	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*	MQIP Milk Quality Improvement Program

Title: Direct Microscopic Count for Bacteria or Somatic Cells			
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Direct Microscopic Count for Bacteria or Somatic Cells

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SECTION 1 INTRODUCTION

1.1 Purpose

The direct microscopic method makes it possible for milk or certain milk products to be examined for numbers of bacterial clumps or somatic cells. In the former application, the direct microscopic clump (DMC) count, bacterial clumps may be counted, and, at the same time, an evaluation made of the distinctive morphology and arrangement of bacteria. To a limited extent, cell morphology and configuration of clumps allow the analyst to assess the cause(s) of quality problems. In the latter instance, large numbers of somatic cells indicate mastitis or other abnormal conditions of the udder. Accordingly, the microscopic method as regards somatic cells is referred to as the direct microscopic somatic cell count (DMSCC). As such, it reflects the research findings of several investigators and is applied as one of the officially recognized procedures for confirming somatic cell counts, which were previously estimated by one of several screening tests. Test results are reported in actual counts of bacterial clumps or individual somatic cells.

The microscopic method has limited, but possibly beneficial, application for determining the extent of bacterial contamination in pasteurized fluid milk and cream. Because dead cells lose some of their ability to take up stain and because numbers of bacteria are generally low in pasteurized milk products, this method is not used to determine compliance with finished product standards. But because large numbers of bacteria in uncultured products give evidence of unsanitary conditions, no matter what type of organisms are present or whether they are viable (living) cells, this method does have value as a quality control procedure.

1.2 Scope

This SOP applies to the Milk Quality Improvement Program Laboratory, and is taken in full from Standard Methods for the Examination of Dairy Products, 17th ed.

This method is applicable to raw milk and to dry milk products, with limited application to processed fluid milk products.

1.3 Definitions

- **Direct microscopic counts** and **direct microscopic somatic cell counts** are, with certain limitations, the number of bacterial and somatic cells, respectively, per milliliter or per gram of dairy product.
- **L-W (Levowitz-Weber) stain** is the stain used for both DMC and DMSCC. It contains solvents to dissolve the butterfat from the milk smears and a dye to stain cells.

- The hydrophobic coating on **Angstadt-Weber Milk Smear Slides** delineates the circles on these slides to permit rapid smearing of 0.01 mL of milk over an exact 1 cm^2 area. The slides have 4 hash marks per circle, indicating the exact beginning and ending points for true horizontal and vertical diameter strip counts. These indicator marks eliminate the need to search for your beginning point – the horizontal or vertical apex of each circle. Simply locate the indicator mark and begin counting.
- **Microscopic factors (MFs)** are values by which the average number of bacterial clumps or somatic cells per field is multiplied to calculate the respective count. The area of a single field determines the amount of milk film that can be seen at any one time.

1.4 Safety

Hazard statements for L-W stain:

Highly flammable liquid and vapor. Harmful if swallowed. Harmful in contact with skin. Causes skin irritation. Causes serious eye damage. Harmful if inhaled. May cause allergy or asthma symptoms or breathing difficulties if inhaled. May cause genetic defects. May cause cancer. May damage fertility or the unborn child. Causes damage to organs. Causes damage to organs through prolonged or repeated exposure.

Here is the link to the full SDS.

Only use L-W stain in the fume hood. Cover the stain jar while staining the slide and return the cover to the stain jar as soon as you have removed the stained slide.

SECTION 2 MATERIALS

- Milk sample(s)
  - Raw samples are typical
  - Pasteurized samples will work if they are creamline, i.e., not homogenized.
  - Homogenized samples will not work for this test.
- Angstadt-Weber Milk Smear Slides
- A 10 or 20 µL micropipette and pipette tips
- A bent-point biological dissecting needle
- A level heating block set to 40-45°C
- Forceps
- L-W stain in a stain jar
- Paper towels

- A water bath (DMSCC only)
- 500 mL beaker, glass or plastic
- Glass thermometer
- A compound light microscope with a 100× oil immersion lens
- Immersion oil
- A stage micrometer slide
- A hand tally (or download a phone app)

SECTION 3 PROCEDURES

This section is reproduced faithfully from Chapter 10 of Standard Methods for the Examination of Dairy Products, 17th ed. Notes from the author of this document are in blue, bold text.

1. Preparation of test sample and milk smear:
    a. Warm samples to be tested for a DSMCC to 40°C immediately before transferring them to slides. Do not warm samples to be used for a DMC of bacteria, or for any other purpose.
    b. Mix sample by shaking 25 times in 7 seconds with a 1-foot (30 cm) movement. Allow it to stand until the foam disperses (but no more than 3 minutes) to obtain a virtually foamless test portion.
    c. Legibly and indelibly identify each film as it is prepared; that is, place a number or other symbol on the edged margin of the slide.
    d. Use a separate (new) tip for each sample. Insert tip not more than 1 cm below surface of the sample, avoiding foam, and then depress the plunger to the first stop. Slowly release the plunger completely. With the tip still below dilution surface, depress plunger to the first stop again and slowly and completely release the plunger and then remove the tip from the sample.
    e. Hold the pipeter horizontally and carefully remove the excess milk from exterior of tip by wiping away from the tip. Do not wipe over the tip.
    f. Hold the pipeter vertically and discharge the test portion near the center of the test area **on the Angstadt-Weber Milk Smear Slide** and touch off on a dry spot. Discharge used tips into a biohazard bag.
    g. Spread the milk film with the tip of a bent-point needle (hold the needle as vertically as possible, do not lay down, as a hockey stick).
    h. Dry the milk film on a level surface at 40° to 45°C within 5 minutes, but do not heat rapidly. Protect from contamination by dust, etc. during drying.
2. Staining the milk smear:
    a. Place the stain in a container that can be covered to prevent evaporation while the slides are in the stain solution. Repeated use of stain in an uncovered vessel may result in the formation of precipitate.
    b. Submerge slides of the fixed, dried films, singly or in multiples, into the stain for 2 minutes. Remove and drain off the excess stain by resting the edge of the slide in a near vertical position on absorbent paper.
    c. Dry the slides thoroughly. **i.e., Allow the slides to dry thoroughly while resting on absorbent paper.**
    d. Rinse the dried, stained slides in 3 changes of tap water at 35° to 45°C. **Use a thermometer.**
    e. Drain the slides and allow them to air dry completely before examining the films under the microscope. **Do NOT try to cheat and use bibulous paper like you would for a Gram stain slide. These smears are much more delicate, and you will ruin them and have to start over again from the very beginning.**

Notes:

Proceed as above in preparing and staining cream films. When small numbers of raw milk films are to be stained, flooding the slides may be more practical than submerging them. Care must be taken to limit flooding exposure so that evaporation does not progress to the point where precipitation of dye occurs. Always work with L-W stain in the fume hood.

Discard used stain whenever the solution becomes contaminated or otherwise unsuitable. When solution is not in use, keep containers tightly closed to prevent evaporation. This applies to surplus supplies as well as to working batches of stain. Avoid using containers or closures that may dissolve or disintegrate and thus contaminate staining solutions. Store solutions in a relatively dark, cool place but do not refrigerate.

3. Examining films for bacteria or somatic cells:
    a. **See DMC and DMSCC interpretation guides with numerous example photomicrographs appended to the end of this document. These incredibly helpful guides were prepared by Cornell Dairy Extension Senior Extension Associate Emeritus Steve Murphy.**
    b. To obtain estimates of the bacteria or somatic cell count per milliliter, examine each film with an oil-immersion objective after placing 1 drop of immersion oil on the film.
    c. When counting bacteria **(DMC)**, count as separate clumps any 2 single cells or clumps of cells (apparently of the same type) that are separated by a distance equal to or greater than twice the smallest diameter of the 2 cells nearest each other. Regardless of the proximity to each other of the cells of different types, count each type as a separate unit.
    d. When making DMSCCs, count only those somatic cells with an identifiable stained nucleus. The nucleus is stained dark blue (bovine) or blue or blue-green (caprine). For polymorphonucleated cells, count as a single cell any that has two or more discernible nuclear lobes; for other somatic cells, count any that has a nucleus that appears to be essentially intact. Do not count if the nucleus is less than 8 microns in diameter. If in doubt about a cell, which may in fact be only a fragment, do not count it.
    e. Determining the counting factor: This counting method uses as boundaries a single strip that runs the width of the microscopic field and across the diameter of the milk film. A single strip factor (SSF) must first be calculated:
    $Area\ of\ a\ single\ strip\ (mm^2) = 11.28\ \ mm \times D\ in\ mm$
    where D is the diameter of the field. The field diameter is measured using a stage micrometer calibrated in 0.01-mm divisions.
    f. Determine the number of single strips in the 0.01-mL milk film by dividing 100 mm^2 (area of the 0.01-mL milk film) by the strip area:
    $$Number\ of\ single\ strips = 100\ mm^2 / area\ of\ single\ strip$$
    g. Convert the number of single strips in 0.01 mL of milk to a 1-mL basis by multiplying the number of single strips in 0.01 mL by 100:

$SSF = Number\ of\ strips\ in\ 0.01\ mL \times 100$

h. Example:
$Diameter\ of\ a\ microscopic\ field = 0.160\ mm$
$Length\ of\ strip\ (diameter\ of\ a\ 1 - cm^2\ circle = 11.28\ mm$
$Number\ of\ single\ strips\ in\ area\ of\ milk\ film\ (0.01\ mL\ of\ milk): 100 \div 1.80 = 55.6$
$SSF = 55.6 \times 100 = 5{,}560$

i. Counting procedure: To make a single-strip count, focus on the film edge in the oil-immersion field that appears to be at the maximum horizontal or vertical excursion. Traverse the entire diameter of the milk film, counting those cells within the strip and also those cells touching one edge of the strip (top or bottom if reading horizontally, or left or right if reading vertically). Do not count bacteria or somatic cells that touch the other edge. During scanning of the strip, continually make fine focusing adjustments.

j. Expression of Results:
$Count\ per\ milliliter = Number\ of\ somatic\ cells\ and/or\ bacterial\ clumps\ in\ a\ single\ strip \times SSF$

k. Example:
Assuming an SSF of 5,560, if an analyst counts 84 cells or clumps in a field-wide strip, the count is computed as follows:
$Count\ per\ milliliter = 84 \times 5{,}560 = 467{,}040$
Round off the result to 2 significant figures. The reported count becomes 470,000 per milliliter.

4. Test Report and Interpretation:
   a. Report counts only to the first 2 left hand digits of the estimate, followed by the appropriate number of zeroes. If the third digit is 5, round according to the following rules:
      i. If the second digit is odd, round up, and raise the second digit by 1.
         1. For example, 235 becomes 240.
      ii. If the second digit is even, round down, and delete the 5 and report the second digit as is.
         1. For example, 225 becomes 220.
      iii. A way to summarize these rules is to round the second number to the nearest even digit when the third digit is 5.
   b. **We're not a reference lab, so this isn't really important. For research purposes, you can round (or not round) the results however you choose.**

A gentle note on interpretation: Listen, milk has a lot going on. There's a bunch of stuff in there that kinda sorta looks like it might be bacterial cells. It isn't. It never is. Bacterial cells in a direct microscopic bacterial count look exactly like bacterial cells in a Gram stain – that is, they are strongly pigmented, have sharply defined edges, and are clearly either rods or cocci beyond any possible twist of the imagination. If you see real bacterial cells in a direct microscopic bacterial count, you will know right away beyond any shadow of doubt. If there's any question in your mind about what some random clump of stuff is, then it's not bacteria. That's all.

