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Procedures for the Microbiological Examination of Production Batch Preparations of the Nuclear Polyhedrosis Virus (Baculovirus) of the Gypsy Moth, *Lymantria dispar* L.

FOREST SERVICE GENERAL TECHNICAL REPORT NE-38
1978
FOREST SERVICE, U.S. DEPARTMENT OF AGRICULTURE
NORTHEASTERN FOREST EXPERIMENT STATION
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MANUSCRIPT RECEIVED FOR PUBLICATION 14 DECEMBER 1976

Abstract

Procedures for the microbiological examination of production batch preparations of the nuclear polyhedrosis virus of the gypsy moth are described. They include methods to count total bacteria and to detect fecal coliform and primary pathogenic bacteria in the preparations. These procedures are consistent with safety guidelines established by the U.S. Environmental Protection Agency (EPA) and they are adaptable for use in the examination of other insect virus preparations.

The work herein reported was funded in part by a U.S. Department of Agriculture sponsored program entitled "The Expanded Gypsy Moth Research and Development Program."

INTRODUCTION

SAFETY GUIDELINES for virus preparations used for the control of forest insects established by the U.S. Environmental Protection Agency (EPA) state that "The fungal and bacterial contamination of the technical virus due to production procedures must not exceed the limits which can be maintained under sanitary manufacturing conditions. More importantly, human and mammalian pathogens (e.g., *Salmonella, Shigella,* and *Vibrio*) and microorganisms producing toxins known to effect mammals must be absent from the technical products" (EPA 1975).

The nuclear polyhedrosis virus (NPV) of the gypsy moth (*Lymantria dispar* L.) is produced[1] from laboratory stock larvae that have been hatched from surface treated eggs and reared under sanitary conditions on an artificial diet (ODell and Rollinson 1966). At a susceptible stage, larvae are given NPV in their diet. Polyhedral inclusion bodies are harvested at 4°C from moribund and dead larvae through a series of screens, then washed and centrifuged to yield an aqueous suspension which is dried to yield the technical virus product.

Since primary human pathogens are not represented in the flora of naturally occurring gypsy moth larvae (Podgwaite and Cosenza 1966, Podgwaite and Campbell 1970, 1972), the most likely sources of contamination of a manufactured virus product by these agents are (a) direct contact with human or lower vertebrate carriers or their excrement or (b) airborne contamination. If sanitary techniques are practiced, this contamination is not likely to occur. However, since aseptic techniques cannot be practiced at all stages in the production, it is necessary to examine the product for pathogens or for microorganisms that indicate a source of contamination.

The following procedures are based upon EPA guidelines (EPA 1975), but also provide a method to detect persistent pathogens not specifically mentioned in the guidelines. The procedures are based on standard microbiological methods described in a variety of manuals and texts, and are designed to provide:

1. Total aerobic, anaerobic and bacterial spore count per gram of the technical product.
2. A count of coliform bacteria per gram of the technical product.
3. Detection of fecal coliform bacteria in the technical product.
4. Detection of primary pathogenic bacteria in the technical product, e.g., *Salmonella, Shigella, Vibrio, Streptococcus* (group A), *Staphylococcus* (coagulase positive), *Clostridium.*
5. Detection of toxic substances or pathogenic microorganisms in the technical product by inoculation of a warm-blooded vertebrate.

MATERIALS AND PROCEDURES

A complete list of materials,[2] including laboratory equipment, glassware and supplies, media and chemicals necessary to detect and count microorganisms in technical virus preparations is in the Appendix. A dilution and plating scheme is shown in Figure 1.

I. Stock Virus Preparation[3]

A. Materials

Technical virus powder
Analytical microbalance
125 ml Erlenmeyer flask
Tris buffer
Sodium dioctyl sulfosuccinate
Waring blender
Vortex
Spatula
Weighing paper

[1] Lewis, F.B. 1976. Procedure for the production of the nucleopolyhedrosis virus of the gypsy moth, *Lymantria dispar* L. Unpubl. rept. Forest Insect and Disease Laboratory, U.S. Dep. Agric. For. Serv., Northeast. For. Exp. Stn., Hamden, Conn.

[2] The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture or the Forest Service of any product or service to the exclusion of others that may be suitable.

