

(2) Discontinuation of any of the conditions described in paragraph (a)(1)(i) of this section, or repeated outbreaks of pullorum or typhoid occur in hatchery supply flocks described in paragraph(a)(1)(ii) of this section, or if an infection spreads from the originating premises, the Service shall have grounds to revoke its determination that the State is entitled to this classification. Such action shall not be taken until a thorough investigation has been made by the Service and the Official State Agency has been given an opportunity for a hearing.

[40 FR 1504, Jan. 8, 1975. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 54 FR 23957, June 5, 1989]

Subpart F—Special Provisions for Ostrich Breeding Flocks and Products

SOURCE: 63 FR 40010, July 27, 1998, unless otherwise noted.

§ 145.61 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Ostrich. Birds of the species *Struthio camelus*, including all subspecies and subspecies hybrids.

§ 145.62 Participation.

Participating flocks of ostriches, and the eggs and chicks produced from them, shall comply with the applicable general provisions of subpart A of this part and the special provisions of this subpart.

(a) Started poultry shall lose their identity under Plan terminology when not maintained by Plan participants under the conditions prescribed in § 145.5(a).

(b) Hatching eggs produced by primary breeding flocks shall be fumigated or otherwise sanitized (see § 147.22 of this chapter).

§ 145.63 Terminology and classification; flocks and products.

Participating flocks, and the eggs and baby poultry produced from them, that have met the respective requirements specified in this section may be

designated by the following terms and their corresponding designs illustrated in § 145.10.

(a) *U.S. Pullorum-Typhoid Clean.* A flock in which freedom from pullorum and typhoid has been demonstrated to the Official State Agency under the criteria in paragraph (a)(1) or (a)(2) of this section. (See § 145.14(a) relating to the official blood test for pullorum-typhoid where applicable.)

(1) It has been officially blood tested within the past 12 months with no reactors.

(2) It is a multiplier or primary breeding flock in which a sample of each bird in flocks of 30 or fewer birds, a minimum of 30 birds from flocks up to 300 birds, or 10 percent of all birds from flocks exceeding 300 birds has been officially tested for pullorum-typhoid within the past 12 months with no reactors: *Provided*, That a bacteriological examination monitoring program for ostriches acceptable to the Official State Agency and approved by the Service may be used in lieu of annual blood testing: *And provided further*, That when a flock is a multiplier breeding flock located in a State which has been deemed to be a U.S. Pullorum-Typhoid Clean State for the past 3 years, and during which time no isolation of pullorum or typhoid has been made that can be traced to a source in that State, a bacteriological examination monitoring program or a serological examination monitoring program acceptable to the Official State Agency and approved by the Service may be used in lieu of annual blood testing.

(b) [Reserved]

PART 146 [RESERVED]

PART 147—AUXILIARY PROVISIONS ON NATIONAL POULTRY IMPROVEMENT PLAN

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AUTHORITY: 7 U.S.C. 429; 7 CFR 2.22, 2.80,
and 371.2(d).

SOURCE: 36 FR 23121, Dec. 3, 1971, unless
otherwise noted. Redesignated at 44 FR 61586,
Oct. 26, 1979.

Subpart A—Blood Testing Procedures

§ 147.1 The standard tube agglutina- tion test.¹

(a) The blood samples should be col-
lected and delivered as follows:

(1) The blood samples should be
taken by properly qualified and author-
ized persons only, and in containers
provided by the laboratory. The con-
tainers should be stout-walled test
tubes, preferably $\frac{3}{8}$ by 3 inches, with-
out lip, or small well-selected medicine
vials, which have been thoroughly
cleaned and dried in a hot-air drying
oven. If stoppers are used, they should
be thoroughly cleaned and dried.

(2) Sufficient blood should be pro-
cured by making a small incision in
the large median wing vein with a
small sharp lancet and allowing the
blood to run into the tube, or by the
use of a small syringe (with 20 or 21
gauge needle) which is properly cleansed
between bleedings with physiological
saline solution. To facilitate the sepa-
ration of the serum, the tubes should
be placed in a slanted position until
the blood has solidified. After the blood
has completely clotted, they should be
packed and shipped by mail (special deli-
very), rapid express, or by messenger,
to the laboratory. All labeling must be
clear and permanent, and may be done
with a suitable pencil on etched por-
tions of the tube, or by means of fast-
gum labels.

(3) The blood samples must reach the
laboratory in a fresh and unhemolyzed
condition. Hemolyzed samples should
be rejected. It is imperative, therefore,

¹The procedure described is a modification
of the method reported in the Proceedings of
the U.S. Live Stock Sanitary Association,
November 30 to December 2, 1932, pp. 487 to
491.

to cool the tubes immediately after slanting and clotting, and unless they reach the laboratory within a few hours, to pack them with ice in special containers, or use some other cooling system which will insure their preservation during transportation. In severe cold seasons, extreme precautions must be exercised to prevent freezing and consequent laking. The samples must be placed in cold (5° to 10° C.) storage, immediately upon arrival at the laboratory.

(b) The antigen shall consist of representative strains of *S. pullorum* which are of known antigenic composition, high agglutinability, but are not sensitive to negative and nonspecific sera. The stock cultures may be maintained satisfactorily by transferring to new sloped agar at least once a month and keeping at 18° to 25° C. (average room temperature) in a dark closet or chest, following incubation for from 24 to 36 hours at 37° C. The antigenic composition and purity of the stock cultures should be checked consistently.

(c) A medium which has been used satisfactorily has the following composition:

Water	1,000 cc.
Difco beef extract	4 gm. (0.4 percent).
Difco Bacto-peptone	10 gm. (1.0 percent).
Difco dry-granular agar.	20 gm. (2.0 percent).
Reaction—pH 6.8 to 7.2.	

(d) Large 1-inch test tubes, Kolle flasks, or Blake bottles should be streaked liberally over the entire agar surface with inoculum from 48-hour slant agar cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated 48 hours at 37° C., and the surface growth washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5° to 10° C.) in tightly stoppered bottles.

(e) Thiosulfate-Glycerin (TG) medium may be used as an alternate medium for the preparation of tube agglutination antigen. The TG medium, formerly used for the preparation of

stained, whole-blood antigen, is described in more detail in the article by A. D. MacDonald, Recent Developments in Pullorum Antigen for the Rapid, Whole-Blood Test, Report of the Conference of the National Poultry Improvement Plan, pages 122-127, 1941. This medium provides a tube antigen of excellent specificity and greatly increases the yield of antigen from a given amount of medium. The TG medium has the following composition:

Beef infusion	1,000 cc.
Difco Bacto-peptone	20 gm. (2.0 percent).
Sodium thiosulfate	5 gm. (0.5 percent).
Ammonium chloride	5 gm. (0.5 percent).
Glycerin, U.S.P. (95 percent) ..	20 cc. (2.0 percent).
Difco dry-granular agar.	30 gm. (3.0 percent).
Reaction—pH 6.8 to 7.2.	

Large 1-inch test tubes, Kolle flasks, Blake bottles, or Erlenmeyer flasks should be seeded over the entire agar surface with inoculum from 24-hour beef infusion broth cultures prepared from the stock cultures of the selected strains. The antigen-growing tubes or bottles should be incubated 96 hours at 37° C., and the surface growth washed off with sufficient phenolized (0.5 percent) saline (0.85 percent) solution to make a heavy suspension. The suspension should be filtered free of clumps through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. The antigen then should be centrifuged. The mass of bacteria should be removed from the centrifuge tubes or bowl and resuspended in saline (0.85 percent) solution containing 0.5 percent phenol. After the bacterial mass has been uniformly suspended in the diluent, it should be again passed through a cotton pad in a Buchner funnel without the aid of suction. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5° to 10° C.) in tightly stoppered bottles.

(f) The diluted antigen to be used in the routine testing should be prepared from the stock antigen by dilution of the latter with physiological (0.85 percent) saline solution containing 0.25 percent of phenol to a turbidity corresponding to 0.75-1.00 on the McFarland nephelometer scale. The hydrogen-ion concentration of the diluted antigen should be corrected to pH 8.2 to 8.5 by the addition of dilute sodium hydroxide. New diluted antigen should be

prepared each day and kept cold. The diluted antigen may be employed in 2 cc. quantities in 4 by ½-inch test tubes, or 1 cc. quantities in smaller tubes, in which the final serum-antigen mixtures are made and incubated. The distribution of the antigen in the tubes may be accomplished by the use of long burettes, or special filling devices made for the purpose.

(g) The maximum serum dilution employed must not exceed 1:50 for chickens, nor 1:25 for turkeys. The available data indicate that 1:25 dilution is the most efficient. In all official reports on the blood test, the serum dilutions shall be indicated. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum-delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37° C.

(h) The results shall be recorded as:

- N, or - (negative) when the serum-antigen mixture remains uniformly turbid.
- P, or + (positive) when there is a distinct clumping of the antigen, and the liquid between the agglutinated particles is clear.
- S, or ? (suspicious) when the agglutination is only partial or incomplete.
- M, or missing, when samples listed on the original record sheet are missing.
- H, or hemolyzed, when blood samples are hemolyzed and cannot be tested.
- B, or broken, when sample tubes are broken and no serum can be obtained.

(Some allowance must always be made for the difference in sensitiveness of different antigens and different set-ups, and therefore, a certain amount of independent, intelligent judgment must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of *S. pullorum*.)

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[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, as amended at 59 FR 12799, Mar. 18, 1994]

§ 147.2 The rapid serum test.²

(a) The procedure for the collection and delivery of blood samples in the rapid serum test is the same as that described in § 147.1(a).