A second note on interpretation: The volume of milk that actually gets counted in a direct microscopic bacterial count is so infinitesimally small that you are unlikely to find a single bacterial cell or clump in any milk sample of good quality or even of marginal quality. This estimation technique, with emphasis on the word estimation, is effective only for the most egregiously, disgustingly contaminated samples. And even in those, there may still only be a few cells visible in the fields that you count.

SECTION 4 TROUBLESHOOTING

As in other methods of enumerating bacteria or somatic cells, results of the direct microscopic method are to be considered as estimates only. In general, the most important factors in determining accuracy, precision, and reproducibility are the training and skill of the analysts. Even with exacting techniques, however, replicate estimates may vary appreciably. The extent of variations in counts made by different analysts as well as by the same analyst has been reported for certain screening and confirmatory methods. Among factors responsible for such variation are inaccuracy in measuring 0.01-mL quantities of sample, faulty preparation and staining of slides, failure of some bacteria to stain, the minute amount of milk actually examined in counting, irregularity in the distribution of bacteria or somatic cells in the films, failure to count a sufficient number of cells, poor microscopy due to inadequate or excessive illumination of the microscope, poor focusing or improper use of colored filters, failure to dry the films on a level surface, eye fatigue, analyst inexperience, and errors in observation and calculation. **Just do the best you can, it will be ok.**

SECTION 5 REFERENCES

Fitts, J. E., D. Laird, and R. T. Marshall. 2004. Direct Microscopic Methods for Bacteria or Somatic Cells. Pages 269-280 in Standard methods for the examination of dairy products. 17th ed. H. M. Wehr and J. F. Frank, ed. American Public Health Association, Washington, DC.

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	2020-12-10	sjr267	Original SOP
Version 2			
Version 3			

The Microscopic Examination of Milk
Characterization of Milk Bacteria and Cells

The microscope has been used to observe and count bacteria and somatic cells in raw milk since the early 1900's. It has proven to be a valuable tool to the dairy industry. The milk smear procedure in use today is outlined in detail in the most recent edition of *Standard Methods for the Examination of Dairy Products* (i.e., SMEDP, 17th ed) and other references. For somatic cell counting, the Direct Microscopic Somatic Cell Count (DMSCC) is considered an official reference method used for regulatory purposes for direct milk counts and/or for calibration of approved electronic instruments. The regulatory procedure for somatic cells is outlined in detail in the most recent FDA 2400 Form. With the exception of the type of cells counted, the 2400 form procedure can be used for bacteria as well. While the Direct Microscopic Clump Count (DMCC) for bacteria is not considered an official test for bacteria counts, it is used throughout the dairy industry to estimate bacteria colony forming units (i.e., "clumps") in raw milk samples taken from the farm, the tank truck or the plant storage facility. The DMCC is most widely used to screen incoming raw milk supplies (i.e., tank-trucks) to determine whether the milk has an acceptable or legal bacterial load and has become accepted in some states as a legal method for rejection of unacceptable milk.

In addition to providing estimated counts of bacteria and somatic cells, the direct microscopic method has also been used as a trouble-shooting guide in attempts to identify the general types of bacteria present in a milk sample. Narrowing down the predominant types of contaminants in a sample can sometimes provide a lead as to the potential source or cause of a microbial defect. Guides with photographs of bacterial types were available as early as 1929 with a pamphlet develop by Dr. Breed, one of the originators of the DMC methods. Another brochure that is well known in the dairy industry in the northeast is the Vermont Extension Bulletin *Milk Under the Microscope,* which was developed by Atherton and Dodge in 1970. These brochures, which are no longer in print, presented pictures of bacterial types commonly seen in milk that were associated with potential inadequacies in the dairy farm production methods, including dirty equipment, poor cow hygiene procedures, mastitis and poor cooling. While these guides were useful, they were most effective when counts in the milk were high. It should also be point out that certain types of bacteria from very different sources can appear very similar under a direct microscopic smear resulting in guess work at best. In many cases however, it may be possible to narrow down the potential causes of high bacteria counts using the microscope as a tool.

The following pages present microscopic images and a summary of certain types of bacteria that are commonly associated with raw milk and dairy products. While pictures of these organisms as they appear with the DMCC are presented, caution must be used when using the microscope alone when trying to characterize the types of bacteria and other microorganisms associated with milk defects. Further information on these general groups of organisms is included with the photographs that would help the user further identify what they see under the microscope and perhaps on an agar plate. Auxiliary tests that might be used with colonies from an agar plate, including the Gram stain, spore stain, catalase test and oxidase test are also described. The groups discussed include:

Prepared by: S. C. Murphy, Sr. Extension Associate
Department of Food Science, Cornell University

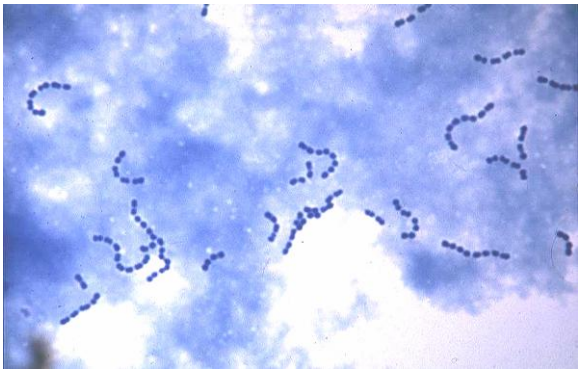


Fig. 1. Short chains of cocci from poor cooling and/ or dirty equipment. Mastitis source possible.

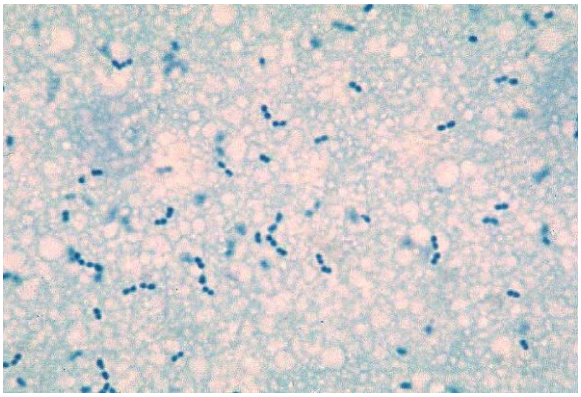


Fig. 2. Pairs of cocci from poor cooling and/or dirty equipment. Mastitis source possible.

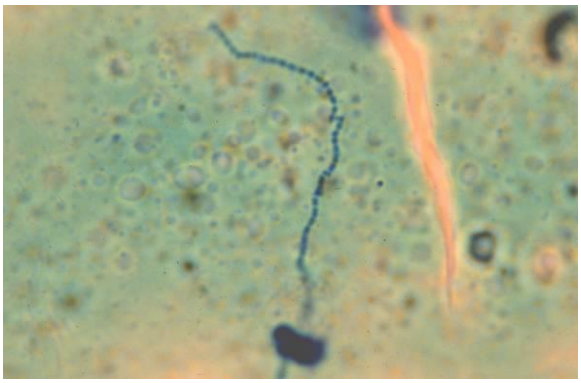


Fig. 3. Long chains of *S. agalactiae* as the result of mastitis infection, association with somatic cells.

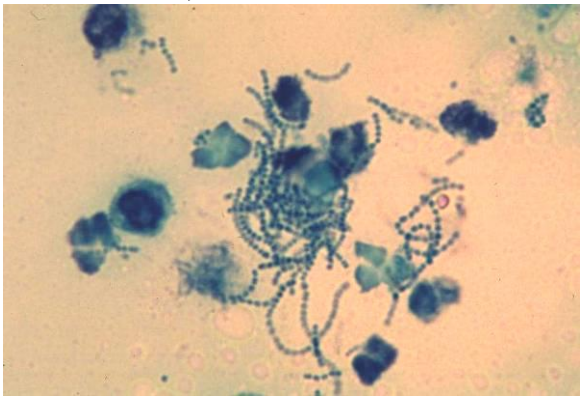


Fig. 4. Severe case of *S. agalactiae* infection from an individual cow showing close association with cells.

SPHERICAL COCCI; PAIRS OR CHAINS

When large numbers of pairs (diplococci) or short chains (streptococci) of spherical bacteria (cocci) are observed in raw milk, possible causes include poor cooling and/or dirty equipment. Environmental streptococci that cause mastitis may also appear as short chains or pairs while very long chains are typical of *Streptococcus agalactiae*. When mastitis is the cause, bacterial cells may be observed in association with somatic cells (leukocytes). With mastitis and poor cooling, milk smears may appear as mostly one type of bacteria, while high counts from dirty equipment would be more likely to contain a mixture of bacterial types (including rods, cocci in clusters).

Technical Information:

Gram-positive, cocci, 0.5-1.2 microns in diameter
Occur in pairs or chains of varying length
Catalase negative, oxidase usually negative
Colonies on SPC agar usually white, small, subsurface
Some strains survive pasteurization, most do not
Some strains may grow slowly under refrigeration

Streptococcus, ***Lactococcus*** and ***Enterococcus*** species are the most common Gram-positive, catalase-negative cocci that occur in milk. Cells are generally seen in pairs (diplococci) or in chains of varying lengths. They are easily recognized in milk smears; although distinguishing between specific types may be difficult.

Lactococcus (lactic streptococci) are involved in dairy fermentations (e.g., cheese) as well as in the spoilage of dairy products. **They are common in nature and the dairy environment and are often associated with plant materials including feeds and bedding materials. They may also thrive on milk soil of poorly cleaned equipment.** These organisms do not grow or grow slowly, under refrigeration, although they grow very well in milk at higher temperatures. **Poor cooling, especially when temperatures exceed 50-60°F, often results in proliferation of these organisms, seen in milk smears as pairs and/or chains of cocci. These organisms may be responsible for "sour" (high acidity) or "malty" defects in milk.**

Enterococcus (fecal streptococci) are often associated with fecal matter, although they survive well in other environments. They appear similar to *Lactococcus*. They may be associated with poor cooling, dirty equipment and in rare cases mastitis.

Streptococcus strains considered as common causes of mastitis include contagious strains (*S. agalactiae*), spread from cow to cow, and environmental strains (*S. uberis, S. dysgalactiae*) contracted from the environment (e.g., bedding). ***S. agalactiae*** **often appears in long chains, which may or may not, be seen associated with somatic cells. Other mastitis streptococci may be seen as pairs or chains of varying lengths resembling organisms in the above two figures (1 & 2).**

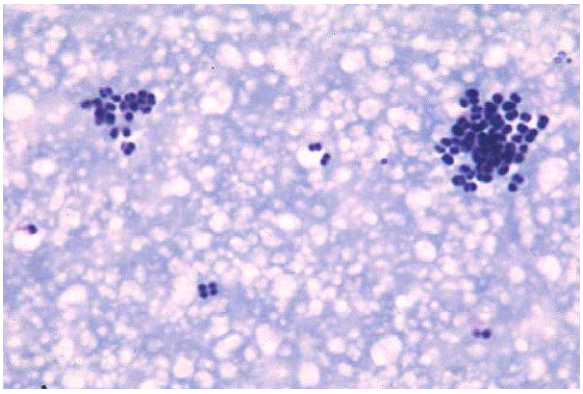


Fig. 5. *Micrococcus* in cluster formation associated with persistent soils on milk equipment.

