U.S. FOOD & DRUG
ADMINISTRATION

Bacteriological Analytical Manual

Chapter 2: Microscopic Examination of Foods

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Introduction

If there is reason to suspect that a food has caused food poisoning or has undergone microbial spoilage, the original product or a low serial dilution of it should be used to prepare a slide for direct microscopic examination. The Gram stain reaction and cellular morphology of the bacteria on the slide may indicate the need for other types of examination. A microscopic examination must be made, even though the food may have undergone heat treatment and the microorganisms involved may no longer be viable. Large numbers of Gram-positive cocci on the slide may indicate the presence of staphylococcal enterotoxin, which is not destroyed by the heat treatments that destroy enterotoxigenic *Staphylococcus aureus* strains. Large numbers of sporeforming, Gram-positive rods in a frozen food specimen may indicate the presence of *Clostridium perfringens*, an organism that is sensitive to low temperatures. Other Gram-positive, sporeforming rods such as *Clostridium botulinum* or *Bacillus cereus* may also be present in the food. When the microscopic examination of suspect food discloses the presence of many Gram-negative rods, consider the symptoms and incubation periods reported for the illness under investigation and select the specific examination method for isolating one or more of the following genera: *Salmonella*, *Shigella*, *Escherichia*, *Yersinia*, *Vibrio*, or *Campylobacter*.

Direct Microscopic Examination of Foods (Except Eggs)

A. Equipment and materials

1. Glass slides, 25 × 75 mm, with etched portion for labeling; 1 slide for each blended food sample (10^{-1} dilution)
2. Wire loop, 3-4 mm, platinum-iridium or nichrome, B&S gauge No. 24 or 26
3. Gram stain reagents (R32)
4. Microscope, with oil immersion objective lens (95-100×) and 10× ocular

5. Immersion oil
6. Methanol
7. Xylene

B. Procedure

Prepare film of blended food sample (10^{-1} dilution). Air-dry films and fix with moderate heat by passing films rapidly over Bunsen or Fisher burner flame 3 or 4 times. Alternatively, air-dry films and fix with methanol 1-2 min, drain excess methanol, and flame or air-dry (this is particularly helpful for foods with a high sugar content). Cool to room temperature before staining. De-fat films of food with high fat content by immersing films in xylene 1-2 min; then drain, wash in methanol, drain, and dry. Stain film by Gram-staining procedure (R32). Use microscope equipped with oil immersion objective (95-100×) and 10× ocular; adjust lighting systems to Koehlor illumination. Examine at least 10 fields of each film, noting predominant types of organisms, especially clostridial forms, Gram-positive cocci, and Gram-negative bacilli.

Direct Microscopic Examination of Eggs (2)

A. Equipment and materials

1. Microscope, with 10× oculars and oil immersion objective (1.8 mm or 90-100×)
2. Microscope slides, 25 × 75 mm or 50 × 75 mm
3. Bacteriological pipet or metal syringe, to deliver 0.01 ml
4. North aniline (oil)-methylene blue stain (R49)
5. 0.1 N lithium hydroxide (R39)
6. Physiological saline solution, 0.85% (sterile) (R63)
7. Butterfield's phosphate-buffered dilution water (R11)
8. Xylene
9. Ethanol, 95%

B. Procedure for liquid and frozen eggs

1. Thaw frozen egg material as rapidly as possible to prevent increase in number of microorganisms present. Thaw below 45°C for 15 min with continuous agitation

in thermostatically controlled water bath. Using bacteriological pipet or metal syringe, place 0.01 ml undiluted egg material on clean, dry microscope slide. Spread egg material evenly over area of 2 sq cm (circular area of 1.6 cm diameter is preferred). Add drop of water to each film for uniform spreading.

2. Let film dry on level surface at 35-40°C. Immerse in xylene up to 1 min; then immerse in 95% ethanol up to 1 min. Stain film 1 min in North aniline (oil)-methylene blue stain (10-20 min preferred; exposure to 2 h does not overstain). Wash slide by repeated immersions in beaker of water, and thoroughly air-dry before examining (do not blot). Count microorganisms observed in 10-60 fields. Multiply average number per field by microscopic factor and by 2, since 2 sq cm area was used. Carry out subsequent operations and observe precautions as directed in "Direct Microscopic Method for Bacteria," *Standard Methods for the Examination of Dairy Products* (1). Express final results as number of bacteria (or clumps) per g of egg material.

