If less than one half a plate is swabbed (more than one**
sample is swabbed on Bi-plate side) the sensitivity (ability to detect infected cows) decreases dramatically potentially giving false negative results (the cow is infected but nothing grows). Once the plate has been swabbed, place the lid back on the plate. Place plate in the incubator upside down (agar is face down or place the plate on its lid) in a 37°C incubator for 18 to 24 hours. Be sure to label each sample (on the bottom of the plate). After 18-24 hours skip to the Day 2: Reading plates section.

**Tri-plates**

Using the saturated swab spread the milk sample evenly on the surface of each sector on the Tri-plate. Make sure that the entire surface of the agar has been covered. Be sure to re-dip the swab between each section. This will help ensure accurate results. Once the plate has been swabbed, place the lid back on the plate. Place the plate in the incubator upside down (agar is face down or place the plate on its lid) in a 37°C incubator for 18 to 24 hours. Be sure to label each sample (on the bottom of the plate). After 18-24 hours skip to reading the Day 2: Reading plates section below.

Plating methods used in microbiologic analyses of milk samples are somewhat quantitative (a saturated cotton swab contains at least 0.1ml). They are intended to meet the need for recognition of specific microorganisms while giving some relative information as to the number of organisms present in the sample.

**Day 2: Reading Plates:**

**Bi-plates**

If clumped milk was plated it should appear as an irregular mass on the plate. Bacteria will appear round, shiny or flat. When using the Bi-plate system all you have to do is look for growth on one side of the plate. If you have growth on the red colored (Factor media) side a Gram-positive organism is causing the problem. If a clear area is seen around the colony (figure 1) *Staphylococcus aureus* is causing the problem. If growth occurs on the pinkish colored (MacConkey media) side you have a Gram-negative
bacteria growing. If no growth occurred put the plates back in the incubator and look at them tomorrow.

If you are using the Bi-plates you need go no further.

**NOTE:** Dispose of plates after use by incineration (burning).

If interested: compare your growth on the red side to figure 1. If a large zone of clearing around some of the colonies is observed it maybe *Staph. aureus*. Send the plate and milk sample to the Laboratory for Udder Health for identification. If you have growth on the pinkish colored media you may wish to look at the figures 6,7,8 to tentatively identify the organisms causing the problems.

**REMEMBER:** The Bi-plates will differentiate between Gram-positive and Gram-negative bacteria and identify *Staphylococcus aureus*. They contain the Factor (Gram-positive only) and MacConkey (Gram-negative) media.

**Tri-plates**

Milk cultures can be difficult to interpret. A plate swabbed with milk from one quarter should have predominant growth of a single organism because a quarter is rarely infected with more than one organism at a time. (All the growth on the plate should look the same [shiny, round, large, small and the same color]. Milk clumps will appear as irregular masses.) Growth of numerous organisms as evidenced by different colony morphologies (size, shape, and color) indicates contamination or poor sample collection technique. Composite samples may have more than one organism present. If there is more than one organism present, look for the most numerous organism first. This is probably the one causing the primary problem. If you are unsure of what an organism is, it can be sent to the Minnesota Veterinary Diagnostic Laboratory for identification. Once the organisms have been tentatively identified return the plates to the incubator for an additional 18 hours of incubation. Re-examine the plates after 36-48 hours of growth.

**Tri-plates**

(Compare the colonies you observe to those in the figures 1,2,3,4,5, 5a, 5b)
Uncontaminated milk samples from a healthy, normal mammary gland contains little or no bacteria. The object when reading plates is to identify and isolate all possible mastitis pathogens. Visually examine the plates, after 18-24 hours of incubation, for the presence of bacteria as evidenced by the presence of colonies.

If there is no growth, reincubate the plates for an additional 24 hours. If no growth is observed after a total of 48 hours the samples can be recorded as a "No Growth".

**Factor Media** (red colored media used primarily for the identification of *Staph. aureus* and *Staph. species*):

NOTE: Staphylococcus will only grow on the red colored media. If you have growth on both the red and deep burgundy media, streptococci are probably causing the problem.

If growth is observed, determine if it is significant by scoring the plate as a low (L=1-10) organisms present, moderate (M=11-50) organisms present or high (H>50) organisms present. Look at the flow chart, it is intended to give you an overview of the tests you may need to perform to identify the organism(s) present. First, look for *Staphylococcus aureus* colonies. On a Factor plate they will appear as creamy, greyish-white or golden-yellow colonies with a clear area of hemolysis around the colony (figure 1). At 24 hours the area of hemolysis (clear around the colony) maybe small. These can be tentatively identified as *Staph. aureus*. By 48 hours the zone of hemolysis is often much larger.

