these plates after 24 and 48 hours of incubation. The contents of the gallbladder can be cultured separately by inoculating 10-ml of VI and TBG broth with cotton swabs and incubating at 37°C for 24 hours. Plate on BG agar and incubate at 37°C. Examine these plates after 24 and 48 hours of incubation. If contamination with pseudomonas or proteus is a problem, make platings on BG sulfapyridine (BGS) agar.

(5) Where field samples are directly inoculated into enrichment broths and a delay of several days occurs before they reach a laboratory, or if recovery of low numbers or organisms is expected from a primary culture, a secondary enrichment culture should be prepared. Subculture a week-old primary culture by transferring 1-ml of inoculum into a fresh tube 10-ml of enrichment broth. This secondary enrichment should be incubated at 37°C for 24 hours before plating. (See paragraph (b)(1) of this section.) TBG broth is recommended for this procedure.

(6) Make a composite sample of the following parts of grossly normal or diseased tissues from the digestive tract: Crop wall, duodenum, jejunum (including remnant of yolk-sac attachment), both ceca, cecal tonsils, and rectum-cloaca. Aseptically collect 10–15 g of each organ or tissue, or whatever lesser amount is available, and grind or blend them completely in 10 times their volume of TBG broth. Transfer 10-ml of a composite sample of a suspension from the digestive tract into 100-ml of TBG broth, and incubate flasks at 42°C for 24 hours. Cultures may be incubated at 37°C if 42°C incubators are not available. The higher incubation temperatures for TBG broth reduce populations of competitive contaminants common in gut tissue. Plate on BG agar and incubate at 37°C. Examine the plates after 24 and 48 hours of incubation. If contamination with pseudomonas or proteus is a problem make platings on BGS agar.

(7) If preferred, individual cotton swab samples may also be taken from the upper, middle, and lower intestinal tract (including both ceca and the rectum-cloaca). Deposit swabs in 10-ml of TBG broth and incubate and plate as described in paragraph (b)(6) of this section.

(8) Transfer suspect colonies to triple-sugar-iron (TSI) agar and lysine-iron (LI) agar and incubate at 37°C for 24 hours.

(9) Cultures revealing typical reactions of salmonellae on TSI and LI agar slants should be transferred to any of the following appropriate biochemical tests for final identification: Dextrose, lactose, sucrose, mannitol maltose, dulcitol, malonate, gelatin, urea broth, citrate, lysine decarboxylase, ornithine decarboxylase, methyl red and Voges-Proskauer, KCN, salicin broths, indole, and hydrogen sulfide. Motility or nonmotility is demonstrated by inoculating a suitable semisolid medium.[10] The Analytical Profile Index for Enterobacteriaceae (API) system may be utilized for identification if feasible. For arizonae identification, make readings daily up to 10 days. An O-nitrophenyl-beta-D-galactopyranside (ONPG) disc may be used to identify slow lactose fermenters.[11]

(10) All salmonella cultures should be serologically typed.

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§ 147.12 Procedures for collecting environmental samples and cloacal swabs for bacteriological examination.

Information concerning the pen arrangement and number of birds per pen should be obtained from the owner so that the required number of samples per pen and per flock can be determined. A means of identifying each sample by pen of origin should be provided. The vehicle transporting the

[10] Formulation for the semisolid motility medium can be obtained from: *Isolation and Identification of Avian Pathogens*, American Association of Avian Pathologists, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348—1692, 1980.

[11] ONPG discs are available from: Baltimore Biological Laboratories, Cockeysville, MD 21030.

personnel taking the samples should be left as far as practical from the poultry pens. Sanitary precautions, including personal cleanliness, should be observed during the sampling procedure. The hands should be carefully washed with a sanitizing soap prior to the sampling. Outer clothing, including gloves, should be changed between visits to different premises so that clean clothing is worn upon entering each premises.

The used and clean apparel should be kept separate. Boots or footwear should be cleaned and disinfected between visits to different premises. Disposable caps should be provided and discarded after use on each premises. After collection, the samples should be protected from drying, light, and excessive temperatures and delivered to the laboratory within one day. If delivery is delayed, samples should be refrigerated.

(a) *For egg- and meat-type chickens, waterfowl, exhibition poultry, and game birds.* All samples and swabs described in this paragraph shall be cultured in accordance with illustration 2 of § 147.11, including delayed secondary enrichment. All salmonellae recovered shall be serogrouped or serotyped.