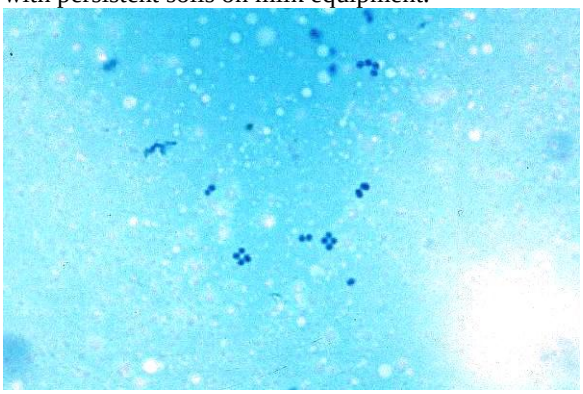


Fig. 6. *Micrococcus* in tetrad formation associated with persistent soils on milk equipment.

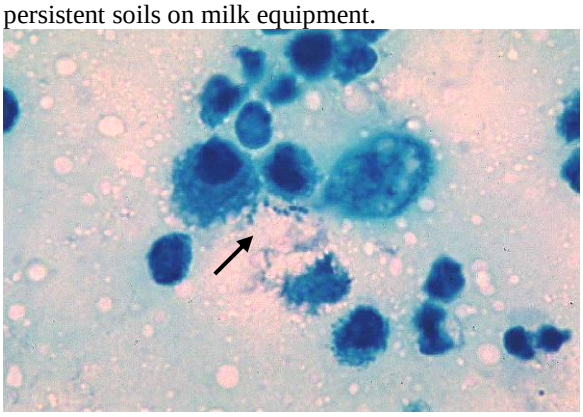


Fig. 7. *Staphylococcus aureus* associated with somatic cells in milk from an infected cow.

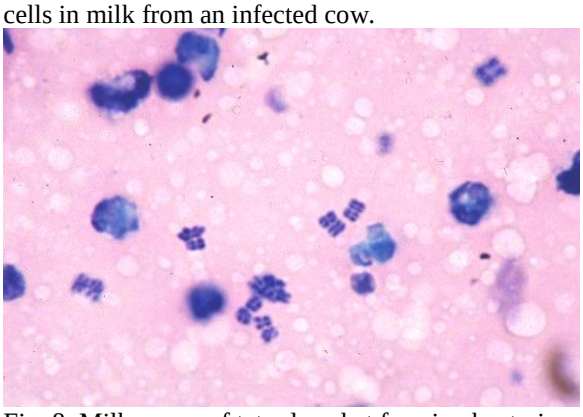


Fig. 8. Milk smear of tetrad packet forming bacteria associated with mastitis.

SPHERICAL COCCI; CLUSTERS, PAIRS OR TETRADS

Cocci in clusters (staphylococci) or in 2 x 2 arrangements (tetrads) are most often associated with persistent poor cleaning (e.g., milk stone, films) or in some cases mastitic cows. These organisms may also be seen as single cells or pairs, which may make it difficult to distinguish them from the streptococci group, although the cells may be larger. In general, they are rarely observed in raw milk as sole contaminants, or in high numbers, except perhaps when a cow with a *Staphylococcus* infection is shedding large numbers with its milk. The most common bacteria in this group include strains of ***Micrococcus*** and ***Staphylococcus*** (Staph.).

Technical Information - ***Micrococcus***:

Gram-positive cocci, 0.5-2.0 microns diameter
Occur as clusters, tetrads or pairs
Catalase positive, **oxidase positive**
Colonies on SPC usually opaque, white, yellow, orange
Some strains survive pasteurization
Some strains may grow slowly under refrigeration (rare)

Micrococcus spp. are common in milk as part of the natural flora of the cow, although they are generally present in low numbers. **Increased numbers of some strains have been associated with milk stone, cracked or old rubber parts and other areas of persistent poor cleaning. These strains are often thermoduric (i.e., survive pasteurization) and are generally not considered to be of the natural flora of the cow.** They may be present in the dairy environment, including bedding. *Micrococcus* spp. rarely reach significant numbers in raw milk. However, numbers may reach sufficient levels to influence fresh counts of pasteurized milk, although rarely above legal limits. Some strains may grow slowly in refrigerated milk.

Technical Information – ***Staphylococcus***:

Gram-positive, cocci, 0.5-2.0 microns diameter
Occur as single cells, pairs, clusters, tetrads
Catalase positive, **oxidase negative**
Colonies on SPC agar usually opaque, gray white
Generally do not survive pasteurization
Do not grow under refrigeration

Staphylococcus spp. are also commonly found in raw milk as part of the natural flora of the cow, although generally in low numbers. **Strains of *Staph. aureus* are associated with contagious mastitis and may be shed into milk, although the influence on bulk tank counts is not as common as with mastitic streptococci species.** Other *Staph*. species also cause mastitis. They may be present in the dairy environment, including bedding, although most strains are associated with the skin of the cow and perhaps dairy personnel. **The potential for growing on soiled equipment exists**, but this has not been well documented. **Most strains are not considered thermoduric or psychrotrophic.**

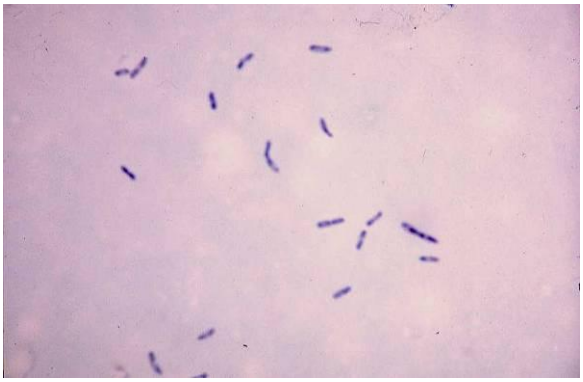


Fig. 9. Pseudomonas strain from raw milk stored at marginal refrigeration for extended period.

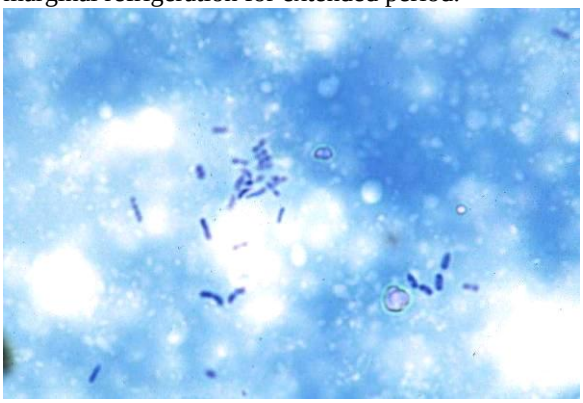


Fig. 10. Coliform bacteria from raw milk, this strain is a smaller rod. Coliform rods will vary in size.

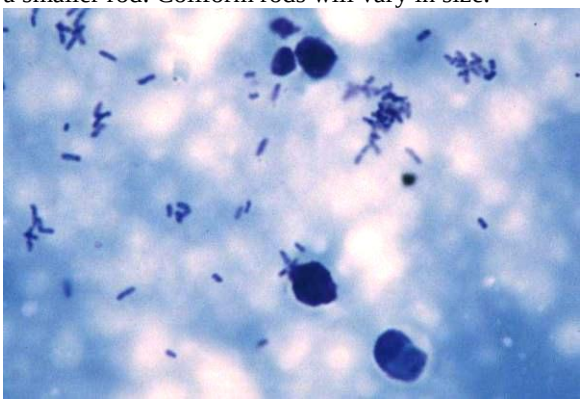


Fig. 11. Coliform bacteria from a mastitic cow with elevated SCC and clinical signs.

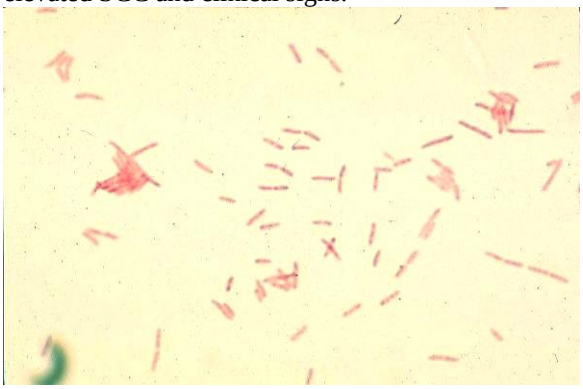


Fig. 12. **Gram-stain** of *Pseudomonas* spp. isolated from raw milk. Cells stain red with Gram-stain.

SHORT - MEDIUM RODS; SINGLES, PAIRS

Rod shaped bacteria (bacilli) are common in raw milk, although it is often difficult to distinguish between types. **Those types most often associated with high counts in raw milk are primarily Gram-negative rods and include psychrotrophs (e.g., *Pseudomonas*) and coliform bacteria**. **In milk smears, these appear as short to medium rods, growing singly or in pairs.** Some strains exhibit bi-polar staining (stain darker on the ends). **These organisms in raw milk are most often associated with contamination due to poor cleaning and sanitation procedures as well as environmental sources and poor pre-milking hygiene. Marginal cooling may cause psychrotrophic strains to increase.**

Gram-negative bacteria are widespread in nature. *Pseudomonas* spp. are often associated with untreated water supplies, although they are common in the environment and will grow well on soiled milking equipment. Coliform bacteria and Gram-negative bacteria in general, have been found in high numbers in bedding materials. Poor pre-milking hygiene procedures may be a source of these organisms although high counts directly from soiled teats would be rare. While coliforms are often associated with manure, some strains thrive in the environment and on poorly cleaned equipment as do other Gram-negatives. **Counts in raw milk resulting from soiled and/or poorly sanitized equipment may be low. However, *Pseudomonas* strains that contaminate milk from this source are most often responsible for high Preliminary Incubation (PI) counts.** Extended refrigeration selects for psychrotrophic bacteria, which often dominate raw milk at the plant.

Certain coliform bacteria and other Gram-negative rods are common causes of mastitis and may be shed into the milk from infected cows, although this is not as common as with mastitic streptococci. Certain strains of *Pseudomonas* may cause mastitis, but these are relatively rare and the influence of shedding into the milk has not been well documented.