(b) The selection and maintenance of suitable strains of *S. pullorum* and the composition of a satisfactory medium are described in § 147.1 (b) and (c).

(c) Large 1-inch test tubes, Kolle flasks, or Blake bottles are streaked liberally from 48-hour slant-agar cultures prepared from stock cultures of the selected strains.

(d) The antigen-growing tubes or bottles should be incubated 48 hours at 37° C., and the surface growth washed off with a very slight amount of 12 percent solution of sodium chloride containing 0.25 to 0.5 percent phenol, filtered through lightly packed sterile absorbent cotton placed in the apex of a sterile funnel.

(e) The washings should be adjusted (using 12 percent sodium chloride containing 0.25 to 0.5 percent phenol) so that the turbidity is 50 times greater than tube 0.75 of McFarland's nephelometer, or to a reading of 7 mm. by the Gates nephelometer.

(f) The individual strain antigens should be tested with negative sera for their insensitivity and with positive sera for high agglutinability in comparison with known satisfactory antigen. The antigens of the separate strains should be combined in equal volume-density and stored in the refrigerator (5° to 10° C.) in tightly stoppered bottles.

(g) The tests should be conducted on a suitable, smooth plate. The serum-antigen dilution should be made so that the dilution will not exceed 1:50 when compared to the standard tube agglutination test. When testing turkey blood samples, it is desirable to use a serum-antigen dilution equivalent to the 1:25 in the tube method. The serum should be added to the antigen and mixed thoroughly by use of the tip of the serum pipette. Most strong positive reactions will be plainly evident

²The procedure described is a modification of the method reported by Runnels, Coon, Farley, and Thorpe, *Amer. Vet. Med. Assoc. Jour.* 70 (N.S. 23): 660-662 (1927).

within 15 to 20 seconds. The final reading should be made at the end of 2 or 3 minutes. Heating the plate at approximately 37° C. will hasten agglutination. Before reading, the plate should be rotated several times.

(h) The results shall be recorded as described in § 147.1(h).

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[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, as amended at 59 FR 12799, Mar. 18, 1994]

§ 147.3 The stained-antigen, rapid, whole-blood test.³

(a) The description of the preparation of antigen is not herein included because the antigen is a proprietary product produced only under license from the Secretary of Agriculture.

(b) A loop for measuring the correct quantity of blood can usually be obtained from the manufacturer of the antigen. A satisfactory loop may be made from a piece of No. 20 gage nichrome wire, 2½ inches long, at the end of which is fashioned a loop three-sixteenths of an inch in diameter. Such a loop, when filled with blood so that the blood appears to bulge, delivers 0.02 cc. A medicine dropper whose tip is adjusted to deliver 0.05 cc. is used to measure the antigen. A glass plate about 15 inches square, providing space for 48 tests, has proved satisfactory for this work. The use of such a plate enables the tester to have a number of successive test mixtures under observation without holding up the work to wait for results before proceeding to the next bird.

(c) A drop of antigen should be placed on the testing plate. A loopful of blood should be taken up from the wing vein. When submerged in the blood and then carefully withdrawn, the loop becomes properly filled. On looking down edge-wise at the filled loop, one observes that the blood appears to bulge. The loopful of blood then should be stirred into the drop of antigen, and the mixture spread to a diameter of about 1 inch. The loop then should be rinsed in

³The procedure described is a modification of the method reported by Schaffer, MacDonald, Hall, and Bunyey, Jour. Amer. Vet. Med. Assoc. 79 (N. S. 32): 236-240 (1931).

clean water and dried by touching it to a piece of clean blotting paper, if necessary. The test plate should be rocked from side to side a few times to mix the antigen and blood thoroughly, and to facilitate agglutination. The antigen should be used according to the directions of the producer.

(d) Various degrees of reaction are observed in this as in other agglutination tests. The greater the agglutinating ability of the blood, the more rapid the clumping and the larger the clumps. A positive reaction consists of a definite clumping of the antigen surrounded by clear spaces. Such reaction is easily distinguished against a white background. A somewhat weaker reaction consists of small but still clearly visible clumps of antigen surrounded by spaces only partially clear. Between this point and a negative or homogeneous smear, there sometimes occurs a very fine granulation barely visible to the naked eye; this should be disregarded in making a diagnosis. The very fine marginal clumping which may occur just before drying up is also regarded as negative. In a nonreactor, the smear remains homogeneous. (Allowance should be made for differences in the sensitiveness of different antigens and different set-ups, and therefore, a certain amount of independent, intelligent judgment must be exercised at all times. Also, the histories of the flocks require consideration. In flocks where individuals show a suspicious agglutination, it is desirable to examine representative birds bacteriologically to determine the presence or absence of *S. pullorum*.)

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[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, as amended at 59 FR 12799, Mar. 18, 1994]

§ 147.4 The tube agglutination test for *S. typhimurium*.

(a) The procedure for the collection and delivery of blood samples in the tube agglutination test for *S. typhimurium* is the same as that described in § 147.1(a).

(b) The "O" antigen should be prepared as follows:

(1) The antigen shall consist of a representative nonmotile strain of *S.*

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typhimurium which is of known antigenic composition and high agglutinability but is not sensitive to negative and nonspecific sera. Strain P 10 meets these requirements.

(2) The stock culture is maintained on 1 percent nutrient agar deeps, which have been incubated for 18-24 hours at 37° C. They are stored at room temperature.

(3) A satisfactory medium used for growing the organism is veal infusion agar (Difco). It is dispensed in 50 ml. amounts into 500 ml. medicine bottles, with screw caps, and sterilized at 15 pounds pressure for 20 minutes. The bottles are then laid flat upon an even surface until the medium has solidified.

(4) The inoculum used for preparation of "O" antigen is a nonmotile strain of *S. typhimurium*. The organism is grown in veal infusion broth (Difco) for 18-24 hours at 37° C.; then plated, for single colony isolation, on veal infusion agar plates. These plates are incubated for 18-24 hours at 37° C. After incubation, single colonies are picked and transferred to veal infusion agar slants, which are incubated for 18-24 hours at 37° C. After this, the cultures are tested for smoothness by using a 1:500 dilution of acriflavine.

(5) Smooth cultures are inoculated into flasks containing veal or beef infusion broth which is incubated for 18-24 hours at 37° C. The incubated broth suspension of organisms is dispensed into the antigen bottles containing veal infusion agar. The suspension is distributed evenly over the agar surface by gently tilting the bottles from side to side. The inoculated bottles are then laid flat, agar side down, for 10-20 minutes. They are subsequently incubated, agar side upward, for 24-48 hours at 37° C. before harvesting.

(6) The harvesting of the organism consists of washing the growth from each antigen bottle with 0.5 percent phenolized physiological saline. The bacterial suspension from each bottle is filtered through sterile milk pad filters into a large sterile container or through a thin layer of absorbent cotton in a Buchner funnel with the aid of suction. To each 100 ml. of the bacterial suspension is added additional phenol to make the final concentration

0.5 percent. The concentrated antigen is tested for sterility at intervals after 24 hours. After sterility is proved, the stock antigen is standardized to determine the density according to the McFarland nephelometer scale.

(7) The diluted antigen to be used in routine testing is prepared from stock antigen, by diluting with 0.25 percent phenolized saline, and is standardized to a turbidity corresponding to 0.75-1.00 of the McFarland nephelometer scale.

(c) The maximum serum dilution employed for the "O" antigen tube test must not exceed 1:25. In all official reports on the blood test, the serum dilutions should be indicated. The sera should be introduced into the agglutination tubes in the desired amounts with well-cleaned serological pipettes or special serum delivery devices which do not permit the mixing of different sera. The antigen and serum should be well mixed before incubation. The serum and antigen mixture must be incubated for at least 20 hours at 37° C.

(d) The results shall be recorded as described in § 147.1(h).

§ 147.5 The microagglutination test for pullorum-typhoid.

Routinely, the microagglutination test is applied as a single-dilution test and only a single 18-24 hour reading is made.

(a) The procedure for the collection and delivery of blood samples in the microagglutination test is the same as that described in § 147.1(a). A method that has proven advantageous is to transfer the serum samples from the blood clot to a microplate as described in "Applied Microbiology," volume 24, No. 4, October 1972, pages 671-672. The dilutions are then performed according to paragraphs (d) or (e) of this section.

(b) Stained microtest antigen for pullorum-typhoid is supplied as concentrated stock suspension and must be approved by the Department.⁴ Directions for diluting will be provided with the antigen. The stock as well as the

⁴Information as to criteria and procedures for approval of concentrated stock suspension of stained microtest antigens may be obtained from the National Poultry Improvement Plan, Veterinary Services, APHIS, USDA, 1498 Klondike Road, Suite 200, Conyers, GA 30094.

diluted antigen prepared each day should be kept sealed in the dark at 5° to 10° C. when not in use.

(c) Available data indicate that a 1:20 dilution for the microagglutination test is most efficient for the detection of pullorum-typhoid agglutinins in both chickens and turkeys. In all official reports on the blood test, the serum dilutions shall be indicated.

(d) The recommended procedure for the 1:20 dilution in the microagglutination test is as follows:

(1) Add 100 microliters (0.10 cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) Using a microdiluter or a multimicrodiluter handle fitted with twelve 10 microliter microdiluters, transfer 10 microliters (0.01 cc.) of the serum sample from the collected specimen to the corresponding well of the microplate. This is accomplished by touching the surface of the serum sample with the microdiluter and then transferring and mixing with the diluent in the microplate well. The microdiluter is removed, blotted, touched to the surface of the distilled water wash, and again blotted. Other acceptable methods of serum delivery are described in "Applied Microbiology," volume 21, No. 3, March 1971, pages 394-399.