(1) *Environmental samples.* Fecal material, litter, dust, or floor litter surface or nest box drag swab samples to be submitted for bacteriological examination shall be collected in accordance with the procedures described in paragraphs (a)(1), (a)(2), or (a)(3) of this section:

(i) *Procedure for sampling in broth.* Authorized laboratories will provide capped tubes 1 to 2 cm in diameter and 15 to 20 cm in length that are two-thirds full of a recently made, refrigerated, sterile enrichment broth (Hajna or Mueller-Kauffmann Tetrathionate Brilliant Green) for each sample. Sufficient tubes shall be taken to the premises to provide at least one tube per pen or one tube per 500 birds, whichever is greater. At least one sterile, cotton-tipped applicator will be needed for each tube. The dry applicator is first placed in or drawn through fresh manure (under roost, near water troughs, fecal droppings, or diarrhetic droppings). After each streaking, place the cotton-tipped applicator in the tube of broth and swirl the applicator to remove the collected material. Withdraw the applicator from the tube and use it to take additional specimens by streaking on or through areas where defecation, trampling of feces, or settling of dust is common; e.g., on or near waterers, feeders, nests, or rafters, etc. When the volume of material collected equals approximately 10 percent of the volume of the broth (usually 10–12 streakings), place the applicator in the tube and break the stick in half, leaving the lower or cotton-tipped half in the broth and retaining the upper half for future disposal. Replace the cap on the inoculated tube and continue the sampling procedure in other areas of the pen.

(ii) *Procedure for sampling in dry containers.* Place a sample of fecal material, litter, or dust in a sterile, sealable container. The sample shall consist of several specimens of material taken from a representative location in the pen or house. Collect at least 10 g (approximately a heaping tablespoonful) of material for each sample. Collect the specimens in each sample with a sterile tongue depressor or similar uncontaminated instrument. The samples shall vary in type and consistency. Half of the samples shall be comprised of material representing defecated matter from a large portion of the flock; i.e., trampled, caked material near waterers and feeders. The minimum number of samples to be taken shall be determined by the following: Five samples from pens or houses of up to 500 birds; Ten samples from pens or houses of 500 to 2,500 birds; Fifteen samples from pens or houses with more than 2,500 birds. The samples may be pooled to not fewer than five samples at the laboratory as long as the volume of material collected equals approximately 10 percent of the volume of the broth.

(2) *Cloacal swabs.* Cloacal swabs for bacteriological examination shall be taken from each bird in the flock or from a minimum of 500 birds in accordance with the procedure described in paragraph (a)(2)(i) of this section.

(i) *Procedure for taking cloacal swabs.* The authorized laboratory will provide sterile capped tubes or other suitable

containers and cotton-tipped applicators for use in taking the cloacal swabs. Insert the cotton-tipped applicator into the cloaca and rectum in such a manner as to ensure the collection of fecal material. Place the swab and adhering fecal material in the tube and break the stick in half, keeping the upper half of the stick for future disposal. The cloacal swabs may be combined in the sterile tubes in multiples of five or in combinations specified by the authorized laboratory.

(ii) [Reserved]

(3) *Drag-swabs*. Utilization of drag swabs (DS) involves the exposure of gauze pads, a key component of a DS sampler, to the surface of random, flock-representative floor litter and nest box areas. The sampler pads shall be sterile and slightly moist to promote adherence of particulate material, and impregnated with double-strength skim milk[12] to protect salmonella viability during sample collection, batching, storage, and shipment. Floor litter surface DS sample results tend to reflect the salmonella carrier/shedder status of a flock. Nonetheless, other environmental samples as described in paragraphs (a)(1)(i), (a)(1)(ii), or (a)(3)(iv) of this section shall also be periodically collected.

(i) *Drag-swab sampler assembly*. Drag-swab (DS) samplers may be assembled using two 3- by 3-inch sterile gauze pads; size 20 wrapping twine; and paper clips, staples, or similar fasteners. Fold each gauze pad in half and attach one pad to a 2-foot-long (60 cm) piece of twine and the other to a 1-foot-long (30 cm) piece of twine. To attach a pad to the twine with a paper clip, bend the end wires of the paper clip slightly and push them through the fabric of the folded pad, thus securing the clips to the folded pads; then securely tie the twine to the free rounded end of the paper clip. To attach a pad to the twine with a staple, staple the twine to the pad near the center of the fold, applying the staple at a right angle to the twine and parallel to the fold. (A pre-tied knot in the free end of the twine will prevent the twine from slipping under the staple during use.) Once the pads and the twine have been attached, securely connect the free ends of both lengths of twine to a small loop tied at the end of a 5-foot-long piece of twine. The resulting assembly resembles the letter Y, with a long vertical stem and two diagonal branches of different lengths with a gauze pad securely attached to the end of each branch. Wrap the twine around each two-pad DS sampler to produce a small bundle. Autoclave the assembled DS sampler bundle and transfer it with sterile forceps or other aseptic method to a resealable sterile bag. Aseptically add 15 mL of double-strength skim milk to the bag and massage the milk into the gauze pads. Seal the bags and store at -20 °C.