Technical Information – ***Pseudomonas,*** *related bacteria*:

Gram-negative rods, 0.5-1.0 by 1.5 – 5.0 microns
Occur singly or in pairs, some strains stain bi-polar
Catalase positive, **oxidase-positive**
Colonies on SPC usually translucent, various pigments
- Large circular surface colonies are common
- Some strains produce diffusible pigments (green)

Do not survive pasteurization
Most common psychrotroph

Technical Information – ***Coliform,*** *related bacteria*:

Gram-negative rods, 0.5-1.0 by 1.5 – 5.0 microns
Occur singly or in pairs, some strains stain bi-polar
Catalase positive, **oxidase negative**
Colonies on SPC usually translucent, often mucoid
- Large surface colonies are common

Colonies on Coliform media (VRBA) are dark red
Do not survive pasteurization
Some strains are psychrotrophic

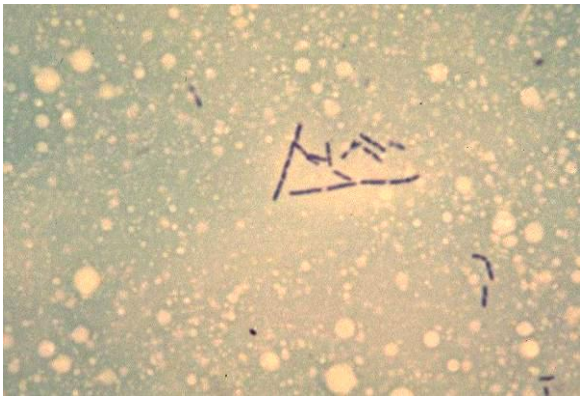


Fig 13. Milk smear of Gram-positive *Bacillus* strain demonstrating similarities to Gram-negative rods.

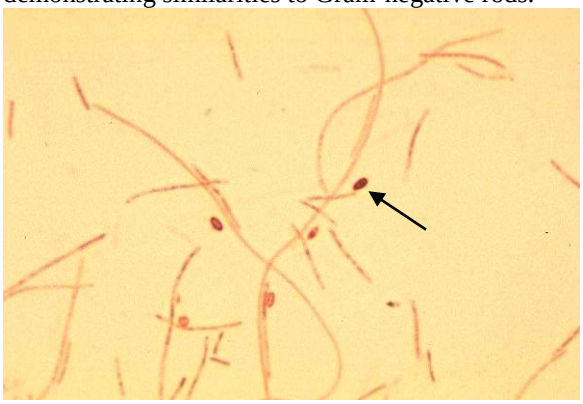


Fig. 14. **Spore Stain** of Gram-positive *Bacillus* strain demonstrating vegetative growth and spores.

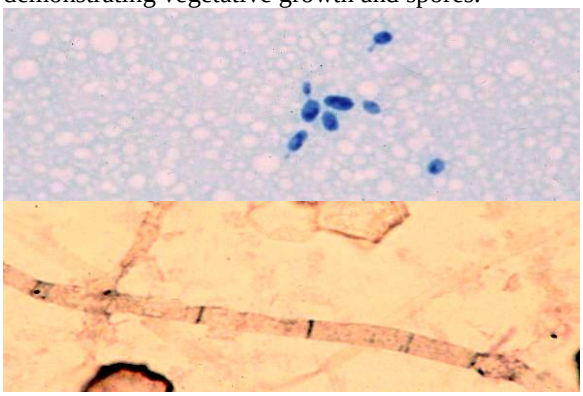


Fig. 15. Milk smear of budding yeast (top) and mold filament (bottom) from aged milk soil.

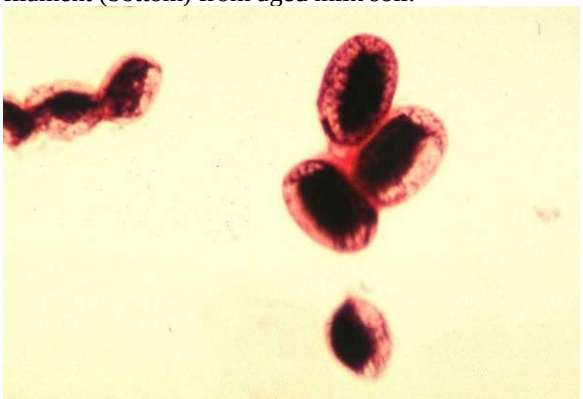


Fig 16. **Gram-stain** of *Prototheca* isolated from milk of a cow with mastitis.

GRAM-POSITIVE RODS; VARIOUS LENGTHS AND SIZES

Gram-positive rod shaped bacteria generally do not occur at high numbers in raw milk although a few strains may be significant contaminants. *Bacillus*, *Paenibacillus*, *Microbacterium* and *Lactobacillus* are the most common. These organisms are common in the dairy environment, although they generally do not grow as rapidly in milk as the previously discussed groups. *Bacillus* and *Paenibacillus* spp. are found more frequently in both raw and pasteurized milk. These are spore-formers, most of which survive pasteurization. Some strains of Gram-positive rods are psychrotrophic, especially the spore formers, although they do not grow as quickly as Gram-negative psychrotrophs that do not survive pasteurization. However, they do have the potential to become significant spoilage organisms when post-pasteurization contamination is prevented. These tend to be hardy organisms that are more likely to survive during cleaning and sanitation procedures and other extreme conditions (e.g., drying).

Technical Information – ***Gram-positive Rods***:

Gram-positive rods, various widths, lengths
Occur singly, in pairs or as end-to-end chains
Rods of some strains have blunt or squared ends
Catalase, oxidase, colony morphology varies
Some strains produce spores (i.e., *Bacillus*, *Paenibacillus*)
Some strains survive pasteurization
Some strains are psychrotrophic

YEAST AND MOLDS

Yeast and molds generally do not occur in large numbers in raw milk though if milk soil remains within uncleanable areas of the milking system, these organisms, especially yeasts, may become significant. Some yeast may be involved in mastitis though this is relatively rare. Yeast, which are much larger than most bacteria, are spherical to oval and generally exhibit budding reproduction. Molds most often appear as large filaments. Both yeast and molds will grow on SPC agar though growth rates are generally slower for most molds. Both types of organisms are common in nature.

PROTOTHECA

***Prototheca* spp. are achlorophyllic (do not contain chlorophyll) algae (microscopic plants) that can cause mastitis in dairy animals.** Primary sources include soil, plants, water (esp. stagnant) and feces. This organism appears as large spherical cells that may be easily mistaken for somatic cells. They also grow on SPC agar and have been responsible for high counts in milk.

AUXILIARY TESTS FOR BACTERIAL CHARACTERIZATION FROM AGAR PLATES

The microscopic examination of raw milk is often used to trouble-shoot high bacteria counts. While milk smears may provide evidence of microorganisms associated with poor cooling and mastitis, other causes of high counts may not be as clear. In many cases, further characterization is required. Generally this requires isolation of the suspect organism(s) from an agar plate (see p. 8 for isolation-streaking procedure). The Gram-stain is one of the first procedures used when classifying bacteria. Other tests include the endospore stain, the catalase test and the oxidase test. The results of these tests are listed under the technical information in the groups of bacteria previously described.

STAINING PROCEDURES FOR COLONIES ON AGAR PLATES

A. Making a Smear from Agar Plates (for isolation/streaking procedure, see p. 8):

1. Flame sterilize transfer loop and cool on a clear sterile area of the agar plate or Petrifilm™. Alternatively, use sterile disposable inoculating loops.
2. Touch desired colony with the loop or needle picking up a small amount of microbial growth. Thoroughly mix growth with a drop of sterile buffered water on a slide to form a thin film.
3. Allow the film on the slide to air dry, then heat fix the slide by passing three times through the flame. The slide should feel warm, but not hot, when placed against the back of your hand. Do Not Over-Heat.
4. Stain the smear with: Levowitz-Weber or Simple Stain (Gram Crystal Violet)
   Gram-Stain Procedure, Endospore Stain Procedure
5. Blot the slide dry and observe under the microscope (oil immersion lens, 100x).

B. Gram Stain Procedure:

Reagents for the Gram stain include crystal violet, Gram's iodine, decolorizer, and safranin. Prepared kits with directions for use are available from laboratory supply companies. General procedure:

1. Prepare a thin smear of the organism, allow to air dry, and then fix with heat. **Ideally the culture should be 18 - 24 hr. old**. Smear may be made from plate or broth cultures (e.g., nutrient broth).
2. Cover smear with crystal violet solution for 1 minute then rinse gently with water.
3. Cover smear with Gram iodine solution for 1 minute or longer. Rinse gently with water.
4. Apply decolorizer solution just until it runs clear (no more color, ~ 20 seconds). Quickly rinse off remaining decolorizer with water. **DO NOT OVER-DECOLORIZE**.
5. Remove excess water. Counter-stain with safranin solution for 1 minute. Rinse with water and blot dry without rubbing.
6. Examine cells microscopically: Cells Stain **Blue = Gram-positive**
   Cells Stain **Red = Gram-negative**

C. Potassium Hydroxide (KOH) Method for Gram Reaction:

1. Place one drop of 3 % KOH on a clean glass slide.
2. Remove bacterial growth from a colony with an applicator stick or sterile loop.
3. Mix growth with KOH and pull away slowly.
4. **Gram-negative will be ropy or thread-like. Gram-positive will not.**

D. Endospore (Spore) Stain (cold method): Older cultures tend to have more spores present, strain dependent. Some require special media to form spores. Spores may be observed free or within cells.

1. Prepare a thin smear of the organism in question, allow to air dry thoroughly. **Fix the smear by passing through a flame 20 times.**
2. Flood the smear with **7.6 % malachite green** (aqueous solution, 7.6. g/100 ml water) for 15-30 minutes (30 minutes may be needed).
3. Rinse gently with water until clear. Counter-stain with **safranin** for 30 seconds. Rinse, blot dry.
4. **Endospores stain green, while the remainder of the cell stains light red**.

BIOCHEMICAL TESTS USED TO CHARACTERIZE BACTERIA

A. Catalase Test:

The catalase test determines if an organism can degrade peroxides (i.e., H_2O_2) to oxygen and water. The enzyme catalase is present in certain bacteria as a protective feature to destroy toxic peroxides. When hydrogen peroxide is added to a culture of bacteria that has the catalase enzyme, visible bubbles of oxygen are liberated during its degradation. Most Gram-negative bacteria common in dairy products are catalase-positive. The catalase test is most useful in distinguishing between certain Gram-positive bacteria (catalase-positive *Micrococcus* versus catalase-negative *Streptococcus* or *Lactococcus*).

Procedure:

1. From an agar plate, transfer a small amount of a colony to the surface of a clean dry slide with a sterile loop or applicator stick. Alternatively, the test can be done directly on a colony.
2. Add one drop of 3 - 5 % Hydrogen Peroxide.
3. **Visible bubbles indicate a positive catalase test**. No bubbles indicate a negative catalase test. Certain strains are weakly positive; a low-powered microscope may be required.

   Catalase-positive: visible bubbles with hydrogen peroxide
   Catalase-negative: no bubbles formed with hydrogen peroxide

B. Oxidase Test:

The oxidase test determines the presence of the enzyme cytochrome-c oxidase (important in cell respiration or electron transport) and is used primarily to distinguish between different groups of Gram-negative bacteria. Members of the family Enterobacteriaceae, which includes the coliform group, *Salmonella*, *Shigella*, and *Proteus*, are oxidase-negative. Gram-negative bacteria that are oxidase-positive include the most commonly occurring psychrotrophs in milk belonging to the genus *Pseudomonas*. *Alcaligenes* and *Flavobacterium* are other genera that are oxidase-positive. The oxidase test is also used to differentiate *Micrococcus* (ox-pos) from *Staphylococcus* (ox-neg).