(3) Dilute the microtest antigens with 0.50 percent phenolized saline and add 100 microliters (0.1 cc.) to each microplate well.

(4) Seal each plate with a plastic sealer or place unsealed in a tight incubation box as described in "Applied Microbiology," volume 23, No. 5, May 1972, pages 931-937. Incubate at 37°C. for 18-24 hours.

(5) Read the test results as described in paragraph (f) of this section.

(e) The recommended procedure for a microagglutination test titration is as follows:

(1) Add 50 microliters (0.05cc.) of 0.85 percent physiological saline to each well of the microplate.

(2) To the wells representative of the lowest dilution in the titration, add an additional 50 microliters (0.05 cc.) of 0.85 percent physiological saline making a total of 100 microliters in these wells.

(3) Transfer each serum sample as described in §147.5(d)(2) of this section to

the first well containing 100 microliters (0.10cc.) in the titration, which represents the lowest dilution.

(4) Make twofold serial dilutions of each serum by transferring 50 microliters (0.05cc.) of diluted serum from one well to the next using twelve 50 microliter microdiluters fitted in a multimicrodiluter handle. When transfers have been made to all of the wells of the desired series, the 50 microliters remaining in the microdiluters are removed by blotting, touching the microdiluters to the surface of the distilled water wash, and blotting again.

(5) Dilute the desired microtest antigen with 0.50 percent phenolized saline and add 50 microliters (0.05 cc.) to each microplate well.

(6) Seal each plate with a plastic sealer or place the unsealed microplates in a tight incubation box and incubate at 37° C. for 18-24 hours.

(7) Read the test results as described in paragraph (f) of this section.

(f) Read the test results with the aid of a reading mirror. Results are interpreted as follows:

(1) N, or - (negative) when the microplate well has a large, distinct button of stained cells; or

(2) P, or + (positive) when the microplate well reveals no antigen button; or

(3) S, or ? (suspicious) when the microplate well has a small button. Suspicious reactions may tend to be more positive than negative [\pm] or vice versa [\mp] and can be so noted if desired.

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[41 FR 48726, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 57 FR 57342, Dec. 4, 1992; 59 FR 12799, Mar. 18, 1994; 59 FR 67617, Dec. 30, 1994; 61 FR 11521, Mar. 21, 1996; 63 FR 3, Jan. 2, 1998]

§ 147.6 Procedure for determining the status of flocks reacting to tests for *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, and *Mycoplasma meleagridis*.

The macroagglutination tests for *Mycoplasma* antibodies, as described in "Standard Methods for Testing Avian Sera for the Presence of *Mycoplasma Gallisepticum* Antibodies" published by the Agricultural Research Service,

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USDA, March 1966, and the micro-agglutination tests, as reported in the Proceedings, Sixteenth Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, 1973, shall be the official tests. Procedures for isolation and identification of Mycoplasma may be found in Isolation and Identification of Avian Pathogens, published by the American Association of Avian Pathologists and §§147.15 and 147.16.

(a) The status of a flock for Mycoplasma shall be determined according to the following criteria:

(1) If the tube agglutination or the serum plate test is negative, the flock qualifies.

(2) If the tube agglutination or the serum plate test is positive, the hemagglutination inhibition (HI) test and/or the Serum Plate Dilution (SPD) test shall be conducted. *Provided*, that for egg-type and meat-type chicken and waterfowl, exhibition poultry, and game bird flocks, if more than 50 percent of the samples are positive for either *Mycoplasma gallisepticum*, *M. synoviae*, or both, the HI and/or the SPD test shall be conducted on 10 percent of the positive samples or 25 positive samples, whichever is greater. The results of the HI and/or SPD tests must be followed by the action prescribed in paragraphs (a)(3), (a)(4), and (a)(5) of this section.

(3) If the tube agglutination or serum plate tests are positive and HI and/or the SPD tests are negative, the flock shall be retested in accordance with paragraph (a)(6) of this section.

(4) If HI titers of 1:40 or SPD titers of 1:5 are found, the flock shall be considered suspicious and shall be retested in accordance with paragraph (a)(6) of this section.

(5) If HI titers of 1:80, positive enzyme-labeled immunosorbent assay (ELISA) titers, or SPD titers of 1:10 or higher are found, the Official State Agency shall presume the flock to be infected. If the indicated titers are found, tracheal swabs from 30 randomly selected birds shall be taken promptly and cultured individually or a PCR-based procedure conducted on these specimens for Mycoplasma, and additional tests conducted in accordance with paragraph (a)(6) of this sec-

tion before final determination of the flock status is made.

(6) Fourteen days after the previous bleeding date, all birds or a random sample comprised of 75 birds shall be tested by the serum plate or tube agglutination test. Tested birds shall be identified by numbered bands.

(7) If the tube agglutination test or serum plate test is negative for the Mycoplasma for which the flock was tested, the flock qualifies.

(8) If the tube agglutination or serum plate test is positive on the retest, the HI and/or SPD test shall be conducted on the reacting samples.

(9) On the retest, if the tube agglutination or serum plate tests are positive at the same or higher rate and the HI or SPD tests are negative, the flock shall be considered suspicious and shall be retested in accordance with paragraph (a)(6) of this section.

(10) On the retest if HI titers of 1:80 and/or SPD titers of 1:10 or higher are found, the flock shall be considered infected: *Provided*, That, at the discretion of the Official State Agency, additional tests may be conducted in accordance with paragraph (a)(6) of this section before final determination of the flock status is made.

(11) If HI titers of 1:80 and/or SPD titers of 1:10 or higher are found on the second retest, the flock shall be considered infected for the Mycoplasma for which it was tested.

(12) If the tube agglutination or serum plate tests are found on the second retest to be positive at the same or higher rate and the HI and/or SPD tests are negative, the flock should be considered infected: *Provided*, That if the status of the flock is considered to be equivocal, the Official State Agency may examine reactors by the in vivo bio-assay, PCR-based procedures, and/or culture procedures before final determination of the flock status is made.

(13) If the in vivo bio-assay, PCR-based procedures, and culture procedures are negative, the Official State Agency may qualify the flock for the classification for which it was tested.

(14) If the in vivo bio-assay, PCR-based procedures, or culture procedures are positive, the flock shall be considered infected: *Provided*, That if only the

bio-assay is positive, additional *in vivo* bio-assay, PCR-based procedures, or cultural examinations may be conducted by the Official State Agency before final determination of the flock status is made.

(15) If the *in vivo* bio-assay, PCR-based procedures, or cultures are positive on retest, the flock shall be considered infected for the mycoplasma for which it was tested.

(b) [Reserved]

[40 FR 1504, Jan. 8, 1975, as amended at 41 FR 48726, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 47 FR 21993, May 20, 1982; 50 FR 19900, May 13, 1985; 54 FR 23957, June 5, 1989; 59 FR 12799, Mar. 18, 1994; 61 FR 11521, Mar. 21, 1996; 62 FR 44070, Aug. 19, 1997; 63 FR 3, Jan. 2, 1998]

§ 147.7 Standard test procedures for mycoplasma.⁵

The serum plate agglutination test, the tube agglutination test, and the enzyme-linked immunosorbent assay (ELISA) test should be considered basic screening tests for mycoplasma antibodies. The test selected will depend on preference, laboratory facilities, and availability of antigen. These three tests, though quite accurate, determine flock status rather than individual bird status, since occasional reactions are nonspecific. Under normal circumstances, the rate of such nonspecific reactions is low. Nonspecific reactions may occasionally be high, particularly after the use of erysipelas bacterin in turkeys and where mycoplasma antibodies are present for closely related mycoplasma other than for the species being tested. The hemagglutination inhibition (HI) test is too cumbersome for routine screening use. Positive reactions are extremely accurate however, and are useful in evaluating serum samples that react with the ELISA, plate, and/or tube antigens. The test should be conducted with 4 HA units. Titers of 1:80 or greater for both chicken and turkey

sera are considered positive, while a 1:40 or 1:20 titer would be strongly suspicious and additional tests should be required.

(a) *Serum plate agglutination test.* (1) The serum plate agglutination test for mycoplasma is conducted by contacting and mixing 0.02 ml of test serum with 0.03 ml of serum plate antigen on a glass at room temperature. The standard procedure is:

(i) Allow antigen and test serums to warm up to room temperature before use.

(ii) Dispense test serums in 0.02 ml amounts with a pipette or standardized loop (rinsed between samples) to 1½ inch squares on a ruled glass plate. Limit the number of samples (no more than 25) to be set up at one time according to the speed of the operator. Serum should not dry out before being mixed with antigen.

(iii) Dispense 0.03 ml of antigen beside the test serum on each square. Hold antigen dispensing bottle vertically.

(iv) Mix the serum and antigen, using a multimixing device if large numbers are to be run at one time.

(v) Rotate the plate for 5 seconds. At the end of the first minute, rotate the plate again for 5 seconds and read 55 seconds later.

(2) A positive reaction is characterized by the formation of definite clumps, usually starting at the periphery of the mixture. Most samples that are highly positive will react well within the 2-minute test period. Reactions thereafter should be considered negative, although partial agglutination at 3 and 5 minutes may warrant further retesting. High-quality antigen contacted with negative serum will usually dry up on the plate without visible clumping. Whenever samples are run, the antigen should be tested against known positive and negative control serums. Standard reference antigens and negative and positive titered sera are available from the National Veterinary Services Laboratories (NVSL), P.O. Box 884, Ames, Iowa 50010.