(ii) *Procedures and applications for DS samplers*. DS samplers shall be completely thawed prior to use. Complete pad/twine/fastener assemblies shall be used to sample floor litter surfaces; nest box surfaces may be sampled using 3- by 3-inch sterile gauze pads impregnated with double-strength skim milk in the manner described in paragraph (a)(1) of this section. In either instance, the Plan participant collecting the samples shall wear a fresh pair of disposable sterile gloves for each flock or house sampled. Each sampler bag shall be marked with the type of sample (floor litter or nest box surface) and the identity of the house or flock from which the sample was taken.

(iii) *Floor litter sampling technique*. For flocks with fewer than 500 breeders, at least one DS set (two DS pads) shall be dragged across the floor litter surface for a minimum of 15 minutes. For flocks with 500 or more breeders, a minimum of two DS sets (four DS pads) shall be dragged across the floor litter surface for a minimum of 15 minutes per DS set. Upon completion of dragging, lower each DS pad by its attached twine into a separate, resealable sterile bag. Alternatively, each DS set of two pads may be lowered by its attached twine into the storage/transport bag from which the DS set was originally taken. Remove the twine from the pad or DS set by grasping the pad or DS set

[12] Obtain procedure for preparing double strength skim milk from USDA-APHIS ''Recommended Sample Collection Methods for Environmental Samples,'' available from the National Poultry Improvement Plan, Veterinary Services, APHIS, USDA, 1498 Klondike Road, Suite 200, Conyers, GA 30094.

through the sides of the bag with one hand while pulling on the twine with the other hand until the connection is broken. Seal the bags and promptly refrigerate them to between 2 and 4 °C. Do not freeze. Discard the twine in an appropriate disposal bag.

(iv) *Nest box sampling technique.* The Plan participant shall collect nest-box samples by using two 3- by 3-inch sterile gauze pads premoistened with double-strength skim milk and wiping the pads over assorted locations in about 10 percent of the total nesting area. Upon completion, place each pad in a separate, resealable sterile bag. Seal the bags and promptly refrigerate them to between 2 and 4 °C. Do not freeze.

(v) *Culturing of litter surface and nest box samples.* When refrigerated to between 2 and 4 °C, pads impregnated with double-strength skim milk may be stored or batched for 5 to 7 days prior to culturing. Pads shipped singly or paired in a single bag shall not be pooled for culturing but shall be separately inoculated into 60 mL of selective enrichment broth.

(b) *For turkeys.* (1) *Environmental samples.* Fecal material, litter, or dust to be submitted for bacteriological examination should be collected in accordance with the procedures described in paragraphs (b)(1)(i) or (b)(1)(ii) of this section:

(i) *Procedure for sampling in broth.* Authorized laboratories will provide capped tubes 1–2 cm in diameter and 15–20 cm in length which are two-thirds full of a recently made, refrigerated, sterile enrichment broth (Selenite Brilliant Green Sulfapyridine or Tetrathionate Brilliant Green) for each sample. Sufficient tubes should be taken to the premises to provide at least one tube per pen or one tube per 500 birds, whichever is greater. At least one sterile, cotton-tipped applicator will be needed for each tube. The dry applicator is first placed or drawn through fresh manure (under roost, near water troughs, cecal droppings, or diarrhetic droppings). After this and each subsequent streaking, the cotton-tipped applicator is placed in the tube of broth and swirled to remove the collected material. The applicator is then withdrawn and is used for taking additional specimens by streaking on or through areas where defecation, trampling of feces, or settling of dust are common; i.e., on or near waterers, feeders, nests, or rafters, etc. When the volume of material collected equals approximately 10 percent of the volume of the broth (usually 10–12 streakings), the applicator is placed in the tube and the stick is broken in half. The lower or cotton-tipped half is left in the broth, and the upper half is retained for future disposal. The cap is then replaced on the inoculated tube, and the sampling procedure is continued in other areas of the pen.