Procedure - Liquid Reagent:

1. Prepare the "oxidase" reagent just before use. Weigh 0.1 gram of para-aminodimethylaniline oxalate and dissolve in 10 ml of distilled water with gentle heating. This reagent is available prepared in sealed vials.
2. Soak an area of filter paper with the "oxidase reagent."
3. Using a wooden applicator stick, toothpick, or platinum loop (do not use standard loop material), apply a portion of bacterial growth from an isolated colony to the moistened area.
4. **If the organism is oxidase-positive the reagent will turn the growth red to black within 2 minutes**. If they are oxidase-negative, no color will develop.

Procedure - Dryslide™ OXIDASE (BBL):

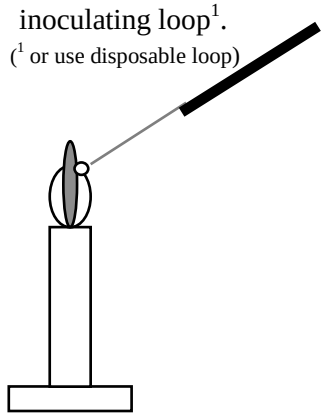
1. Open pouch and remove the number Dryslides to be used. Seal pouch tightly, store at room temperature and use the remainder within one week.
2. Using a wooden applicator stick, toothpick, or platinum loop, apply a portion of bacterial growth from an isolated colony to the reaction area of the slide.
3. **Oxidase-positive, the slide will turn purple within 20 seconds**. If it is oxidase-negative, no color will develop. Disregard color changes after 20 seconds.

References:
1. National Mastitis Council. 1999. *Laboratory Handbook on Bovine Mastitis*. NMC, Madison, WI.
2. Murphy, S.C. and K.J. Boor. 2000. Trouble-shooting sources and causes of high bacteria counts in raw milk. Dairy, Food and Environmental Sanitation. 8:606-611.
3. Hayes, M.C., R.D. Ralyea, S.C. Murphy, N.R. Carey, J.M. Scarlett and K.J. Boor. 2001. Characterization of elevated microbial counts in bulk tank raw milk. J. Dairy Sci. 84:292-298.
4. Marshall, R.T. editor. 1993. *Standard Methods for the Examination of Dairy Products*. 16th Edition APHA, Washington D.C.
5. Atherton, H.V. and W.A. Dodge. 1970. *Milk Under the Microscope*. Vermont Extension Serv, Univ. of Vt.

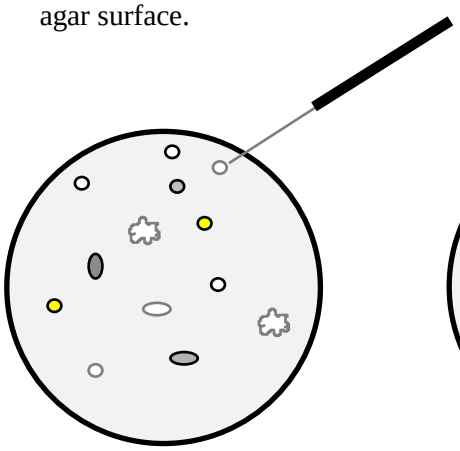
Appendix

Streaking Isolated Bacterial Colonies for Purification and Identification

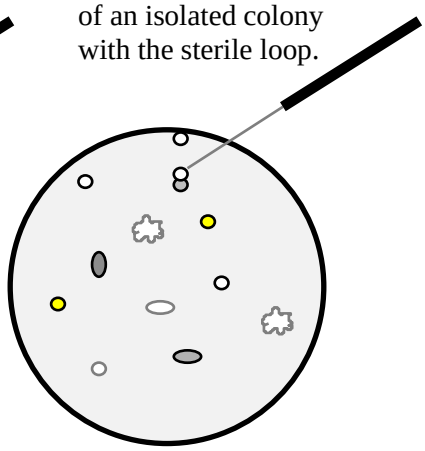
1. Flame sterilize inoculating loop[1].
([1] or use disposable loop)



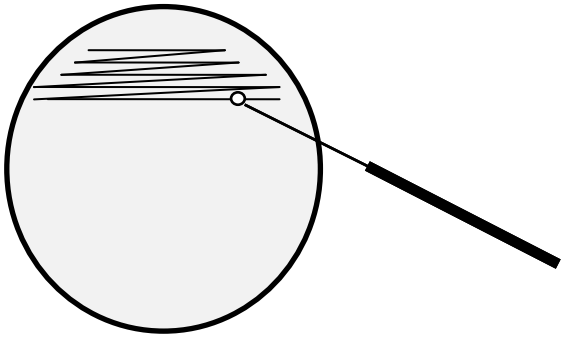
2. Cool loop on sterile agar surface.



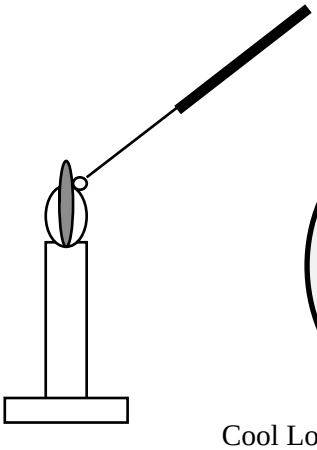
3. Pick up a small amount of an isolated colony with the sterile loop.



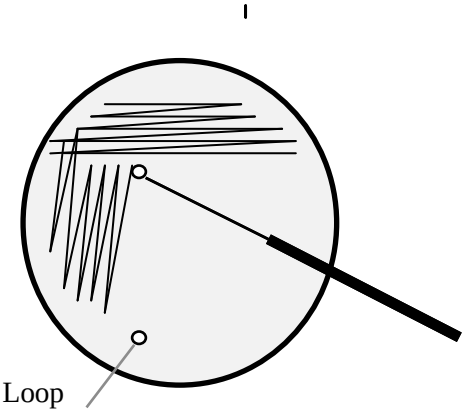
4. Streak the loop across a section of a pre-poured sterile SPC agar plate, back and forth several times over the surface of the plate.



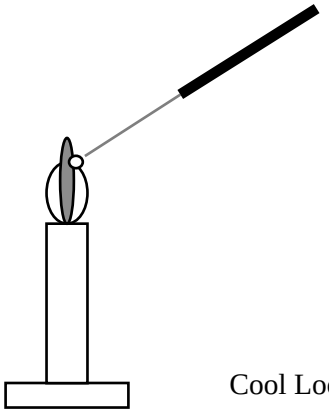
5. Flame sterilize the loop[1], and cool on a sterile section of the agar surface.



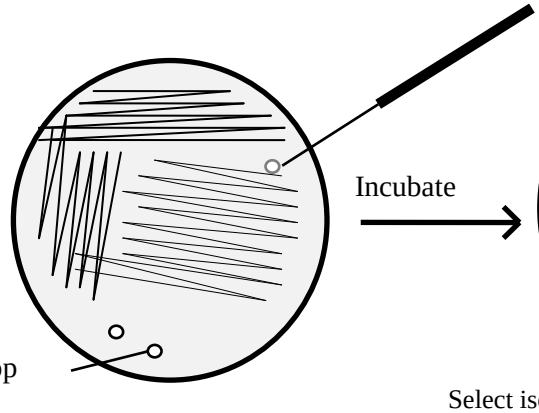
6. Streak into the previous streaked section once or twice, continue streaking across one third of plate



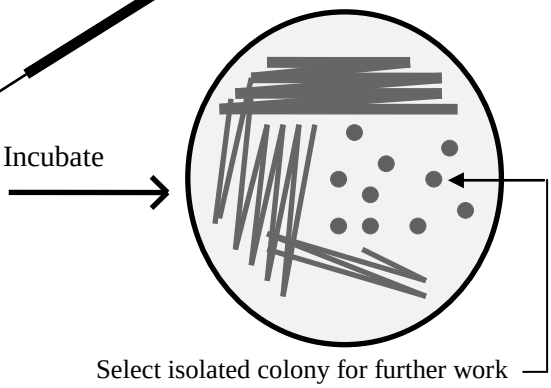
7. Flame sterilize the loop[1] and cool on plate.



8. Streak into previous section once or twice, continue streaking final third of plate.



9. Incubate. Select isolated colonies for further identification.



DIRECT MICROSCOPIC SOMATIC CELL COUNT GUIDELINE

Rules and Examples for Counting Somatic Cells in Milk

The following images are scans of slides prepared by the FDA Laboratory Quality Assurance Team (LQAT) for use in training for the Direct Microscopic Somatic Cell Count (DMSCC) procedure as outlined in Form FDA-2400d. When viewing these images, keep in mind that the focus is fixed, whereas the actual counting procedure requires constant adjustment of the microscope's fine focus. This guideline can be used to help properly identify and count somatic cells in milk and should facilitate standardization of analysts performing the DMSCC procedure.

RULES FOR IDENTIFYING AND COUNT1NG SOMATIC CELLS
DMSCC – Single Strip Procedure (Form FDA-2400d)

1**. Cells possess a nucleus stained dark blue**

The nuclear mass of a cell, composed of one or more nuclear units, should stain dark blue with L-W stain. The intensity of "dark" blue may vary with staining technique and cells. The nuclear mass generally has no recognizable form, though generally appears as a solid blue unit. In some cells it may be granular in appearance. Some distortion may be expected. Count each dark blue mass, which bears resemblance to a typical nucleus. The nuclear mass of a leucocyte is polylobed and the lobes (units) are bridged by nuclear material. These multi-lobed nuclei with bridges are counted as one cell.

The cytoplasm which normally surrounds each nucleus may (a) be stained light blue, (b) not stained and appear as a clear zone or (c) have disintegrated and not be present.

2. **Cells are generally ≥ 8 microns; do not count cells < 4 microns; count fragments only if > 50% of nuclear material is visible.**

The nuclear mass of a countable somatic cell is generally eight microns or greater in size. Cells or fragments (cell that has been obviously damaged or degraded) may appear that are smaller. Do not count cells with nuclei of less than 4 microns. Fragmented cells are counted only if more than fifty percent of the nuclear mass is visible (at least four microns). Do not count cells without a nucleus.

3. **Cluster of cells counted as one unless nuclear units are clearly separated.**

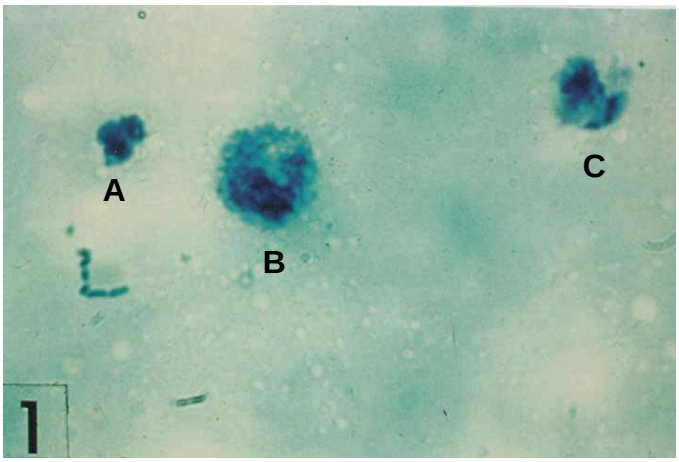
Use the fine focus to determine if nuclear bridges are present. If a clear area can be seen surrounding any of the nuclear masses in the cluster, count each clearly delineated nuclear mass as one cell. If the mass shows no clear differentiation, or there are bridges evident, count those attached as one.