[3] Modified after Martignoni, M. 1970. A production control procedure for nucleopolyhedrosis virus preparations. Unpubl. rep. For. Sci. Lab., U.S. Dep. Agric. For. Serv., Pac. Northwest For. Exp. Stn., Corvallis, Oreg. 14 p.

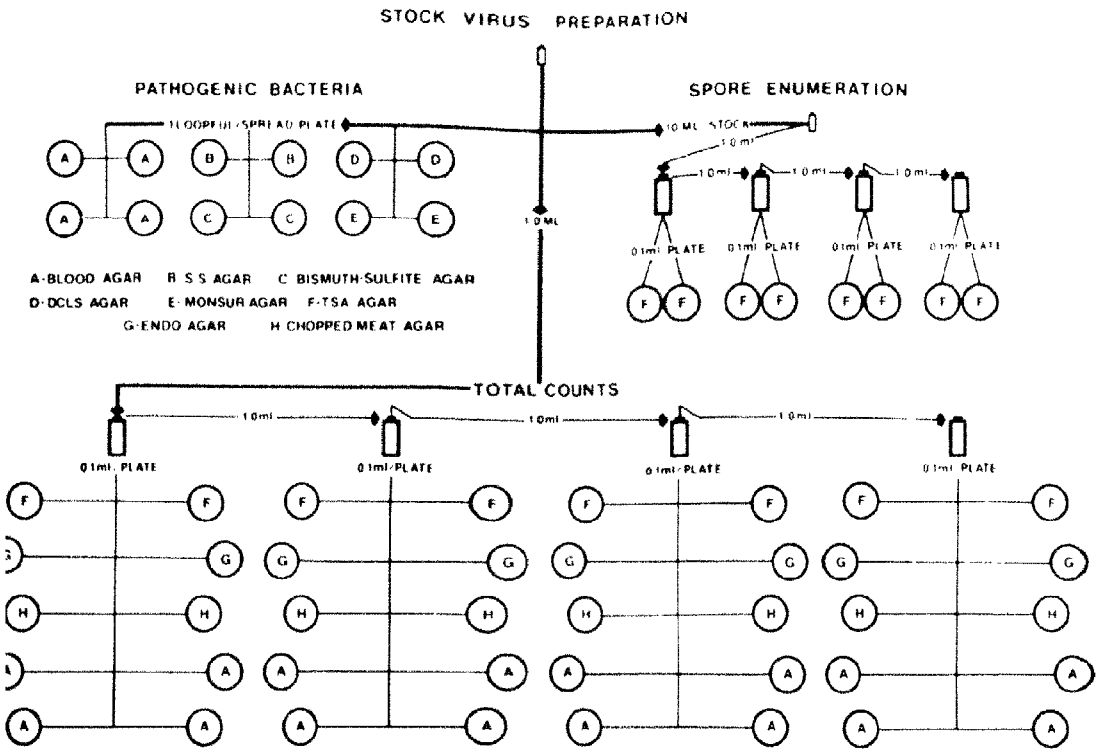

Figure 1.—A dilution and plating scheme for the microbiological examination of batch preparations of the nuclear polyhedrosis virus of the gypsy moth.

B. Method

Prepare the stock virus suspension as follows: Weigh 50 mg of the technical virus powder directly in a sterile 125 ml Erylenmeyer flask. Add 50 ml of sterile 0.01 M tris buffer pH 7.0 with 0.00075 percent sodium dioctyl sulfosuccinate, and mix well. When the material is in suspension, usually within 5 min, pour into a Waring blender and homogenize at low speed for 1 min. Pour the homogenized suspension into the original Erlenmeyer flask and refrigerate immediately. Agitate repeatedly before use (if possible with a mechanical mixer).

II. Buffer Preparation (Am. Pub. Health Assoc. 1967)

A. Materials

Potassium dihydrogen phosphate (KH_2PO_4)
Sodium hydroxide (NaOH)
1 liter Erlenmeyer flasks
1 ml sterile serological pipettes
pH meter
Sterile distilled water

B. Method

Dissolve 34 g of KH_2PO_4 in 500 ml of sterile distilled water, adjust to pH 7.0 with 1 N NaOH solution and dilute to 1 liter with

sterile distilled water. This is a concentrated phosphate buffer solution and it should be stored at 4°C. Add 1.25 ml of this stock solution to sterile distilled water up to 1 liter to yield a standard buffer solution.