(3) Since it is difficult to measure uniform amounts of serum with a calibrated loop, this technique should not be used in conducting an official test.

⁵For additional information on mycoplasma test procedures, refer to the following references: Proc. 77th Annual Meeting, U.S. Animal Health Association, 1973; Isolation and Identification of Avian Pathogens, 3rd Edition; Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens, 1991.

(b) *Serum plate dilution test.* (1) The serum plate dilution (SPD) test may be used to evaluate possible nonspecific reactions, gain additional information to evaluate positive plate tests occurring in an unexpected manner, and/or to evaluate the level of mycoplasma antibodies present in the serum sample. If sufficient serum is available, the following method would provide the dilutions required to conduct the test.

(i) Rack three tubes and put 0.8 ml of phosphate-buffered saline (PBS) in tube 1 and 0.5 ml of PBS in tubes 2 and 3.

(ii) Pipette 0.2 ml of the test serum into tube 1 and discard the pipette.

(iii) With a pipette, mix the serum and PBS in tube 1 and withdraw 0.5 ml and add to tube 2.

(iv) Repeat the process in step (iii), mixing the contents of tube 2 and transferring 0.5 ml to tube 3.

(v) Conduct the test, as described for the serum plate test in paragraph (a), on the undiluted sample and on samples in tubes 1, 2, and 3 after proper mixing of each dilution.

(vi) To assist in the evaluation of the test, conduct concurrent SPD tests using both positive 1:80 and positive 1:160 HI sera for the mycoplasma being tested. The antigen should be pretested for reactivity with standard serum at the 1:5 and 1:10 dilution.

(vii) Interpretation of the SPD test results should be based on the criteria in § 147.6.

(c) *Tube agglutination test.* (1) The mycoplasma tube agglutination test is conducted by mixing 0.08 ml of test serum with 1.0 ml of diluted (1:20) antigen in a tube and allowing the mixture to react for 18-24 hours at 37° C. The diluent will be the standard phosphate-buffered saline with phenol. This solution is made up as follows:

	Grams
Sodium hydroxide (C.P.)	0.15
Sodium chloride (C.P.)	8.5
Potassium dihydrogen phosphate (KH ₂ PO ₄) (C.P.)	0.68
Phenol (Crystal) (C.P.)	2.5
Distilled water to make 1,000 ml	

The pH of the buffered phenolized saline will be 7.1-7.2 if all reagents are accurately measured. The stock tube antigen is diluted 1:20 with buffered

phenolized saline. The procedures for the tube test are as follows:

(i) Rack 12 x 75 mm clean tubes and identify the tubes according to the sample to be tested.

(ii) Add 0.08 ml of the individual test serum to each tube. This will create approximately a 1:12.5 screening dilution of test serum when 1.0 ml of diluted antigen is added. The use of a pipetting device will insure proper mixing of serum and antigen.

(iii) To interpret positive reactions to the 1:12.5 dilution, two additional dilutions may be made by adding 0.04 ml of serum for 1:25 dilution and 0.02 ml of serum for 1:50 dilution, with the addition of 1.0 ml of diluted antigen as indicated in paragraph (c)(1)(ii) of this section.

(iv) Shake racks and incubate test systems for 18-24 hours at 37° C.

(2) Tests are read against a dark background under indirect fluorescent light. Regarded as a positive reaction is a clearing of the supernatant fluid, with visible sediment in the bottom of the tube. Incomplete reactions are suspect. Positive and negative control sera should be incorporated into each day's run of tests. Reactions at 1:25 or greater are considered positive. They should be confirmed by the HI test. Incubation for periods greater than 24 hours may be helpful in evaluating suspicious reactions and need for possible retesting or other diagnostic tests.

(d) *Hemagglutination Inhibition (HI) test.* The mycoplasma HI test is conducted by the constant-antigen, decreasing-serum method. This method requires using a 4-hemagglutination (HA) unit of diluted antigen. Differences in the number of HA units used will change the titers of positive sera markedly. Standard HA antigens for *Mycoplasma gallisepticum*, *M. synoviae*, and *M. meleagridis* are available from NVSL. The antigen has been titrated and diluted to approximately 1:640. The HA titration of each sample should be checked as described in paragraph (d)(2) on initial use or after long storage. To maintain HA activity, the undiluted HA antigen should be stored at -60° to -70° C. The test procedures are illustrated in Tables 2 and 3 of this paragraph.

(1) *Preparation of materials.*

(i) Prepare phosphate-buffered saline (PBS) as follows:

	Grams
Sodium hydroxide (C.P.)	0.15
Sodium chloride (C.P.)	8.5
Potassium dihydrogen phosphate (KH ₂ PO ₄) (C.P.)	0.68
Distilled water to make 1,000 ml	

The pH of the PBS will be 7.1-7.2 if all reagents are accurately measured.

(ii) Collect the turkey or chicken red blood cells (RBC's) in Alsever's solution which has been prepared as follows:

	Grams
Sodium citrate	8.0
Sodium chloride	4.2
Dextrose	20.5
Distilled water to make 1,000 ml.	

The sodium citrate and sodium chloride are dissolved in 800 ml distilled water and sterilized at 15 lbs. pressure for 15 minutes. Dissolve the dextrose in 200 ml distilled water, sterilize by Seitz or other type of filtration and then add aseptically to the sterile sodium citrate and sodium chloride solution.

(iii) From a turkey(s) or chicken(s) known to be free of the mycoplasma being tested, withdraw sufficient blood with a syringe containing Alsever's solution to give a ratio of 1 part blood to 5 parts Alsever's solution (e.g., 8 ml blood in 40 ml of Alsever's solution).

Centrifuge the blood suspension at 1,000 rpm for 10 minutes and remove the Alsever's solution or supernatant with a pipette.

(iv) Wash the RBC's two times in 10 or more parts of Alsever's solution, centrifuging after each washing. Centrifugation is at 1,000 rpm for 10 minutes. The supernatant fluid is removed and the RBC deposit resuspended to give a 25 percent suspension of packed RBC's in Alsever's solution. (In testing either chicken or turkey sera, the homologous RBC system must be used; i.e., use chicken cells when testing chicken serum and turkey cells when testing turkey serum.) If this suspension is kept refrigerated, it should keep for 7 or 8 days after the blood has been collected.

(v) For the test, 1 ml of the 25 percent RBC's is added to 99 ml of buffered saline to make a 0.25 percent RBC suspension.

(2) *Hemagglutination (HA) antigen titration.* The HA stock antigen is stored at -70 ° C in PBS buffer containing 25 percent glycerin (vol/vol) in a concentrated suspension (i.e., 320-640 HA units/ml) in screwtype vials. Under such conditions, potency will be retained for years. There will be a rapid loss of titer if improperly stored. The titer of HA antigen is determined as illustrated in Table 1 and described in paragraphs (d)(2)(i) through (x) of this section.

TABLE 1 Titration of Hemagglutination (HA) Antigen

Reagents (ml)	Tube No.							
	1	2	3	8	9	10	11 ^a
PBS	0.8	0.5	0.5	0.5	0.5	0.5	0.5
Antigen	0.2							
Transfer	0.5→	0.5→	0.5→→	0.5→	0.5→	0.5→	↓ ^c
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ant. dilution	1:5	1:10	1:20	1:640	1:1280	1:2560	
Results ^b	+	+	+	+	-	-	

^a Tube 11, PBS/RBC control.

^b + = HA; - = no HA (sample titer 1:640).

^c Discard 0.5 ml.

(i) Rack a series of 11 chemically clean 12 x 75 mm test tubes. Label the tubes 1-11 left to right.

(ii) Put 0.8 ml of PBS in tube 1 and 0.5 ml of PBS in each of tubes 2-11.

(iii) Add 0.2 ml of antigen to tube 1. This will make a 1:5 dilution of antigen. Discard pipette.

(iv) Mix contents of tube 1 thoroughly with a clean pipette, and transfer 0.5 ml to tube 2. This will make a 1:10 dilution of antigen in tube 2. Discard pipette.

(v) Continue making serial twofold dilutions of antigen, changing pipettes after each transfer, through tube 10. This will result in a series of twofold dilutions ranging from 1:5 to 1:2560. Discard 0.5 ml of antigen dilution from tube 10.

(vi) Add 0.5 ml of 0.25 percent RBC's to tubes 1-11. Tube 11 will serve as PBS/RBC control.

(vii) Shake the rack and incubate at room temperature until the cells in the

PBS/RBC control tube have settled into a compact button at the bottom of the tube.

(viii) If turkey sera is also to be tested for HI titer, repeat steps outlined in paragraphs (d)(2)(i) through (vii) of this section, using 0.25 percent turkey RBC's.

(ix) The end point of the titration is the highest dilution of antigen that produces complete agglutination of the RBC's, as evidenced by the formation of a thin sheet of cells covering the concave bottom of the tube. For example, if complete agglutination is produced through tube 8 (a dilution of 1:640 of antigen), the antigen would be said to titer 640, the reciprocal of the dilution.

(x) Specificity of HA antigen should be determined by conducting HI tests with specific chicken sera of variable HI titers. Specific turkey sera of varying HI titers should be used if turkey sera is also to be tested.

TABLE 2 Hemagglutination Inhibition (HI) Test:

Reagents (ml)	Tube No.							
	1 ^a	2	3	8	9	10	11 ^b
PBS	0.8	0	0			0	0	0.5
8-unit antigen	0	0.5	0			0	0	0
4-unit antigen	0	0	0.5	0.5	0.5	0.5	0.5	0
Test serum	0.2	0	0	0	0	0	0	0
Transfer	0.5→	0.5→	0.5→	...→	0.5→	0.5→	0.5→	0.5↓ ^c
0.25% RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Serum dilution	1:5	1:10	1:20	...	1:640	1:1280	1:2560	

^a Tube 1. Serum control.