4. **Count cells touching only the top or the bottom edge of a horizontal strip (or left or right edge for vertical edge of the strip).**

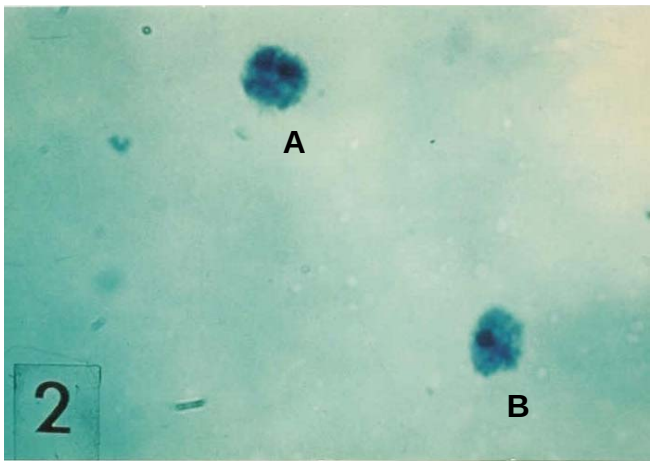
Nuclear masses that touch the edge or go outside of the strip boundaries should only be counted from one side of the strip to avoid counting an area larger than specified. Count cells that touch only one boundary of the strip; for horizontal strip counting, select either the upper or the lower edge; for vertical strips, select either the left or the right edge. Do not count cells that touch the other boundary.

5. ***If in Doubt, Do Not Count Questionable Cells!***

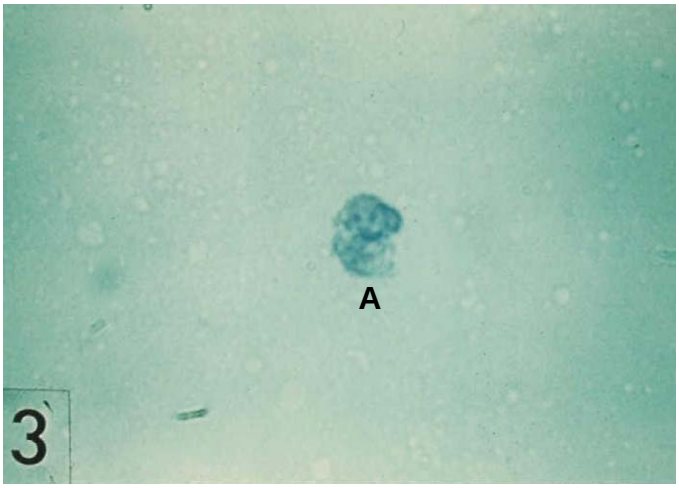
This Guideline was adapted from an FDA-LQAT's DMSCC training slide set and was originally prepared in printable format by James E. Fitts of NYS Department of Agriculture & Markets, Milk Control (04/98). This update was prepared by J. Fitts, Agriculture & Markets, and S. Murphy, Department of Food Science, Cornell University (07/04).



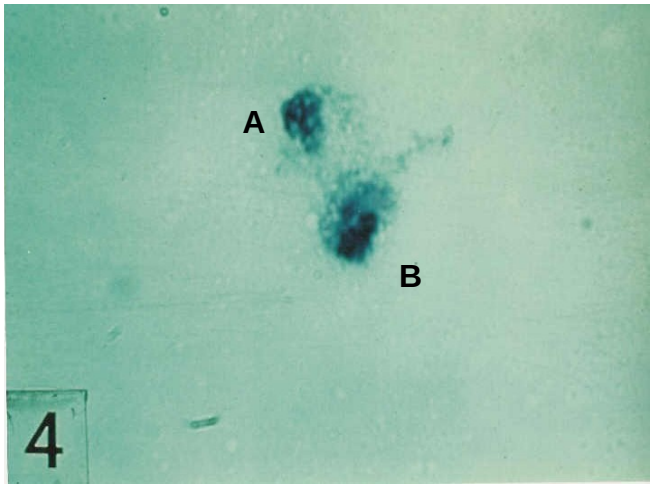
1. "A," "B" & "C" are all countable cells (3 cells).



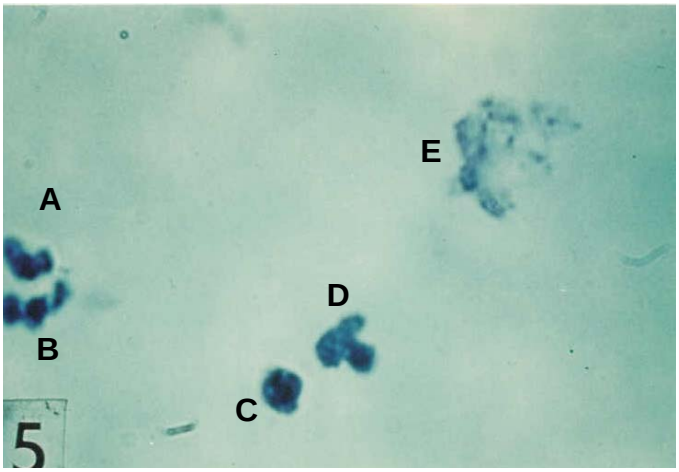
2. "A" & "B" are countable cells (2 cells).



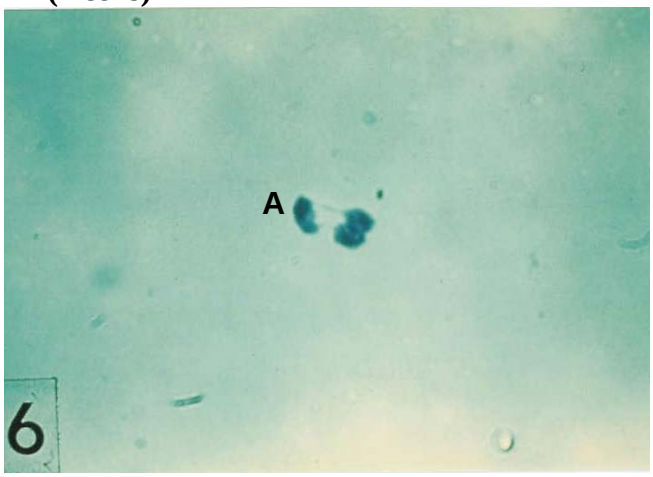
3. "A" is a countable cell (1 cell).



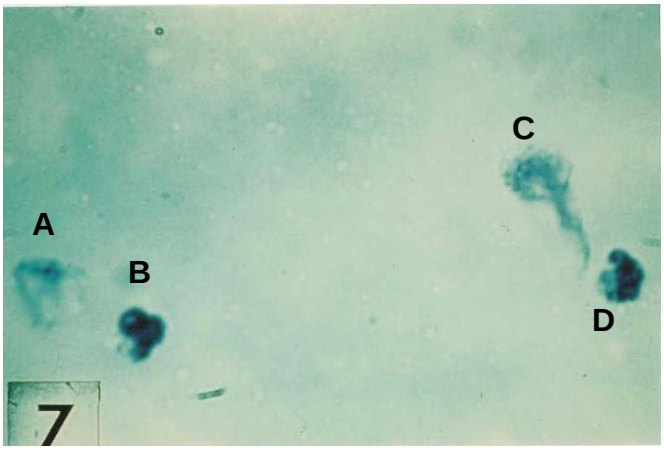
4. "A" & "B" are countable cells, both surrounded by disintegrating cytoplasm. (2 cells)



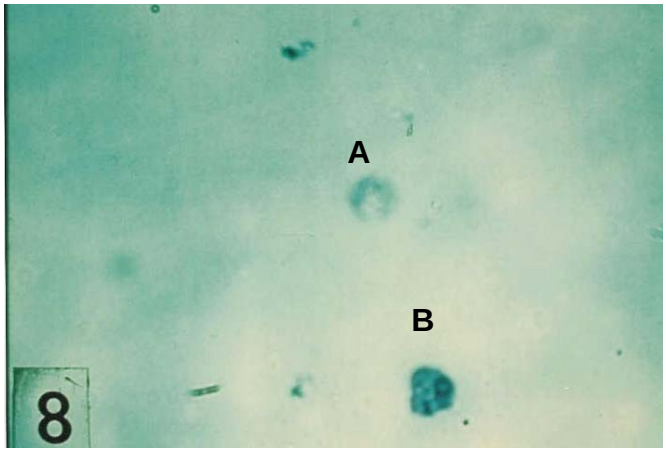
5. "A – D" are typical countable cells, "E" is cellular debris and is not counted. (4 cells)



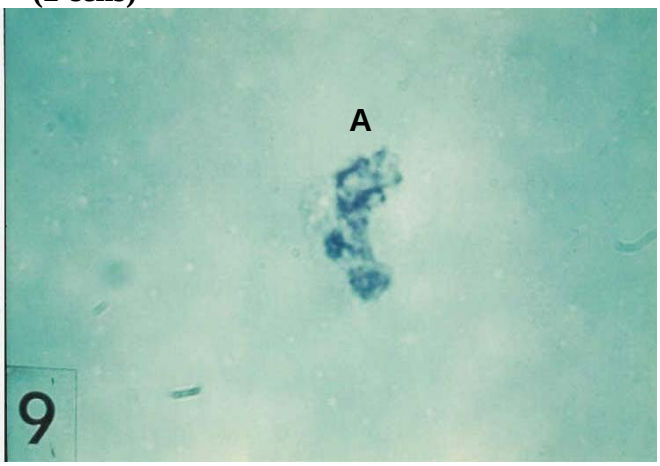
6. "A" is one cell with nuclear lobes connected by a nuclear bridge. Counted as 1 cell. (1 cell)



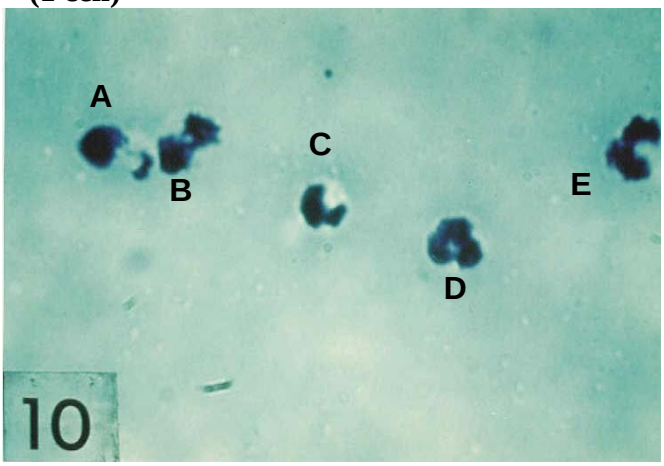
7. "A" & "C" are cytoplasmic debris and are not counted. "B" & "D" are typical cells. (2 cells)