(ii) *Procedure for sampling in dry containers.* A sample of fecal material, litter, or dust is placed in a sterile, sealable container. The sample shall consist of several specimens of material taken from a representative location in the pen or house. At least 10 g (approximately a heaping tablespoonful) of material shall be collected for each sample. The specimens in each sample shall be collected with a sterile tongue depressor or similar uncontaminated instrument. The samples should vary in type and consistency. Half of the samples should be comprised of material representing defecated matter from a large portion of the flock; i.e., trampled, caked material near waterers and feeders. The minimum number of samples to be taken shall be determined by the following:

Five samples from pens or houses of up to 500 birds;
Ten samples from pens or houses of 500 to 2,500 birds;
Fifteen samples from pens or houses with more than 2,500 birds.

The composite samples above may be pooled to not less than five samples at the laboratory as long as the volume of material collected equals approximately 10 percent of the volume of the broth.

(2) *Cloacal swabs.* Cloacal swabs for bacteriological examination are taken from each bird in the flock or from a minimum of 500 birds in accordance with the procedure described in paragraph (b)(2)(i) of this section.

(i) *Procedure for taking cloacal swabs.* The authorized laboratory will provide sterile capped tubes or other suitable containers and cotton-tipped applicators for use in taking the cloacal

swabs. The cotton-tipped applicator is inserted into the cloaca and rectum in such a manner as to insure the collection of fecal material. The swab and adhering fecal material is then placed in the tube and the stick is broken in half, with the upper half retained for future disposal. The cloacal swabs may be combined in the sterile tubes in multiples of five or in combinations specified by the authorized laboratory.

(ii) [Reserved]

(3) *Drag-swabs.* Drag-swabs for bacteriological examination should involve the exposure of at least six unpooled pads per house to promote representative sampling and some element of quantification.

(i) *Drag-swab assembly.* Assemble drag-swab sampling sets from folded-once 3-by-3-inch sterile gauze pads secured with paper clips. Bend end wires of each paper clip slightly to catch into the swab fabric, thus securing the clips to the folded pads. Use two pads, assembled as described to make each drag-swab sampling set. Securely connect one pad through the free rounded end of the paper clip to a 2-ft (0.6 m) length of size 20 fibrous wrapping twine. Similarly connect the other pad to a 1-ft (0.3 m) length of twine. Then securely connect the free ends of both lengths of twine to a small loop tied at the end of a similar 5-ft length of twine. The resulting assembly resembles the letter Y with a 5-ft long vertical stem and two diagonal branches (one 1 ft long and the other 2 ft long), with a folded swab securely attached at the end of each branch. After assembly, place each two-pad drag-swab sampling set into a sterile bag.

(ii) *Procedure for taking drag-swab.* (A) *Floor litter:* The Plan participants should collect two samples as follows: Drag four 3-by-3-inch sterile gauze pads premoistened with double strength skim milk[13] over the floor litter surface for 15 min minimally. Place the gauze pads used to collect the samples in 18-oz whirl-pack bags, two pads per bag with each bag containing 5 ml of double strength skim milk. This will maintain the moistness of the sample during transport. Mark the bags with the type of sample and the house identification.

(B) *Nest-boxes.* The Plan participant should collect one nest-box sample by using two 3-by-3-inch sterile gauze pads premoistened with double strength skim milk. Wipe the two gauze pads used to collect the sample over assorted locations of about 10 percent of the total nesting area. Place the gauze pads used to collect the sample in an 18-oz whirl-pack bag containing 5 ml of double strength skim milk. Mark the bag with the type of sample and the house identification.

(c) *Chick box papers.* Samples from chick box papers may be bacteriologically examined for the presence of salmonella. The Plan participant may collect the samples in accordance with paragraph (c)(1) of this section or submit chick box papers directly to a laboratory in accordance with paragraph (c)(2) of this section.

(1) Instructions for collecting samples from chick box papers:

(i) Collect 1 chick box paper for each 10 boxes of chicks placed in a house and lay the papers on a clean surface.

(ii) Clean your hands and put on latex gloves. Do not apply disinfectant to the gloves. Change gloves after collecting samples from 10 chick box papers or any time a glove is torn.