^b Tube 11. PBS/RBC control.

^c Discard 0.5 ml.

TABLE 3 Antigen Control:

Reagents (ml)	Tube No.				
	1	2	3	4	5
4-unit antigen	1.0	0	0	0	0
PBS	0	0.5	0.5	0.5	0.5
Transfer	0.5→	0.5→	0.5→	0.5→	0.5 ^b ↓
0.25% RBC	0.5	0.5	0.5	0.5	0.5
Unit Antigen/tube	4	2	1	1/2	1/4
Results ^a	+	+	+	-	-

^a + = HA; - = no HA.

^b Discard 0.5 ml.

(3) Reagents for mycoplasma HI test.

(i) Eight-unit antigen (Dilution factor for stock antigen is established by dividing titer by 8; i.e., 640 antigen is diluted 1:80 in PBS to make 8-unit antigen.)

(ii) Four-unit antigen (made by diluting surplus 8-unit antigen 1:2 with PBS).

(iii) PBS at pH 7.0.

(iv) Unknown test serums.

(v) Positive control serum of known titer (should be from the same species as the unknown).

(vi) Negative control serum (should be from the same species as the unknown).

(vii) Solution of 0.25 percent washed RBC's.

(4) Test outline.

(i) Rack 10 chemically clean 12 x 75 mm tubes for each serum, including

controls, to be tested. Identify each row of tubes, and label tubes in each row 1-10, left to right. In row 1, add tube 11 for a PBS/RBC control.

(ii) Put 0.8 ml of PBS in tube 1 of each test row; put 0.5 ml of 8-unit antigen in tube 2 of each test row; put 0.5 ml of 4-unit antigen in each of tubes 3-10 in each test row; and put 0.5 ml of PBS in tube 11.

(iii) Add 0.2 ml of test serum to tube 1. This tube will be the serum control in the test system.

(iv) Mix and make 0.5 ml transfers from tube 1 through tube 10. This will result in serial twofold dilutions of serum starting with 1:5 and ending with 1:2560. Discard 0.5 ml from tube 10.

(v) Rack five tubes in which to set up an antigen control.

(vi) In tube 1, put 1.0 ml of 4-unit antigen; put 0.5 ml of PBS in tubes 2-5.

(vii) Make 0.5 ml serial transfers from tube 1 through tube 5, changing pipettes after each transfer. Discard 0.5 ml from tube 5. This will result in a series of tubes respectively containing 4, 2, 1, 1/2, and 1/4 units of antigen.

(viii) After 20-30 minutes at room temperature to permit antibody-anti-

gen reaction, add 0.5 ml of 0.25 percent washed RBC's to each tube. Shake racks and incubate as for HA titration.

(ix) In this test system, positive serum should inhibit the HA activity of the antigen, while negative serum should have no effect. Inhibition will be evidenced by the formation of a free-flowing bottom of cells in the bottom of the tube. The titer of the serum can be calculated as the reciprocal of the highest dilution of serum that produces complete HI. Controls should read as follows:

(A) Serum control (tube 1). Cells should settle out.

(B) PBS/RBC control (tube 11). Cells should settle out.

(C) Antigen control. HA in tubes 1-3. Cells should settle out in tubes 4-5.

(D) Positive and negative serum control. Positive control should inhibit to its known titer; negative control should have no inhibitory effect.

(x) With this test system and 4 units of antigen, HI titers of 80 or above are considered positive and titers of 40 are strongly suspicious. However, titers of 10 or 20 are usually negative. Sample test results are illustrated in Table 4 in this paragraph.

TABLE 4.—SAMPLE RESULTS OF HI TESTS
[Tube and Serum Dilution]

	1	2	3	4	5	6	7	8	9	10
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Serum A (HI neg.)	-	+	+	+	+	+	+	+	+	+
Serum B (HI 1:40)	-	-	-	-	+	+	+	+	+	+
Serum C (HI 1:160)	-	-	-	-	-	-	+	+	+	+
Serum D (HI 1:20)	-	-	-	+	+	+	+	+	+	+

+, HA.
-, no HA or HI.

(xi) If serological results from agglutination tests complemented by the HI test are inconclusive, cultural examination, bio-assay, or retesting of samples after an interval of at least 21 days may be indicated.

(e) *Procedure for mycoplasma hemagglutination inhibition tests using microtiter technique.* (1) *Procedure No. 1.* The microtiter mycoplasma HI test was developed from the tube HI test described in §147.7(d). Refer to these procedures for preparation of materials not listed below.

(i) *Materials needed.* (A) Microtiter equipment (minimal); i.e., microplates, microdiluters, micropipettes, go-no-go diluter delivery tester, (0.05 ml).

(B) Phosphate-buffered saline (PBS).

(C) Reagents from NVSL; i.e., HA antigen and negative and positive titered sera for the mycoplasma to be tested.

(D) Homologous red blood cells (RBC's) suspension 0.5 percent (2 ml of 25 percent RBC's to 98 ml of PBS) obtained from birds free of the mycoplasma to be tested. (See paragraphs

(d)(1)(ii) through (v) of this section for preparation of RBC's.)

(ii) *Microtiter hemagglutination (HA) antigen titration.* (A) Mark off two rows of 10 wells each for antigen titer (HA is done in duplicate).

(B) Mark last well in each row for cell controls.

(C) Prepare in small test tube (12 x 75 mm) a starting dilution of antigen by combining 0.1 ml antigen with 0.9 ml PBS. This is a 1:10 dilution.

(D) Add 0.05 ml PBS to all wells, including cell controls.

(E) Add 0.05 ml antigen (1:10 dilution) with diluters to the first well in both rows, mix thoroughly, transfer diluter to second well of each row and mix, continuing through the 10th well of each row. With mixture in diluter from last well, check diluter on go-no-go card, then place diluter in distilled water. If diluter checks out, antigen dilution will be 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120.

(F) Add 0.05 ml of 0.5 percent RBC suspension to all wells using a 0.05 dropper.

(G) Seal plate (if plate is to be held over 2 hours); shake and allow to stand at room temperature until cells in cell control gather in compact button. The titer is the highest dilution in which agglutination is complete. The dilution contains 1 HA unit in 0.05 ml.

(H) Prepare a dilution of antigen which contains 8 HA units in 0.05 ml. Example: if the antigen titer is 1:640, then that dilution contains 1 HA unit per 0.05 ml. Then $640 \div 8 = 80$, or a dilution of 1:80 containing 8 HA units. Or $640 \div 4 = 160$, a dilution of 1:160 containing 4 HA units per 0.05 ml.

(iii) *Microtiter HI test.* (A) Prepare two dilutions of antigen, one containing 8 HA units per 0.05 ml and one containing 4 HA units per 0.05 ml. The 4-unit antigen can be prepared from the 8-unit antigen by mixing with equal parts of PBS.

(B) Mark off one row of 8 wells for each test.

(C) Prepare a 1:5 dilution of each sera to be tested in a small test tube (12x75 mm): 0.1 ml serum plus 0.4 ml PBS or 0.05 ml serum plus 0.20 ml PBS.

(D) Add 0.05 ml PBS with the 0.05 ml dropper to the first well in each row.

(E) Add 0.05 ml of 8-unit antigen to well 2 in each row.

(F) Add 0.05 ml of 4-unit antigen to well 3 through 8 for each row.

(G) For each serum to be tested, load 0.05 ml diluter with 1:5 dilution as prepared in paragraph (iii) above and place in first well of row.

(H) Mix well and transfer loaded diluter to well 2. Continue serial twofold dilutions through well number 8.

(I) Well 1 (serum dilution of 1:10) is serum control. Well 2=1:20 dilution; well 3=1:40 dilution; well 4=1:80 dilution; well 5=1:160 dilution; well 6=1:320 dilution; well 7=1:640 dilution; and well 8=1:1280 dilution.

(J) *Antigen control.* (1) Mark off 6 wells for antigen controls.

(2) Add 0.05 ml PBS to wells 2, 3, 4, 5, and 6.

(3) Add 0.05 ml 8-unit antigen to wells 1 and 2.

(4) With empty diluter, mix contents of well 2. Continue serial twofold dilutions through well 6.

(5) Well 1 contains 8 units; well 2 contains 4 units; well 3 contains 2 units; well 4 contains 1 unit; well 5 contains $\frac{1}{2}$ unit; and well 6 contains $\frac{1}{4}$ unit.

(6) Mark off two wells for cell controls and add 0.05 ml PBS to each.

(7) After 20-30 minutes at average room temperature (20°-23°C) to permit antibody-antigen reaction, add 0.05 ml of a 0.5 percent suspension of RBC's to all wells.

(8) Seal all wells (if wells are to be held over 2 hours). Shake the plate thoroughly.

(9) Incubate at room temperature for 30-45 minutes.

(K) *Interpretation:* The HI titer is the highest serum dilution exhibiting complete inhibition of hemagglutination as indicated by flowing of cells when the plate is tilted. Serum having a titer of 1:80 or greater is considered positive. A titer of 1:40 or 1:20 is suspicious.

(2) *Procedure No. 2.* Purpose: To test for antibodies to avian mycoplasma by hemagglutination inhibition (HI). The test uses the constant antigen, titered-sera method for measuring antibodies to *M. gallisepticum*, *M. synoviae*, or *M. meleagridis*.

(i) *Materials needed.*

(A) *M. gallisepticum*, *M. synoviae*, and/or *M. meleagridis* HI antigens.