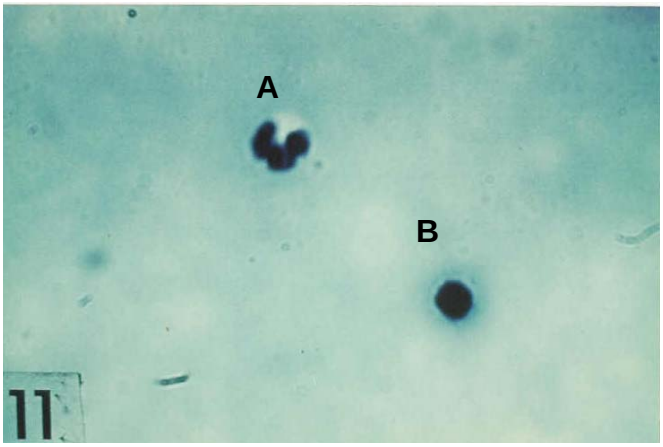
8. "A" is a "ghost" cell (no nuclear material) and not counted. "B" is a countable cell. (1 cell)



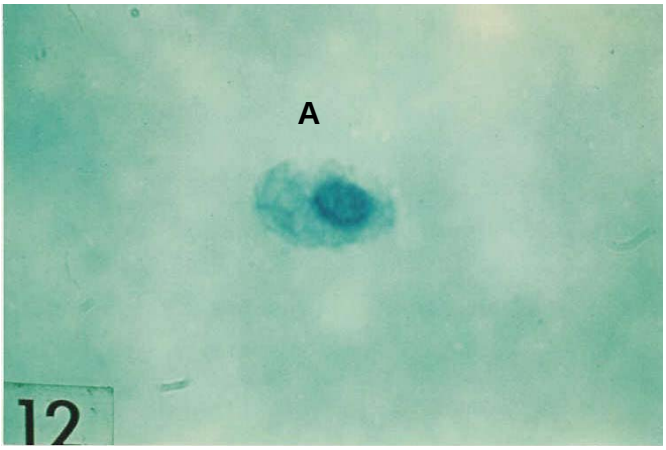
9. "A" is one cell with disintegrating nucleus, countable. (1 cell)



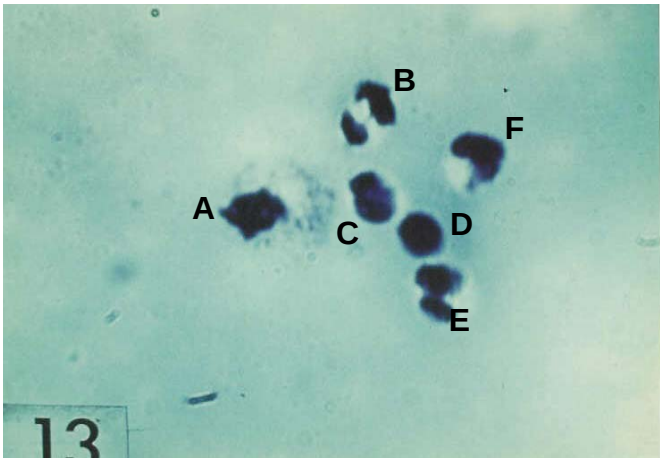
10. "A-E" are typical cells, all with multiple lobes. (5 cells)



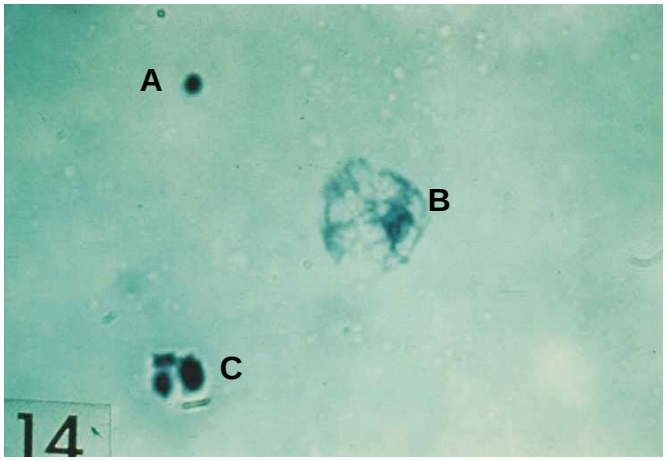
11. "A" is a typical cell. "B" is a typical monocyte. Both are counted. (2 cells)



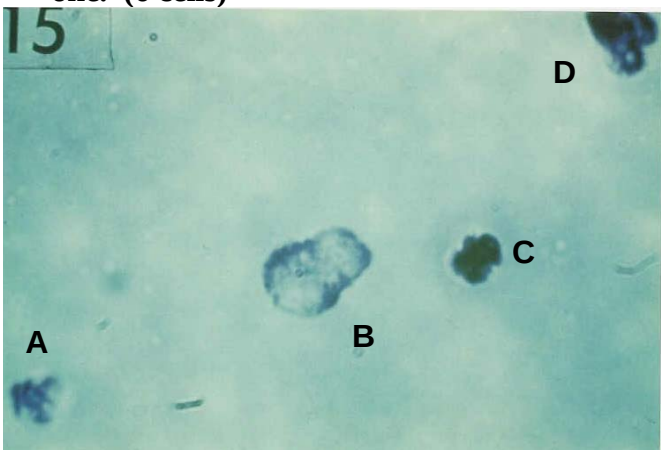
12. "A" is a single cell with large visible cytoplasm. (1 cell)



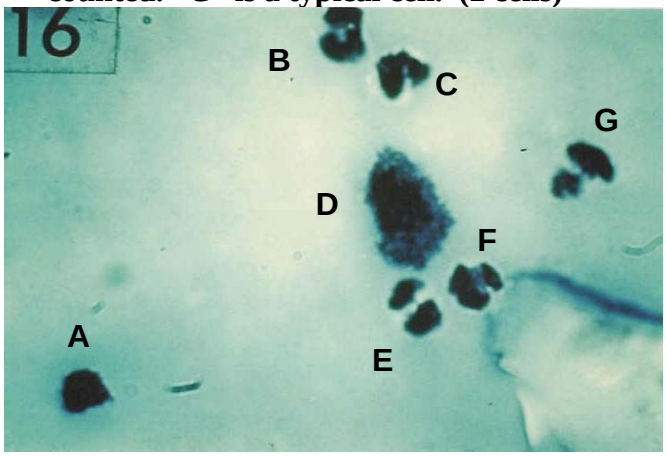
13. All are countable cells. "B" & "E" have bi-lobed nuclei with bridges, each counted as one. (6 cells)



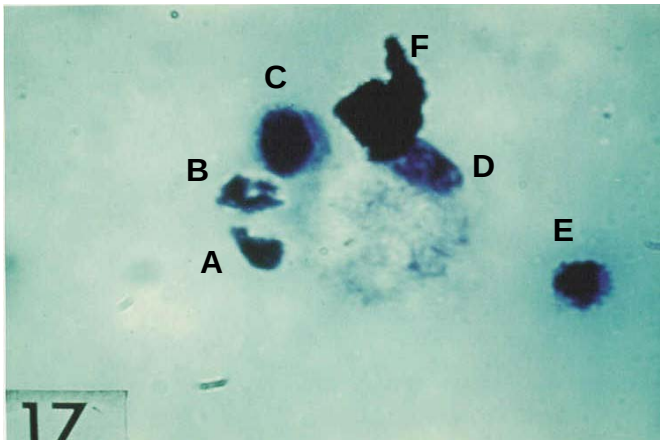
14. "A" is not counted based on size (< 4 microns). "B" though disintegrating, is counted. "C" is a typical cell. (2 cells)



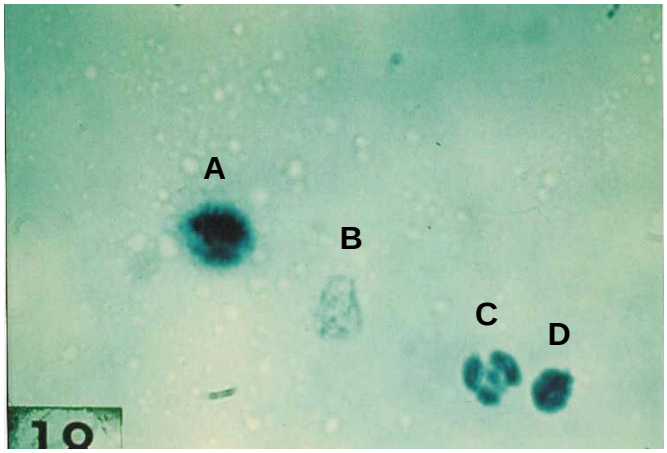
15. "A," "C" & "D" are countable cells. "B" is cytoplasmic debris not counted. (3 cells)



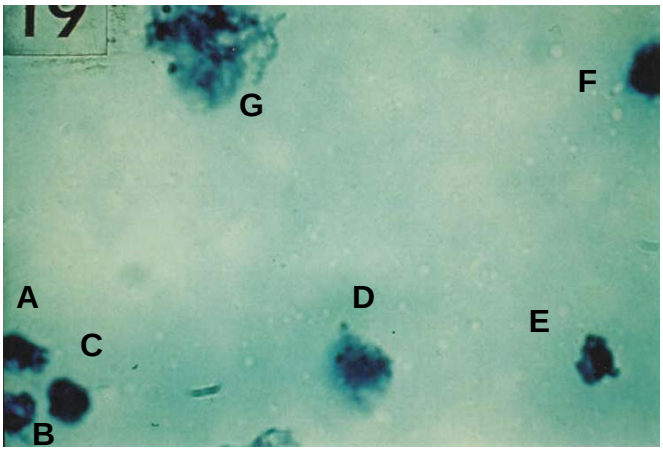
16. "A-G" are all countable cells. (7 cells)



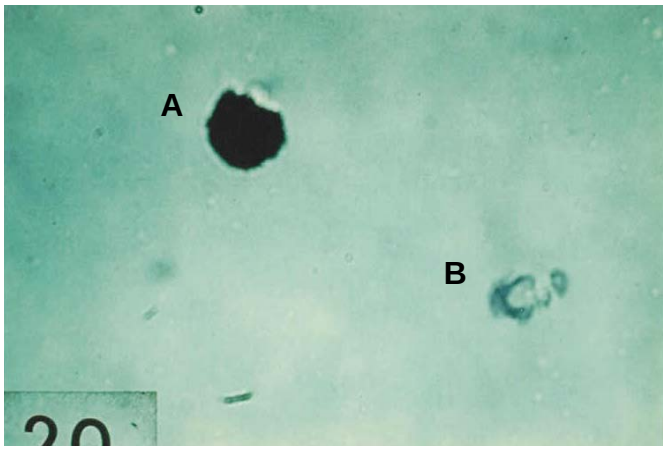
17. "A," "B," "C" & "E" are typical cells (4). "D" is a countable cell with large area of disintegrating cytoplasm (1 cell). "F" is debris, not a countable cell. (5 cells)