**Optional confirmatory testing**: Using a flat toothpick or bacteriological loop gently scrape the colony off the plate and confirm the identification as catalase positive by placing a drop of hydrogen peroxide on a clean glass slide to which one colony is added using the toothpick or loop. The presence of bubbles immediately indicates a positive reaction. **DO NOT PERFORM THE CATALASE TEST ON THE FACTOR PLATE, AS THE BLOOD PRESENT WILL GIVE A FALSE POSITIVE REACTION.** If so desired, you can confirm the identity of this organism as coagulase positive staphylococci (probably *Staph. aureus*) by inoculating 1-2 colonies into approximately 0.5ml rabbit
plasma. Incubate the coagulase reaction at 37°C overnight. Most reactions will gel (figure 1a, 1b) within 4-5 hours.

- **Staphylococcus species** (coagulase-negative or non *Staph aureus*) have an appearance similar to *Staphylococcus aureus*, but they are not hemolytic or have a very narrow zone of complete hemolysis which may be more pronounced after 48 hour of incubation.

**Optional confirmatory testing**: Test these organisms to see if they are catalase positive using hydrogen peroxide as above. If they are catalase positive and they do NOT give a large zone of beta (clear) hemolysis (figure 2) they are considered to be *Staph. species.*

**NOTE**: Yeast is catalase positive also and may appear visually as coagulase negative *Staphylococcus species*. It is important to examine these under the microscope. If large oval cells with small buds are present, the sample should be recorded as yeast.

- **Bacillus species** will appear as large flat dull catalase positive colonies with a large zone of beta (clear) hemolysis surrounding them are probably.

- **Arcanobacterium species** will appear as very tiny, nearly invisible, colonies with clear hemolysis at 24 hours. By 48 hours they will appear as pinpoint white colonies with a narrow clear zone of hemolysis around them.

- **Corynebacterium species** will appear in areas where fat from the milk is present. These are small colonies, which are barely visible at 24 hours. However, they are visible at 48 hours but are very small or tiny. They can be differentiated from the streptococci or arcanobacterium by being catalase positive.

**Optional confirmatory testing**: Next, look for small (size of fine tipped ball point pen), white to translucent colony that may have alpha (green), beta (clear) or no hemolysis around the colonies. Select one of these with the most numerous colonies and perform
the catalase test. It should be catalase negative (not producing bubbles). Proceed to the MTKT plate.

**MTKT Media** (dark burgundy colored media used to detect *Streptococcus species*)

**NOTE:** Only streptococci will grow on the MTKT plate. If you have growth on both the Factor and MTKT media Streptococci of some kind is probably causing the problem.

If growth is observed, let the MTKT plate stand at room temperature for 20-30 minutes. This will allow the esculin reaction to occur. Determine if significant growth has occurred by scoring the plate as a low (L=1-10) organisms present, moderate (M=11-50) organisms present or high (H>50) organisms present.

- **Streptococcus agalactiae** (figure 3) colonies will have a narrow band of beta (clear) hemolysis around the colony and appear bluish-gray. [If suspected send culture plate to the University of Minnesota Laboratory for Udder Health for confirmation.]

- **Streptococcus dysgalactiae** (figure 4) will not have hemolysis around the colony will appear as a bluish-gray colony with no hemolytic reaction and no darkening of the agar around the colony.

- **Streptococcus uberis** (figure 5a) will cause a darkening of the agar around the colony and have either no hemolysis or clear hemolysis around the colony and often have a very dark or black center. It may be necessary to restreak the suspect colony onto another MTKT plate. The growth should appear dark/black in color after 24 hours incubation. If any doubt exists send to the Laboratory for Udder Health for confirmation. *Streptococcus uberis* may have either green hemolysis or no hemolytic reaction on the Factor plate.

- **Enterococcus species** (figure 5b) will appear similar to *Streptococcus uberis* on the MTKT plate except that it will have a greenish-gray-black color. They will also split esculin so the agar will appear darkened around the colony. Enterococcus species
may have either alpha (green) hemolysis or show no hemolytic reaction on the Factor plate.

*Optional confirmatory testing:* Select a suspected Strep. ag. colony and perform the CAMP test or by agglutination using group B antisera, which is commercially available.