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(B) Positive and negative control sera.

(C) Phosphate buffered saline (PBS).

(D) Microtiter plates, 96-well, U-bottom.

(E) 12-channel pipettor (Titerek).

(F) 50 μ L pipettor (Pipetman P200).

(G) Pipette tips.

(H) 0.5 percent homologous red blood cells (RBC's) in PBS (use RBC's from the same species being tested).

(I) Plate-sealing tape.

(J) Mirrored plate reader.

(ii) *Microtiter hemagglutination antigen (HA) titration.*

(A) Perform standard hemagglutination test (HA) on mycoplasma antigen to determine titer of antigen.

(1) Dispense 50 μ L of PBS into each well of 3 rows of a 96-well microtiter plate.

(2) Dispense 50 μ L of stock antigen into the wells of 2 rows.

(3) Perform serial two-fold dilutions (50 μ L) using a 12-channel pipettor. The dilution series will be from 1:2 to 1:4096.

(4) Add 50 μ L of 0.5 percent homologous RBC's to each well of all 3 rows. The row with no antigen serves as an RBC control.

(B) Incubate at room temperature (approximately 30 minutes) until the control RBC's give tight buttons. The HA titer is read as the last well to give a complete lawn (hemagglutination). The desired endpoint is 4 HA units. The well containing the 1:4 dilution should give a complete HA while the 1:8 dilution should show less than complete HA.

(C) Dilute stock antigen to 4 HA units for the HI test. The dilution required to give 4 HA units is calculated by dividing the stock antigen HA titer by 8. (Example: 1:320 HA units \div 8 = 40, dilute stock antigen 1:40.)

(iii) *Hemagglutination inhibition assay.*

(A) Label one column (A to H) of a 96-well, U-bottom microtiter plate for each sample, each positive and negative control sera, antigen backtitration, and RBC control.

(B) Add 40 μ L of PBS to the top row of wells (row A) of the plate.

(C) Add 25 μ L of PBS to all remaining wells of the plate.

(D) Add 10 μ L of each test sera to well A of each column (making a 1:5 sera dilution).

(E) Serially dilute 25 μ L from well A through H using a 12-channel pipettor. Discard the final 25 μ L. Row A = 1:5...row H = 1:640.

(F) With an Oxford doser, add 25 μ L of 4 HA unit antigen to wells B through H. Well A serves as sera control.

(G) Prepare an antigen backtitration by adding 25 μ L of PBS to each well of one column. Add 25 μ L of diluted antigen to well A and serially dilute 25 μ L from wells A to D. This prepares 1:2, 1:4, 1:8, and 1:16 dilutions. (It is recommended that the antigen control backtitration be performed before the diluted antigen is used in the assay. Dilution problems could be detected and corrected before the inappropriately diluted antigen is used in the assay.)

(H) Leave a column of wells blank for an RBC control.

(I) Agitate gently and incubate for 30 minutes at room temperature.

(J) Add 50 μ L of 0.5 percent RBC's to all wells. Note: Do not agitate after RBC's have been added (agitation may result in false positive reactions by causing the RBC's to fall, resulting in "false" buttons).

(K) Cover the plate with sealing tape. Incubate at room temperature for 30 minutes or until control RBC's give a tight button.

(L) Read the reaction on a mirrored plate reader.

(iv) *Results.*

(A) The titer is reported as the reciprocal of the last dilution to give a tight button of RBC's. The final dilution scheme includes the antigen in the dilution calculation and is as follows: B=1:20, C=1:40, D=1:80, E=1:160, F=1:320, G=1:640, H=1:1,280.

(B) For the assay to be valid:

(1) The positive control sera must give a result within one dilution of the previously determined titer.

(2) The negative control sera must be negative.

(3) The backtitration of the antigen must be 1:4 or 1:8.

(4) The RBC control must give tight, non-hemolyzed buttons.

(5) Sera controls (well A of each test sera) must not have non-specific agglutination or hemolysis. If negative, report as "negative with non-specific agglutination or non-specific hemolysis" or "unable to evaluate due to non-specific agglutination or hemolysis" or treat the serum to remove the non-specific agglutination and repeat the test. (See paragraph (e)(2)(v) of this section.)

(v) *Treatment to remove non-specific agglutination.*

(A) *Purpose.* Treatment of serum to remove non-specific agglutination that is interfering with HI assays.

(B) *Specimen.* Serum.

(C) *Materials.* Homologous RBC's (chicken or turkey), 50 percent solution PBS, centrifuge, incubator, 4C (refrigerator).

(D) *Procedure.* (1) Prepare a 1:5 dilution of test serum by adding 50 µL of serum to 200 µL of PBS.

(2) Prepare a 50 percent solution of RBC's by adding equal volumes of packed RBC's to PBS. Mix well.

(3) Add 25 µL of 50 percent RBC solution to the serum dilutions.

(4) Vortex gently to mix.

(5) Incubate at 4 °C for 1 hour.

(6) Centrifuge to pellet the RBC's.

(7) Use the supernatant to perform the HI assay. Modify the dilution scheme in the assay to consider the initial 1:5 dilution prepared in the treatment. For the 1:5 dilution scheme, do not add PBS to row A. Add 50 µL of the 1:5 treated supernatant to row A. Serially dilute 25 µL from rows A through H. This prepares a serum dilution of 1:10 through 1:640 in rows B through H.

[49 FR 19803, May 10, 1984, as amended at 57 FR 57342, Dec. 4, 1992; 59 FR 12799, Mar. 18, 1994; 63 FR 3, Jan. 2, 1998]

§ 147.8 Procedures for preparing egg yolk samples for diagnostic tests.

The following testing provisions may be used for retaining the classification U.S. M. Gallisepticum Clean under § 145.23(c)(1)(ii)(C) and § 145.33(c)(1)(ii)(C), and for retaining the classification U.S. M. Synoviae Clean under § 145.23(e)(1)(ii)(b) and § 145.33(e)(1)(ii)(b) of this chapter.

(a) Under the supervision of an Authorized Agent or State Inspector, the eggs which are used in egg yolk testing must be selected from the premises

where the breeding flock is located, must include a representative sample of 30 eggs collected from a single day's production from the flock, must be identified as to flock of origin and pen, and must be delivered to an authorized laboratory for preparation for diagnostic testing.

(b) The authorized laboratory must identify each egg as to the breeding flock and pen from which it originated, and maintain this identity through each of the following:

(1) Crack the egg on the round end with a blunt instrument.

(2) Place the contents of the egg in an open dish (or a receptacle to expose the yolk) and prick the yolk with a needle.

(3) Using a 1 ml syringe without a needle, aspirate 0.5 ml of egg yolk from the opening in the yolk.

(4) Dispense the yolk material in a tube. Aspirate and dispense 0.5 ml of PBS (phosphate-buffered saline) into the same tube, and place in a rack.

(5) After all the eggs are sampled, place the rack of tubes on a vortex shaker for 30 seconds.

(6) Centrifuge the samples at 2500 RPM (1000 x g) for 30 minutes.

(7) Test the resultant supernatant for *M. gallisepticum* and *M. synoviae* by using test procedures specified for detecting IgG antibodies set forth for testing serum in § 147.7 (for these tests the resultant supernatant would be substituted for serum); except that a single 1:20 dilution hemagglutination inhibition (HI) test may be used as a screening test in accordance with the procedures set forth in § 147.7.

NOTE.— For evaluating the test results of any egg yolk test, it should be remembered that a 1:2 dilution of the yolk in saline was made of the original specimen.

[50 FR 19900, May 13, 1985; 63 FR 3, Jan. 2, 1998]

Subpart B—Bacteriological Examination Procedure

§ 147.10 Laboratory procedure recommended for the bacteriological examination of egg-type breeding flocks with salmonella enteritidis positive environments.

Birds selected for bacteriological examination from egg-type breeding

flocks positive for *Salmonella enteritidis* after environmental monitoring should be examined as described in §147.11(a) of this subpart, with the following exceptions and modifications allowed due to the high number of birds required for examination:

(a) Except when visibly pathological tissues are present, direct culture, §147.11(a)(1) of this subpart, may be omitted; and

(b) Enrichment culture of organ (non-intestinal) tissues using a non-selective broth, §147.11(a)(2) of this subpart, may be omitted.

[59 FR 12801, Mar. 18, 1994]

§ 147.11 Laboratory procedure recommended for the bacteriological examination of salmonella.

(a) For egg- and meat-type chickens, waterfowl, exhibition poultry, and game birds. All reactors to the Pullorum-Typhoid tests, up to at least four birds, should be cultured in accordance with both *direct* (paragraph (a)(1)) and *selective enrichment* (paragraph (a)(2)) procedures described in this section. Careful aseptic technique should be used when collecting all tissue samples.

(1) Direct culture (refer to illustration 1). Grossly normal or diseased liver, heart, pericardial sac, spleen, lung, kidney, peritoneum, gallbladder, oviduct, misshapen ova or testes, inflamed or unabsorbed yolk sac, and other visibly pathological tissues where purulent, necrotic, or proliferative lesions are seen (including cysts, abscesses, hypopyon, and inflamed serosal surfaces), should be sampled for direct culture using either flamed wire loops or sterile swabs. Since some strains may not dependably survive and grow in certain selective media, inoculate *non-selective plates* in addition to two selective plating media. Refer to illustration 1 for recommended bacteriological recovery and identification procedures.⁶ Proceed immediately with collection of organs

and tissues for selective enrichment culture.