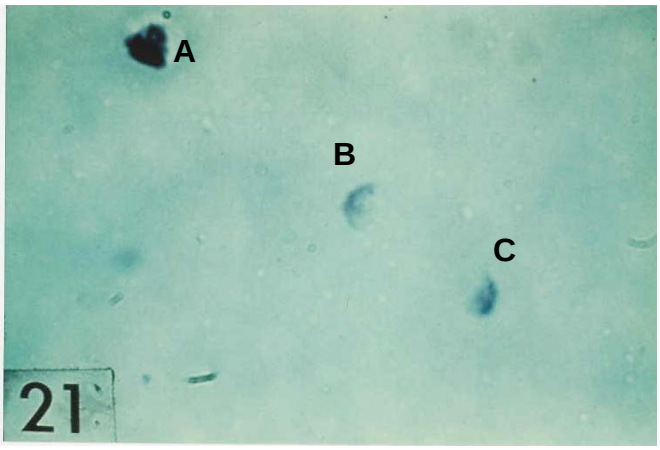
18. "A," "C" & "D" are typical cells. "B" is a ghost cell or debris and not counted. (3 cells)



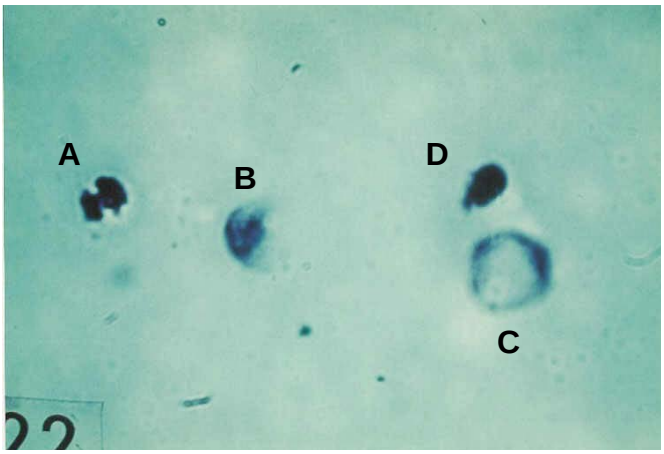
19. "A-G" are typical cells. (7 cells)



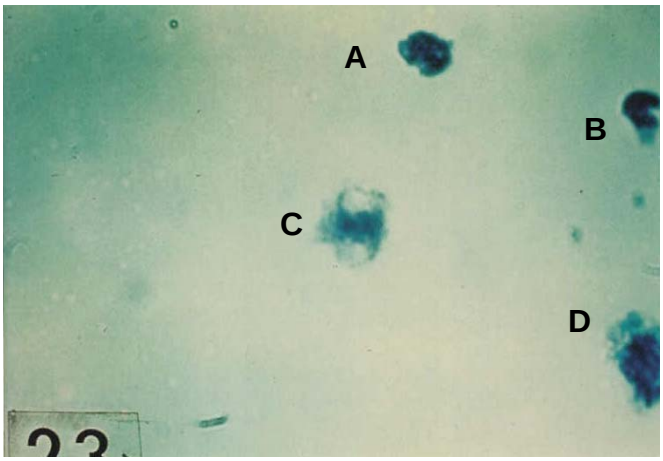
20. "A" & "B" are debris. (0 cells)



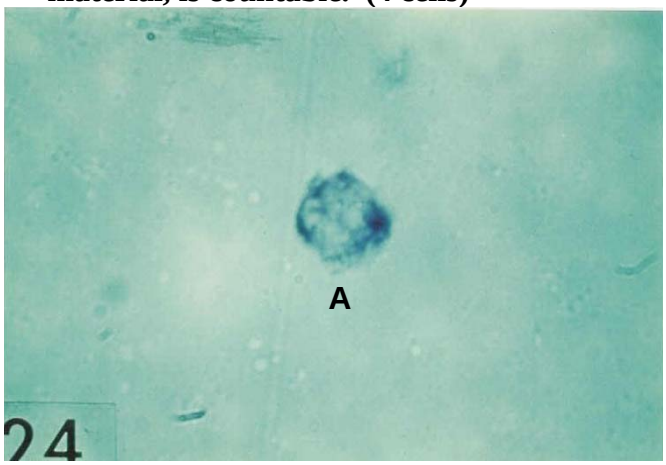
21. "A" is a typical countable cell. "B" & "C" are cytoplasmic debris not counted. (1 cell)



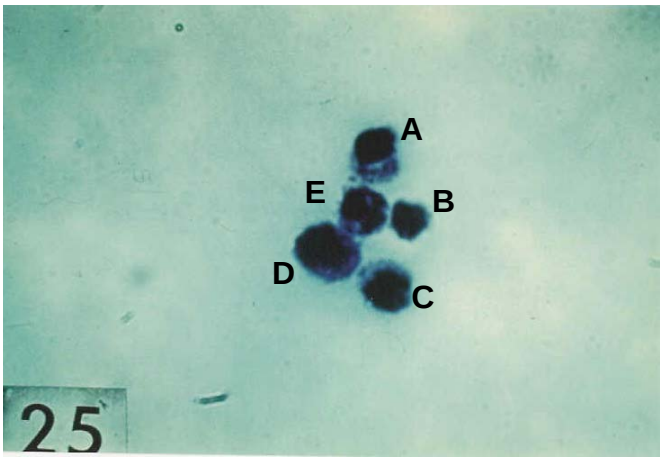
22. "A" & "D" are typical cells. "B" is a fragment greater than 50% (countable) and though "C" has disintegrating nuclear material, is countable. (4 cells)



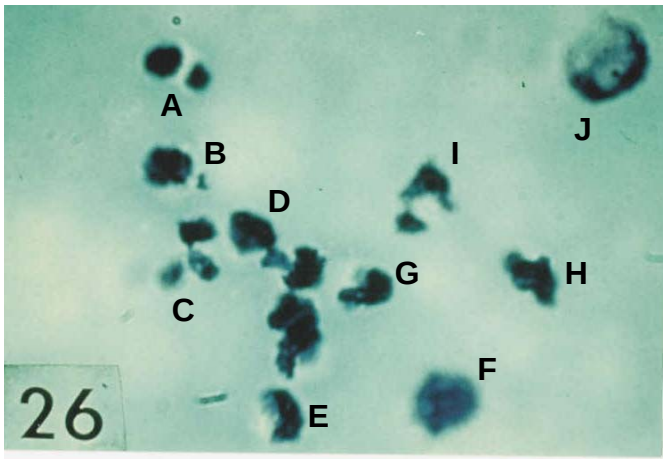
23. "A" & "B" are typical cells. "C" & "D" are countable cells showing early degeneration. (4 cells)



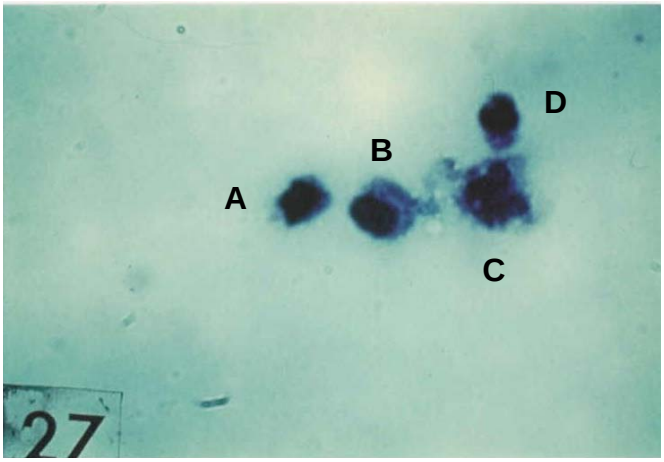
24. "A" is a disintegrating cell, though countable. (1 cell)



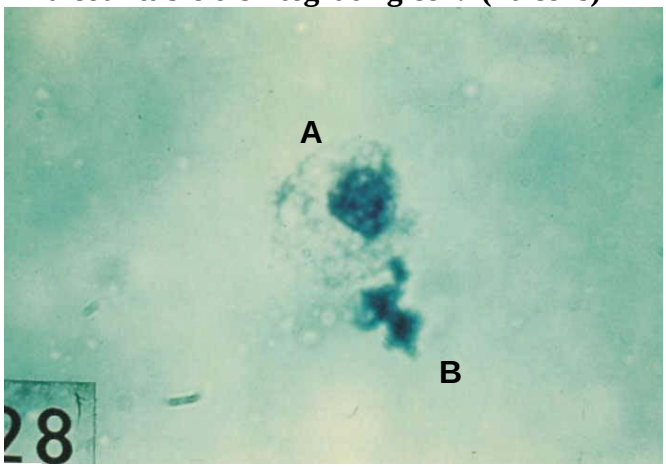
25. "A-E" is a typical clump of cells counted individually. (5 cells)



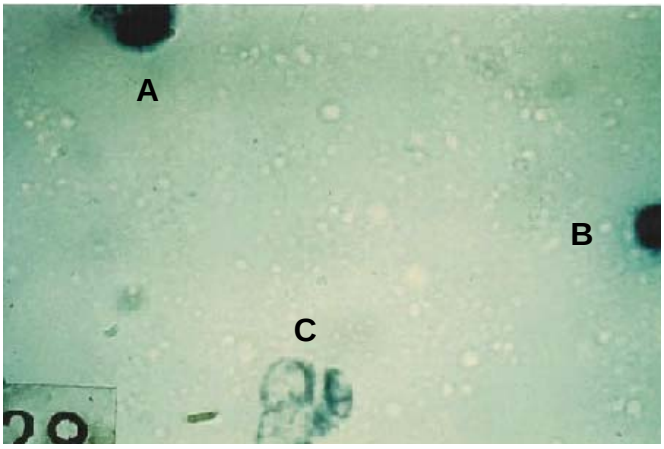
26. "E," "F," "G" & "H" are mononuclear cells. "A," "B" & "I" are bi-lobed cells. "C" is a tri-lobed cell. "D" has 4 nuclear lobes. "J" is a countable disintegrating cell. (10 cells)



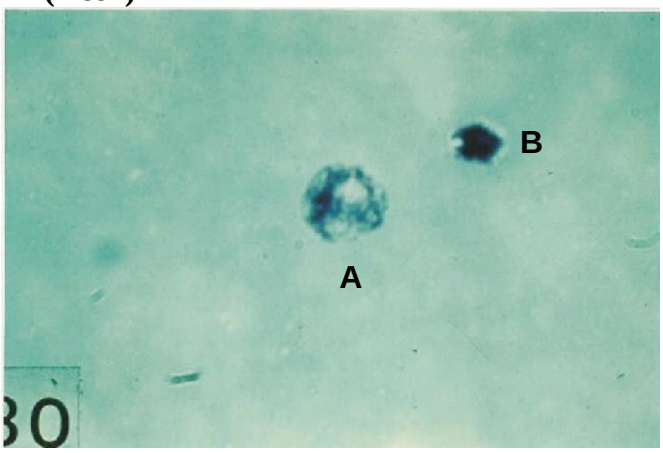
27. "A-D" are all typical countable cells. (4 cells)



28. "A" is a typical cell showing cytoplasmic degeneration. "B" is cellular debris. (1 cell)



29. "A" & "B" are typical cells. "C" is a ghost cell. (2 cells)



30. "A" is a disintegrating cell, though countable. "B" is a typical cell. (2 cells)