C. Procedure for dried egg products

Thoroughly mix sample; prepare 1:10 dilution by aseptically weighing 11 g egg material into sterile, wide-mouth, glass-stoppered or screw-capped bottle. Add 99 ml diluent (R11) or sterile physiological salt solution and 1 sterile tablespoon of sterile glass beads (0.1 N lithium hydroxide may be used as diluent and is preferred for samples of whole egg and yolk products that are relatively insoluble). Thoroughly agitate 1:10 dilution to ensure complete solution or distribution of egg material by shaking each container rapidly 25 times, using up-and-down or back-and-forth movement of about 1 ft arc, within 7 s. Let bubbles escape.

Place 0.01 ml of 1:10 or 1:100 dilution dried egg material on clean microscope slide and spread evenly over 2 sq cm. Proceed as in Direct Microscopic Examination of Eggs, B-2, above. Multiply average number of microorganisms per field by twice the microscopic factor (since 2 sq cm area was used) and multiply by 10 or 100, depending on whether film was prepared from 1:10 or 1:100 dilution. Express final results as number of bacteria (or clumps) per g of egg material.

Care and Use of the Microscope

Caveats

- Never dust a lens by blowing on it. Saliva will inevitably be deposited on lenses and is harmful, even in minute amounts.
- If pressurized air is used for dusting, use an inline filter to trap oil and other contaminants.
- Do not use dry lens tissue on a lens.
- Never use facial tissues to clean lenses. They may contain glass filaments which can scratch lenses. Linen or chamois may be used for cleaning but may not be as convenient as lens tissue. Do not confuse lens tissue with bibulous paper, which should never be used to clean lenses. Follow the manufacturer's recommendation for using

cleaning solvents other than water. Lens mounting glues are often soluble in alcohol. Xylene used sparingly is generally acceptable for serious stains, such as residual oils.

- Never leave microscope tubes open. Always keep them closed with dust plug, eyepiece, or objective, as appropriate.
- Avoid touching lenses. Even light fingerprints, especially on objectives, can seriously degrade image quality.
- Do not attempt to take optics apart for cleaning. Internal optics should not need routine cleaning and should be professionally serviced if needed.
- Use proper immersion liquids on immersion objectives as specified by the manufacturer. Avoid getting immersion liquid on non-immersion objectives; it can damage the lens mounting glue.
- Keep microscopes covered when not in use. Avoid extremes of temperature and high humidity. In work areas with consistently more than 60% relative humidity, store microscopes in circulating air if possible. In very high humidity, optical parts should be stored in tightly covered containers with desiccant and kept very clean to prevent mold growth on the optic coating.
- Do not use the substage diaphragm to control brightness.

Cleaning

- Body of the microscope. Use alcohol or soapy water on cloth to wipe body. Lubricate sliding parts with a petroleum jelly, such as Vaseline, or use a manufacturer-recommended lubricant
- Lenses and optics
- Grit and dust can scratch lenses and coatings.
- Blow away dust with rubber bulb.
- For light cleaning, breathe on lens to fog it, and then use lens tissue as described below. The fogging is basically water and is not harmful.
- For dirtier lenses, use lens cleaner solution, such as that manufactured by Kodak, available from any camera supply store. Remove stubborn stains by using xylene sparingly.
- Use the following procedure to properly clean lenses of microscopes or other optical equipment. Crumple a piece of lens tissue to create many folds to trap dirt without grinding it into the lens. Do not touch the part of the tissue that will be applied to the lens; excessive touching transfers natural oils from the fingers to the lens tissue. Apply a small amount of lens cleaning solution to the lens tissue and blot the tissue against absorbent material to prevent fluid from entering the lens mount. Wipe the lens very lightly to remove gross dirt that was not blown away by the rubber bulb. If necessary, repeat the cleaning process with a new piece of lens tissue and with more pressure to remove oily or greasy residue. Complete the process by using the light cleaning procedure (breath and lens tissue) described above.
- Adjustments. Follow the manufacturer's recommendations for microscope adjustments that can be made by the user. Tension of the coarse focus mechanism can usually be adjusted by the user. Directions for adjusting the lighting and eyepiece follow.

Proper Set-up and Illumination of the Compound Microscope

Eyepieces (oculars) must be adjusted to the user's eyes. Only microscopes with binocular tubes are discussed here. Except for interpupillary distance, other adjustments hold true for microscopes with monocular tubes. To adjust interpupillary distance, lengthen or shorten the distance between centers of oculars to match the distance between centers of pupils of the eyes.