(2) Selective enrichment culture (refer to illustration 2). Collect and culture organ samples separately from intestinal samples, with intestinal tissues collected last to prevent cross-contamination. Samples from the following organs or sites should be collected for culture in selective enrichment broth. A non-selective broth culture (illustration 1) of pooled organs and sites should also be included as described in paragraph (a)(3) of this section.

(i) Heart (apex, pericardial sac, and contents if present);

(ii) Liver (portions exhibiting lesions or, in grossly normal organs, the drained gallbladder and adjacent liver tissues);

(iii) Ovary-Testes (entire inactive ovary or testes, but if ovary is active, include any atypical ova);

(iv) Oviduct (if active, include any debris and dehydrated ova);

(v) Kidneys and spleen; and

(vi) Other visible pathological sites where purulent, necrotic, or proliferative lesions are seen.

(3) From each reactor, aseptically collect 10 to 15 g, or the nearest lesser amount available, from each organ or site listed in paragraph (a)(2) of this section and mince, grind, and blend them completely in 10 times their volume of beef extract broth or a comparable non-selective broth. Organs or sites listed in paragraph (a)(2) of this section may be pooled from the same individual bird. Suspensions should be transferred in 10-ml aliquots to 100 ml of both tetrathionate brilliant green (TBG) (Hajna or Mueller-Kauffmann) broth and a separate non-selective broth and incubated at 37 °C for 24 hours. Refer to illustration 2 for recommended bacteriological recovery and identification procedures, including delayed secondary enrichment and combinations of plating media that significantly suppress the overgrowth of contaminants, such as brilliant green Novobiocin (BGN) and Xylose-Lysine-Tergitol 4 (XLT4). As a supplemental procedure, a colony lift assay may also be used as a screening test to

⁶Biochemical identification charts may be obtained from "A Laboratory Manual for the Isolation and Identification of Avian Pathogens," chapter 1, Salmonellosis. Third edition, 1989, American Association of Avian Pathologists, Inc., Kendall/Hunt Publishing Co., Dubuque, IA 52004-0539.

aid in the detection of group D salmonella suspect colonies on selective and nonselective agar culture plates.

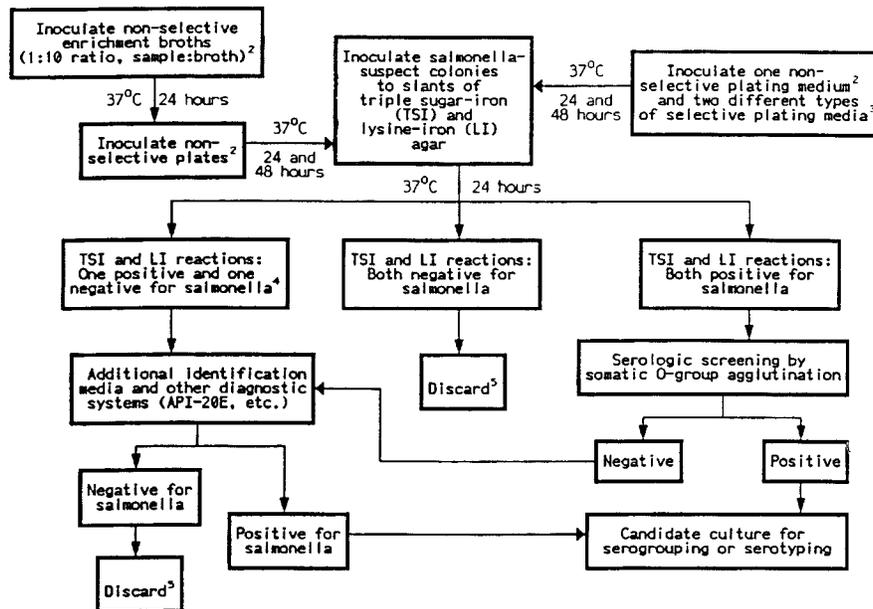
(4) From each reactor, make a composite sample of the following parts of grossly normal or diseased tissues from the digestive tract: Crop wall, duodenum (including portions of the pancreas), jejunum (including remnant of yolk-sac attachment), both ceca, cecal tonsils, and rectum-cloaca. Aseptically collect 10–15 g or the nearest lesser amount available from each specified digestive or intestinal tissue, and mince, grind, and blend them completely in 10 times their volume of TBG broth. The digestive/intestinal tissues may be pooled from the same individual bird. Do not pool tissues from different birds. Transfer 10 ml of the described digestive TBG suspensions into 100 ml of TBG broth, and incubate at 41.5 °C for 24 hours. Cultures may be incubated at 37 °C if 41.5 °C incubators are not available. The higher incubation temperatures for TBG broth re-

duce populations of competitive contaminants common in gut tissue. Refer to illustration 2 and paragraph (a)(5) of this section for recommended bacteriological recovery and identification procedures, including delayed secondary enrichment and combinations of plating media that significantly suppress the overgrowth of contaminants, such as BGN and XLT4, and a colony lift assay to aid in the detection of group D salmonella colonies.

(5) As a supplement to the standard colony pick to triple sugar-iron (TSI) and lysine-iron (LI) agar slants, a group D colony lift assay may be utilized to signal the presence of hard-to-detect group D salmonella colonies on agar culture plates. A system such as the Analytical Profile Index for Enterobacteriaceae (API) may also be utilized to aid cultural identifications.

(6) All isolates culturally identified as *salmonellae* should be serogrouped or serotyped.

ILLUSTRATION 1: *Organ (non-intestinal) tissues.*¹
Pullorum-Typhoid reactors.



¹ All pullorum-typhoid reactors should also be evaluated with selective enrichment broths (refer to illustration 2).

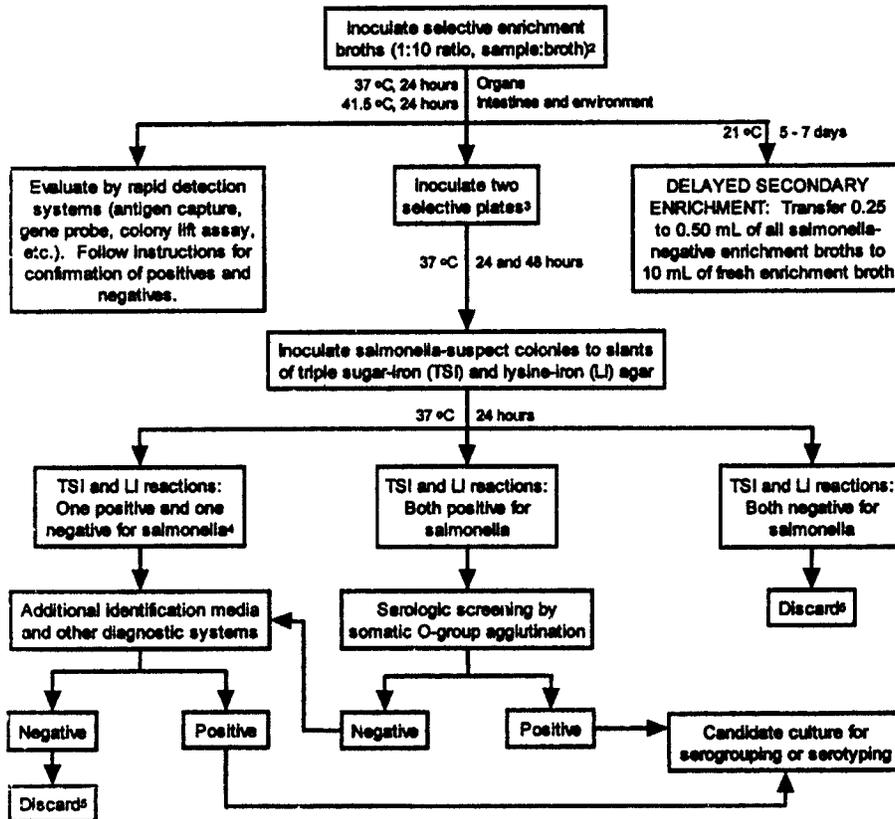
² Beef extract or infusion broths and plates are preferred. Comparable non-selective media may also be used.

³ Inoculate brilliant green (BG) or BG-Novobiocin (BGN) AND another selective media such as xylose-lysine-desoxycholate (XLD) or XLD-Novobiocin (XLDN).

⁴ If combined results with TSI and LI agars, additional identification media, and O-group screening procedures are inconclusive, restreak original colony onto selective plating media to check for purity.

⁵ Reevaluate if epidemiologic, necropsy, or other information indicates the presence of an unusual strain of Salmonella.

ILLUSTRATION 2: *Environmental, organ, and intestinal samples.*¹
Environmental monitoring programs and pallidum-typhoid reactors.



¹ Organ tissues from all reactor birds should also be evaluated without selective enrichment (refer to Illustration 1).

² Hajna TT or Mueller-Kauffmann tetrathionate enrichment broth is preferred over selenites.

³ For enrichment broths of organ samples, inoculate xylose-lysine-desoxycholate (XLD) or XLD-Novobiocin (XLDN) and brilliant green (BG) or BG-Novobiocin (BGN) media. One of the media shall be either XLDN or BGN. For enrichment broths of intestinal or environmental samples, inoculate xylose-lysine-targitol 4 (XLT4) or XLDN and BGN or BG media.

⁴ If combined results with TSI and LI agars, additional identification media, and O-group screening procedures are inconclusive, restreak original colony onto selective plating agar to check for purity.

⁵ Reevaluate if epidemiologic, necropsy, or other information indicates the presence of an unusual strain of Salmonella.