(iii) Saturate a sterile 3-by-3 inch gauze pad with double-strength skim milk (see footnote 11 to this section) and rub the pad across the surface of five chick box papers. Rub the pad over at least 75 percent of each paper and use sufficient pressure to rub any dry meconium off the paper. Pouring a small amount of double-strength skim milk (1 to 2 tablespoons) on each paper will make it easier to collect samples.

(iv) After collecting samples from 10 chick box papers, place the two gauze pads used to collect the samples (i.e., one pad per 5 chick box papers) into an 18 oz. Whirl-Pak bag and add 1 to 2 tablespoons of double-strength skim milk.

(v) Promptly refrigerate the Whirl-Pak bags containing the samples and

[13] Obtain procedure for preparing double strength skim milk from USDA–APHIS ''Recommended Sample Collection Methods for Environmental Samples'' available from the National Poultry Improvement Plan, Veterinary Services, APHIS, USDA, 1498 Klondike Road, Suite 200, Conyers, GA 30094.

transport them, on ice or otherwise refrigerated, to a laboratory within 48 hours of collection. The samples may be frozen for longer storage if the Plan participant is unable to transport them to a laboratory within 48 hours.

(2) The Plan participant may send chick box papers directly to a laboratory, where samples may be collected as described in paragraph (c)(1) of this section. To send chick box papers directly to a laboratory, the Plan participant shall:

(i) Collect 1 chick box paper for each 10 boxes of chicks placed in a house and place the chick papers immediately into large plastic bags and seal the bags.

(ii) Place the plastic bags containing the chick box papers in a clean box and transport them within 48 hours to a laboratory. The plastic bags do not require refrigeration.

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[38 FR 13709, May 24, 1973. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 57 FR 57342, Dec. 4, 1992; 59 FR 12805, Mar. 18, 1994; 59 FR 67617, Dec. 30, 1994; 61 FR 11524, Mar. 21, 1996; 62 FR 44070, Aug. 19, 1997; 63 FR 3, Jan. 2, 1998; 65 FR 8019, Feb. 17, 2000]

§ 147.13 Procedure for bacteriological culturing of eggshells for colon bacilli organisms.

Proper precautions to avoid environmental contamination of the samples during the collection and laboratory process, and proper handling of the samples following collection are essential. Each State Inspector involved in eggshell culture activities must receive instruction in the necessary sanitation procedures, sampling procedures, and sample handling by the authorized laboratory involved. The Official State Agency will maintain a record showing that the required instruction was given to each State Inspector.

(a) *Sample selection*. Forty (40) eggs in the top flats of each of three randomly selected cases of sanitized eggs from each flock will be utilized for each sampling.

(b) *Swab procedure*. A 2.5 centimeter diameter circular area of the large end of each of the eggs will be rubbed with a sterile swab previously moistened with sterile lactose broth, or other suitable liquid media provided by the authorized laboratory. One swab will be used for five eggs, and four swabs will be pooled to each sterile, capped tube provided by the authorized laboratory.

(1) From the tube containing four swabs and lactose broth or other suitable media, 1 ml. will be transferred to 10 ml. lactose in a fermentation tube.

(2) Incubate at 37 °C for 48 hours. The presence of acid, and gas in the amount of 10 percent or more after 24 and 48 hours of incubation, provides a presumptive conclusion of the presence of colon bacilli organisms.

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[41 FR 14256, Apr. 2, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 59 FR 12805, Mar. 18, 1994]

§ 147.14 Procedures to determine status and effectiveness of sanitation monitored program.

The following monitoring procedures[14] may be applied at the discretion of the Official State Agency:

(a) Monitor effectiveness of sanitation program.

(1) Culture the surface of cased eggs periodically for fecal contaminating organisms as described in § 147.13.

(2) Culture a sample of dead-in-shell eggs periodically from each breeding flock for coliforms. Such eggs should also be cultured for the dependable recovery of *salmonellae*. Culturing for the dependable recovery of *salmonellae* should include the use of:

(i) Preenrichment broths supplemented with 35 mg ferrous sulfate per 1,000 ml preenrichment to block iron-binding, *Salmonella*-inhibiting effects of egg conalbumin; and

(ii) Tetrathionate selective enrichment broths, competitor-controlling

[14] Laboratory procedures for monitoring operations proposed here are described in the following two publications: Isolation and Identification of Avian Pathogens, American Association of Avian Pathologists, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348—1692, 1980, and Culture Methods for the Detection of Animal Salmonellosis and Arizonosis, Iowa State University Press, Ames, Iowa 50010, 1976.