III. Standard Plate Count

A. Materials

Stock virus suspension
Trypticase soy agar (BBL)[4]
Standard buffer solution
200 ml milk dilution bottles
1 ml sterile serological pipettes
Sterile petri dishes (15 x 100 mm)
Colony counter
Vortex
Bacteriological incubator
Zephiran chloride disinfectant

B. Dilution method

1. To each of four 200 ml milk dilution bottles with rubber-lined screw caps, add sufficient standard buffer to yield a total volume of 99 ml ± 2% after sterilization.
2. Sterilize the milk dilution bottles at 121°C for 15 min at 15 psi.
3. Label 1 bottle for each of the following:
   10^2-SPC
   10^4-SPC
   10^6-SPC
   10^8-SPC
4. Vigorously shake the stock virus suspension for 1 min (a mechanical vortex or mixer is recommended).
5. Immediately draw off 1 ml of the stock virus suspension with a 1 ml sterile serological pipette, and wipe the exterior of the pipette free of suspension.
6. Immediately transfer the pipette contents to bottle 10^2 and thoroughly flush out the pipette interior by drawing and releasing the diluted suspension.
7. This transfer results in a 1:100 dilution containing 0.01 mg of the technical virus preparation per ml.
8. Cap and shake bottle 10^2 by the standard method: 25 times for 7 sec in an arc of 1 ft.
9. By the same method transfer 1 ml of dilution 10^2 to bottle 10^4. Cap and shake bottle 10^4 as above.
10. Complete this serial transfer with bottle 10^6 and bottle 10^8.

These are suggested plating dilutions. Optimum plating dilutions should be selected as the technical virus preparation dictates.

C. Plating method

1. Disinfect the work area.
2. Set out 8 sterile petri dishes in 2 rows of 4 dishes.
3. Label 1 plate and its replicate for each of the following:
   10^2-SPC
   10^4-SPC
   10^6-SPC
   10^8-SPC
4. Thoroughly shake bottle 10^2, and with a 1 ml sterile serological pipette transfer 0.1 ml of dilution 10^2 to each plate labeled 10^2-SPC. Lift the dish cover only enough to admit the pipette.
5. Shake bottle 10^4, and with a 1 ml sterile serological pipette transfer 0.1 ml of dilution 10^4 to each plate labeled 10^4-SPC.
6. Repeat this technique with dilutions 10^6 and 10^8 for plates labeled 10^6-SPC and 10^8-SPC.
7. Prepare 160 ml of trypticase soy agar according to the manufacturer's specifications and hold at 45° to 50°C.
8. Aseptically pour or pipette 15 to 20 ml of the tempered trypticase soy agar into each petri dish. Gently swirl each dish 10 times in a figure-eight pattern. Make sure that the medium does not spill over the edge of the dish. Keep the covers slightly ajar until the media solidify.
9. Replace the covers. Invert the dishes and place them in an approved bacteriological incubator at 35° ± 1°C for 24 ± 2 h. To provide adequate air circulation, avoid stacking the plates.
10. Return the stock virus suspension and the four milk dilution bottles to the refrigerator. The prepared dilutions will be used for subsequent examinations, and will be referred to as the original milk dilution bottles.
11. Disinfect the work area.

[4] Baltimore Biological Laboratories.

D. Counting method
1. After 24 ± 2 h remove the plates from the incubator.
2. Select plates that show 30 to 300 countable colonies.
3. Place a selected plate on an approved colony counter, and record the plate and the count.
4. If the replicate plates of the same dilution do not meet the established colony limits, the following rules apply:
   a. If the recommended dilutions do not provide countable colonies, select the appropriate dilutions and repeat the SPC procedure.
   b. If only one plate of the same dilution provides the colonies within the established limits while the replicate plate shows less than 30 or more than 300, then the data recorded should be the average of the colonies counted for that dilution.
5. After the initial count has been recorded, incubate the plates an additional 24 h before discarding.
6. Report results with not more than two significant figures as "Standard Plate Count per unit of sample at 35°C;" e.g., a count of 122 is reported as 120, a count of 125 is reported as 130, and a count of 35 is reported as 35.