**MacConkey Media** (pinkish colored media used to detect gram negative bacteria):
Examine the MacConkey agar. If growth is observed, determine if it is significant by scoring the plate as a low (L=1-10) organisms present, moderate (M=11-50) organisms present or high (H>50) organisms present. Pink colonies indicate that lactose fermenters (*Klebsiella species, E. coli, Enterobacter, etc.*) are present. Tan or colorless colonies indicate *Pseudomonas* or *Proteus species* are present.

- **E. coli** (figure 6) appears dry-pink with a dark center (punctated) and the agar medium surrounding the colony may appear pink as well.

- **Klebsiella species** (figure 7) appears as a moist or wet (mucoid) looking light pinkish-white to white in the center of the colony. The edges are generally pink in color. If touched with a loop or toothpick they may string up slightly.

- **Enterobacter species** (figure 8) appear as a faint pink-white colony.

**Non-lactose fermenters:**
On colonies which appear white-tan, green, brownish dry, rough and irregular.

*Optional confirmatory testing:* Perform the oxidase test. Using filter paper and placing a small drop of oxidase reagent on the filter paper. Using a toothpick carefully pick 1-2 colonies and mix it with the oxidase reagent on the filter paper. If the area where you added the colonies turns deep bluish-purple within 60 seconds, the organism is oxidase positive. Do NOT use a loop for this test as it will turn the oxidase reagent purple giving a false positive reaction.
- *Pseudomonas species* (figure 9) are tan or semi-clear appearing colonies and are oxidase positive. In addition, they may have a grape like odor.

REMEMBER: *Pasteurella multocida* or *Mycoplasma species* will not grow on these plates. In you suspect you have a *Pasteurella species* send the milk sample to Quality Milk.

NOTE: Dispose of all plates following local regulations.
Milk Sample

Mix the sample well

Swab Factor, MTKT, Blood Agar and McConkey plates with sterile swab dipped in milk sample.

Incubate for 18 to 24 hours at 37° C.

Examine Culture plates for bacterial growth.

- Growth on Factor
  - Gram positive cocci
    - Catalase (+)
      - White creamy or slightly yellow coloring Probable Staphylococcus spp.
      - Hemolysis reaction
        - Clear zone of hemolysis
          - Probable Staph aureus, hyicus, intermedius (Fig 1)
            - Confirm Coagulase test (+) (Fig. 1b)
              - No hemolysis, Staph spp. (Fig 2)
                - Coagulase (-)
          - Hemolysis (variable), Esculin (+), brown black, non-Strep ag., Enterococcus, Strep uberis (Fig 5a,b)
            - Non-hemolytic, Esculin (-) non-Strep ag. Strep dysgalactiae (Fig 4)
              - Beta (clear) hemolysis, Esculin (-), Strep ag. (Fig 3) [Send to U of MN for confirmation]
        - Hemolysis & Esculin reaction
          - Probable Staph aureus, hyicus, intermedius (Fig 1)
            - Confirm Coagulase test (+) (Fig. 1b)
              - No hemolysis, Staph spp. (Fig 2)
                - Coagulase (-)
          - Hemolysis (variable), Esculin (+), brown black, non-Strep ag., Enterococcus, Strep uberis (Fig 5a,b)
            - Non-hemolytic, Esculin (-) non-Strep ag. Strep dysgalactiae (Fig 4)
              - Beta (clear) hemolysis, Esculin (-), Strep ag. (Fig 3) [Send to U of MN for confirmation]
    - Catalase(-)
      - Tiny grayish - white colonies
        - Probable Streptococcus spp.
          - Hemolysis reaction
            - Hemolysis & Esculin reaction
              - Hemolysis (variable), Esculin (+), brown black, non-Strep ag., Enterococcus, Strep uberis (Fig 5a,b)
                - Non-hemolytic, Esculin (-) non-Strep ag. Strep dysgalactiae (Fig 4)
                  - Beta (clear) hemolysis, Esculin (-), Strep ag. (Fig 3) [Send to U of MN for confirmation]
Figure 1 - *Staph. aureus*

Figure 1a: Coagulase Negative

Figure 1b: Coagulase Positive

Figure 2 - *Staph. species*
Figure 3 - Strep. agalactiae

Hemolysis

Figure 4 - Strep. dysgalactiae

Figure 5 - Enterococcus, Strep. uberis

Dark around colony
Figure 5a: *Streptococcus uberis*

Figure 5b: *Enterococcus species*
Figure 9a - Pseudomonas species
Yellowish Colored Colonies

Figure 9b - Pseudomonas species
Yellow Around Colonies