Adjust the microscope for each eye. One eyepiece, or the tube into which it fits, is usually adjustable. Place a specimen slide on the microscope stage, turn on the illumination, and focus at low magnification. Cover the eyepiece that has the focusing eye tube with a card and, with both eyes open, bring the specimen into focus for the other eye with the fine focus knob. It is important that vision be relaxed by looking up frequently to distant objects or to infinity by staring "through the wall." This will help prevent eyestrain caused by trying to "accommodate" the object, bringing it into focus with the eye at a point closer than infinity. When consistent sharp and relaxed focus has been obtained at one particular point on the slide, switch the card to cover the other eyepiece, but this time use the focusing ring on the open eyepiece to bring the same point on the slide into focus. Follow the same procedure for relaxed viewing.

The next important point is the distance from the eye to the eyepiece. If the eyes are too close or too far away, the field of view will be reduced and the specimen may appear less sharp. From a few inches away, move in slowly until the field appears the widest and sharpest. This distance, from the pupil of the eye to the lens, is the eye point or exit pupil of the microscope.

The focusing of the microscope and adjustment for each eye will correct for most conditions of near or farsightedness, eliminating the need to wear corrective eyeglasses during microscope work. Even moderate astigmatism will not hamper most microscope use; however, for more serious astigmatism, for certain other conditions of the eyes, or if preferred, prescription eyeglasses should be worn. Correction for astigmatism in eyeglasses can easily be determined by holding the eyeglasses at arm's length and rotating them while looking at an object through one lens at a time. If the length or width of the object changes while doing this with either lens, then there is an astigmatism correction and wearing the glasses during microscope work may be recommended. Special high-point eyepieces are made with a longer exit pupil distance to easily accommodate the extra distance needed when eyeglasses are worn. These eyepieces are identified in some way by the manufacturer.

To take advantage of the optimal resolution and illumination of the microscope, a technique known as Koehlor illumination is used. NOTE: Some settings may have been preset by the manufacturer and will not be adjustable.

With a slide specimen on the stage, use a low power (2-10X) objective, and focus with the coarse and fine adjustments. Low magnification provides a larger field of view for easier searching of the specimen. It also provides a greater working distance than higher power objectives, offering more safety against focusing too low and breaking the slide.

The microscope may have an auxiliary swing-in (or swing-out) lens in the condenser. Follow manufacturer's recommendations for correct use of the swing-in/swing-out lens with various objectives. Use the coarse and fine adjustments to focus the specimen. To obtain Koehlor illumination, close the lamp (field) iris diaphragm, if present, at the base of the microscope, and bring into focus by vertical adjustment of the condenser. Use the centering

screws or knobs on the condenser, if present, to center the focused circle in the field. If not on the condenser, centering screws or knobs for this purpose may be on the base near the lamp diaphragm. Open the lamp diaphragm until it is just past the field of view. This may not be possible with low power objectives of some microscopes until the auxiliary lens in the condenser is correctly adjusted.

If the microscope has no lamp diaphragm, place a piece of paper with a small hole cut in it over the lamp opening and make the same adjustments to focus on its inner edge. Microscopes with mirror and external light source are not discussed here, but the principles are similar. Follow the manufacturer's instructions for adjusting the lamp filament if possible.

Set the substage (aperture) diaphragm next. This adjustment has a crucial effect on the resolution and contrast of the image. The substage diaphragm is opened or closed to 2/3 the size of the field as seen by removing an eyepiece and looking down the tube. To approximate this setting without removing an eyepiece, open the substage diaphragm fully and gradually close it while looking through the microscope until the image gains a sudden increase in sharpness and detail. This should be close to the 2/3 open position; it can be achieved with a little practice and double checking initially by removing the eyepiece and looking down the tube. Replace the eyepiece. If the lighting is too bright, use the rheostat, if provided, to turn it down, or add neutral density or other filters. Do not use the substage diaphragm to control brightness. Resolution will suffer if it is stopped down (closed) too far or opened too much. Although stopping down gives more contrast, it impairs resolution, and spurious details are formed by diffraction lines or fringes.

Repeat the procedures for Koehlor illumination with each objective used.

For phase-contrast microscopy, follow the same basic steps. Do not use the substage diaphragm but make adjustments to bring the phase annulus and annular diaphragm into coincidence. Refer to the manufacturer's instructions. Use of a green filter is recommended.

References

1. American Public Health Association. 1985. Standard Methods for the Examination of Dairy Products, 16th ed. Chapter 10. APHA, Washington, DC.

2. *Official Methods of Analysis of AOAC International* (2000) 17th Ed., AOAC International, Gaithersburg, MD, USA , Official Method **940.37F**

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