IV. Sporulating Bacteria Count

A. Materials

Trypticase soy agar (BBL)
Sterile glass test tube with screw cap (16 x 150 mm)
10 ml sterile serological pipette
Constant-temperature water bath
200 ml milk dilution bottles with 99 ml standard buffer
1 ml sterile serological pipettes
Sterile petri dishes (15 x 100 mm)
Zephiran chloride disinfectant

B. Method
1. Disinfect the work area.
2. Remove the stock virus suspension from the refrigerator and shake it by the standard method.
3. With a 10 ml sterile serological pipette, immediately transfer 10 ml of the stock virus suspension to a sterile glass test tube.
4. Incubate the suspension in a constant temperature water bath at 65° ± 1°C for 30 min.
5. Label 4 milk dilution bottles as described in Sec. III, B, but substitute "spore" for "SPC."
6. Remove the test tube from the water bath; with a sterile 1 ml serological pipette, immediately transfer 1 ml of the suspension to bottle 10^2-spore. (Wipe the exterior of the pipette clean before submerging it in the standard buffer and flush out the interior of the pipette before removing it from the bottle.)
7. Complete the dilution procedure (Sec. III, B) and the plating method (Sec. III, C); incubate and record the data according to the Standard Plate Count (Sec. III, D).
8. Report the results as "Total spore count per unit of sample at 35°C," i.e., per gram of technical virus powder.
9. Disinfect the work area.

V. Anaerobic Bacteria Count

A. Materials

5% defibrinated sheep blood agar
Chopped meat glucose agar
4 original milk dilution bottles with prepared dilutions (Sec. III, B)
1 ml sterile serological pipettes
Carbon dioxide (CO_2) "Gas Packs" (BBL)
Hydrogen-carbon dioxide (H–CO_2) "Gas Packs" (BBL)
Anaerobic jars
Zephiran chloride disinfectant

B. Method
1. Disinfect the work area.
2. Prepare 480 ml of 5% difibrinated sheep blood agar according to manufacturer's specifications, and temper to 45° to 50°C.
3. Remove the 4 original milk dilution bottles (Sec. III, B) from the refrigerator and allow them to reach room temperature.
4. Arrange 24 sterile petri dishes in 4 rows of 6 dishes; label 1 row for each of the following:

10^3-ANA
10^5-ANA
10^7-ANA
10^9-ANA

5. Thoroughly shake bottle 10^2; with a 1 ml sterile serological pipette, transfer 0.1 ml of the 10^2 dilution to each 10^3-ANA dish. Cover each dish and set aside.
6. Repeat the above technique with bottle 10^4 and the 10^5-ANA dishes, bottle 10^6 and the 10^7-ANA dishes, and bottle 10^8 and the 10^9-ANA dishes.
7. Pour 15 to 20 ml of blood agar into the first four petri dishes in each row. Gently swirl each plate as directed in Sec. III, C, and allow the media to dry with dish covers slightly ajar.
8. Replace the covers, invert the blood agar plates, and incubate two plates of each dilution at 35°C in a 10% CO_2 atmosphere using an anaerobic jar with a CO_2 "Gas Pack" generator.
9. Incubate the two remaining replicate plates of blood agar of each dilution under strict anaerobic conditions, using an anaerobic jar with an H-CO_2 "Gas Pack."

   Incubate the blood agar plates under both conditions to detect anaerobic organisms by comparing the morphologies of the colonies.
10. Prepare 160 ml of chopped meat glucose agar according to the manufacturer's specifications, and temper to 45° to 50°C.
11. Aseptically pour or pipette 15 to 20 ml of chopped meat glucose agar into the two remaining dishes for each dilution. Allow the medium to dry with covers ajar; replace the covers and invert plates; incubate plates under anaerobic conditions described above.
12. All plates are incubated at 35°C for 48 ± 2 h.
13. Record the plate and the count data, following the SPC procedure (Sec. III, D).
14. Examine plates for pathogenic anaerobic bacteria according to standard anaerobic bacteriology methods (Sutter et al. 1975).
15. Report counts as "Total anaerobic microorganisms per unit of sample."
16. Disinfect the work area.

VI. Coliform Count

A. Materials

Endo I agar (BBL)
4 original milk dilution bottles with prepared dilutions
Lauryl tryptose broth (BBL)
Standard test tubes (16 x 150 mm)
API[5] enteric diagnostic strips
Sterile petri dishes (15 x 100 mm)

B. Method

1. Disinfect the work area.
2. Remove the 4 original milk dilution bottles (Sec. III, B) from the refrigerator and allow them to reach room temperature.
3. Label and prepare plates following SPC directions (Sec. III, C). Substitute endo I agar for trypticase soy agar. Substitute "coli" for "SPC" on labels.
4. Incubate all plates at 35° ± 1°C for 24 ± 2 h.
5. Remove the plates from the incubator after 24 ± 2 h.
6. Place the selected plates (30 to 300 colonies) on an approved colony counter.
7. Count all dark pink to red colonies (lactose fermenters) and all colonies with a metallic sheen.
8. Report counts as "Total coliform colonies per unit of sample."
9. To confirm the presence of coliforms, inoculate lauryl tryptose broth with a loopful of the suspect colony and incubate 48 ± 3 h at 35° ± 0.5°C (Sec. VII, B). If gas is produced, the presence of coliforms is presumed, and should be confirmed by an API enteric diagnostic strip prepared according to the manufacturer's instructions.
10. Disinfect the work area.

VII. Detection and Confirmation of Fecal Coliforms

A. Materials

EC broth (BBL)
Durham fermentation tubes
Standard test tubes (16 x 150 mm)

[5] Analytab Products, Inc.

APPENDIX

Materials Required to Detect and Count Microorganisms in Technical Virus Preparations

Some of the materials listed below are not specifically described in the materials and procedures section, yet are required either for basic taxonomic procedures or for a more extensive examination of the technical virus powder.

Laboratory equipment

1. Constant-temperature water bath with cover
2. Constant-temperature bacteriological incubator
3. Autoclave or suitable sterilization apparatus
4. Refrigerator (4°C)
5. pH meter
6. Analytical microbalance
7. Negative pressure hood
8. Compound light microscope (oil immersion 100X)
9. Waring blender
10. Colony counter
11. Mechanical vortex

Glassware and supplies

1. 200 ml milk dilution bottles with rubber-lined screw caps
2. Sterile petri dishes (15 x 100 mm)
3. 1 ml and 10 ml sterile serological pipettes with 0.1 ml gradations
4. Anaerobic jars
5. Hydrogen-carbon dioxide (H-CO_2) "Gas Packs" (BBL)
6. Carbon dioxide (CO_2) "Gas Packs" (BBL)
7. Durham fermentation tubes
8. Standard test tubes (16 x 150 mm)
9. Mechanical pipette suction apparatus
10. API enteric diagnostic strips
11. Microscope slides
12. 125 ml Erlenmeyer flask
13. 1 liter Erlenmeyer flask
14. Spatula
15. Weighing paper
16. Bacteriological loop (platinum)
17. Test tube rack
18. Record book
19. Marking pencil

Media and chemicals

1. Bismuth-sulfite agar (BBL)
2. Chopped meat glucose agar (BBL)
3. 5% defibrinated sheep blood agar (BBL)
4. Deoxycholate citrate lactose sucrose agar (BBL)
5. EC broth (BBL)
6. Endo I agar (BBL)
7. Enterococcus confirmatory agar (BBL)
8. Eosin methylene blue agar (BBL)
9. Ethyl violet azide broth (BBL)
10. Indole-nitrate medium (BBL)
11. Koser citrate medium (BBL)
12. Lauryl tryptose broth (BBL)
13. MacConkey agar (BBL)
14. M-FC broth (BBL)
15. Milk-protein hydrolysate agar (BBL)
16. Monsur agar
17. M.R.-V.P. medium (BBL)
18. Salmonella-Shigella agar (BBL)
19. Thiosulfate citrate bile salts sucrose agar (BBL)
20. Trypticase soy agar (BBL)
21. Violet red bile agar (BBL)
22. Alpha-napthol
23. Kovac's reagent
24. Methyl red reagent
25. Potassium dihydrogen phosphate (KH_2PO_4)
26. Potassium hydroxide (KOH)
27. Sodium dioctyl sulfosuccinate
28. Sodium hydroxide (NaOH)
29. Sterile distilled water
30. Tris buffer (2-amino-2-hydroxymethyl-1, 3- propanediol)
31. Zephiran chloride disinfectant

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