(b) For turkeys—(1) *Bacteriological examination of Salmonella reactors and necropsy specimens.* Grossly normal or diseased liver, heart, pericardial sac, spleen, lung, kidney, pancreas, peri-

toneum, drained gallbladder, oviduct, misshapen ova, testes, inflamed or unabsorbed yolk sac, and other visibly pathological tissues where purulent, necrotic, or proliferative lesions are

seen (including cysts, abscesses, hypopyon, and inflamed serosal surfaces), should be directly cultured by means of a flamed wire loop or with sterile swabs.⁷ Careful aseptic technique must be utilized throughout the process of collecting tissues. Selective media should not be relied upon to deal with contaminants, since some strains may not dependably survive and grow in certain selective media. Inoculate veal infusion (VI) and brilliant green (BG) agar plates. Incubate the plates for 24 and 48 hours at 37°C. The digestive system should always be cultured separately (see paragraph (b)(7) of this section) after other anatomical organs and systems have been collected and cultured.

(2) *Bacteriologic examination of environmental and other contaminated specimens.* (i) Culture a representative sample of the specimen in tetrathionate Hajna (TTH) selective broth (TT Mueller-Kauffmann or selenite-cystine is also acceptable) as a temperature of 41–42 °C for 24 hours. Note: Do not use selenite-cystine if double strength skim milk is used as a preservative for the sample.

(ii) Inoculate an agar late of brilliant green novobiocin (BGN) and an agar plate of xylose-lysine-tergitol 4 (XLT4), incubate at 37 °C for 24 hours, and retain culture tubes at room temperature for 5–7 days for possible reculturing of the negative tubes using 0.25 ml in TTH.

(iii) Inoculate *Salmonella* suspect colonies to slants of triple sugar-iron (TSI) and lysine-iron (LI) agar and incubate at 37 °C for 24 hours. Five colony picks per plate should be taken unless 50 percent or more of the plates have *Salmonella*-like colonies. In that case, the number of picks may be reduced to three per plate.

(iv) Conduct serologic screening of cultures revealing typical reactions of *Salmonella* on TSI and LI agar slants using somatic O-group antisera agglutination or transfer for further identification to appropriate biochemical

tests such as: 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 non-motility is demonstrated by inoculating a suitable semisolid medium. The Analytical Profile Index API 20E⁸ for Enterobacteriaceae (APE) system may also be used for further identification if desired.

(v) Serotype all *Salmonella* group D cultures at the National Veterinary Services Laboratory.

(3) The following organs should be aseptically collected for culture:

(i) Heart (apex, pericardial sac, and contents if present.);

(ii) Liver (portions exhibiting lesions or, in grossly normal organs, the drained gallbladder and adjacent liver tissues.);

(iii) Ovary-Testes (entire inactive ovary or testes, but if ovary is active, use own judgment and include any atypical ova.);

(iv) Oviduct (if active, include any debris and dehydrated ova.);

(v) Pancreas and kidneys; and

(vi) Spleen.

(4) Aseptically collect 10–15 g or whatever lesser amount is available of each organ or site listed in paragraph (b)(3) of this section from each reactor, and grind or blend them completely in 10 times their volume of VI broth. Organs may be processed individually or in combinations where appropriate. Suspensions should be transferred in 10-ml aliquots to 100-ml of both VI and tetrathionate brilliant green (TBG) broth and incubated at 37°C for 24 hours. Plate the VI broth on VI and BG agar and plate the TBG broth on BG agar and incubate at 37°C. Examine 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

⁷Culture media preparation and biochemical identification charts can be obtained from *Culture Methods for the Detection of Animal Salmonellosis and Arizonosis*, Committee on Salmonellosis and Arizonosis, AAFLD, 1976 Iowa State University Press, Ames, IA 50010.

⁸We use trade names solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.

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 non-motility is demonstrated by inoculating a suitable semisolid medium.⁹ 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.¹⁰

(10) All salmonella cultures should be serologically typed.

(Approved by the Office of Management and Budget under control number 0579-0007)

[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 47 FR 21994, May 20, 1982; 50 FR 19900, May 13, 1985; 57 FR 57342, Dec. 4, 1992; 59 FR 12801, Mar. 18, 1994; 61 FR 11521, Mar. 21, 1996; 63 FR 3, Jan. 2, 1998]

§ 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 personnel taking the samples should be left as far as practical from the poultry pens. Sanitary precautions, including

⁹Formulation 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.

¹⁰ONPG discs are available from: Baltimore Biological Laboratories, Cockeysville, MD 21030.

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 milk¹¹ 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 pretied 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 through the sides of the bag with one hand while pulling on the twine with the other hand until the connection is

¹¹Obtain 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.

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 milk¹² 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 transport them, on ice or otherwise refrigerated, to a laboratory within 48 hours of collection. The samples may

¹²Obtain 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.

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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]

§ 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.

(Approved by the Office of Management and Budget under control number 0579-0007)

[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 procedures¹³ 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 plating media (XLT4, BGN, etc.), delayed secondary enrichment procedures, and colony lift assays detailed in

¹³Laboratory 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.

paragraph (a)(5) and illustration 2 of § 147.11.

[41 FR 48726, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 57 FR 57343, Dec. 4, 1992; 59 FR 12805, Mar. 18, 1994; 59 FR 59640, Nov. 18, 1994; 61 FR 11524, 11525, Mar. 21, 1996]

§ 147.15 Laboratory procedure recommended for the bacteriological examination of mycoplasma reactors.¹⁴

(a) Turbinates, trachea, air sacs, sinuses, nasal passages, respiratory exudates, synovial fluid, eggs (including yolk, yolk sacs, membranes and allantoic fluid), should be directly sampled with sterile swabs. Aseptic techniques are very important as some organisms may not be suppressed by the antimicrobial agents used in this procedure. Tissue suspensions from large volumes are sometimes desirable from the sites listed above and occasionally from the oviduct and cloaca. Tissues should be ground or blended completely in 10 times their volume of Mycoplasma Broth Medium (MBM). (See paragraph (f) of this section.) Specimens submitted to referral laboratories in order of preference for recovery of the mycoplasma organisms are: (1) live birds, (2) refrigerated fresh tissues, (3) tissue specimens packed with dry ice.

(b) Inoculate 5-10 ml of MBM with a swab, wire loop or 0.1 ml of the tissue suspension. When evidence of growth is observed (lowered pH or turbidity of broth) transfer each broth culture as needed to maintain the original isolates. Incubate tubes at 37°C for at least 21 days before discarding as negative. When growth is first observed or if no growth occurs by the 4th or 5th day of incubation, inoculate broth culture onto a plate of Mycoplasma Agar Medium (MAM). (See paragraph (g) of this section.) Several cultures may be inoculated on one plate by using a wire loop or a cotton swab. Incubate plates 3-5 days at 37°C in a high humidity

chamber. If preferred, 5 percent CO₂ may be added or a candle jar may be used. Tiny circular and translucent colonies with elevated centers are very suggestive of mycoplasma. Indirect lighting and a low power or dissecting microscope are recommended for observation of the colonies as they are rarely more than 0.2-0.3 mm in diameter.

(c) Isolates must be serotyped.

(1) Isolates may be shipped in MBM with ice packs if shipment will be in transit less than 2-3 days. Longer shipments require freezing of the MBM with dry ice, or shipping MAM slants at room temperature. Isolates must have indications of growth before shipment is made.

(2) Isolates may be stored in MBM at -20°C for 2-3 weeks, or they may be stored at -68°C for several years.

(d) Alternate method of culture: An overlay enrichment culture for fastidious and sensitive mycoplasma, especially for *M. meleagridis* should be included.

(1) Pour 2-3 ml of MAM into a test tube and tilt the tube until a slant (approximately 45°) is obtained. Other containers are acceptable.

(2) Overlay the slant with sufficient MBM, so that the media (including inoculum) covers the agar slope.

(3) Inoculate the culture as indicated in paragraph (b) of this section.

(4) Incubate and examine the overlay as indicated in paragraph (b) of this section.

(e) Preparation of media components:¹⁵

(1) Deionized distilled water suitable for cell culture fluids should be used.

(2) All glassware should be carefully washed with a nonresidue detergent such as Alcojet and rinsed three times in tap water and twice in deionized distilled water.¹⁶

(3) Thallium acetate in a 10 percent solution is added to an approximate final concentration of 1:4000; however,

¹⁴Yoder, H. W., Jr., "Mycoplasmosis." In: Isolation and Identification of Avian Pathogens. (Stephen B. Hitchner, Chairman, Charles H. Domermuth, H. Graham Purchase, James E. Williams.) 1980, pp. 40-42, Creative Printing Company, Inc., Endwell, NY 13760.

¹⁵Trade names are used in these procedures solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.

¹⁶Alcojet is available from: Alconox, Inc., New York, NY 10003.

highly contaminated specimens may require a final concentration of 1:2000.¹⁷ Thallium acetate is added to deionized distilled water first, except as noted in paragraph (e)(4) of this section, to prevent the precipitation of proteins.

(4) Mycoplasma Broth Base, dextrose, phenol red, and cysteine hydrochloride are added to deionized distilled water first if autoclave sterilization is used.¹⁸ Thallium acetate and then the remaining components are added aseptically after cooling the autoclaved media to 45°C or less.

(5) Use sterile deionized distilled water to reconstitute penicillin.

(6) Sterile serum should be inactivated by heating at 56°C for 30 minutes. Swine serum may be used for *M. gallisepticum*, *M. synoviae*, *M. gallinarum*, and *M. meleagridis* isolation; however, horse serum is usually recommended for *M. meleagridis* isolation.

(7) Phenol red should be prepared as a 1 percent solution.

(8) NAD (beta nicotinamide adenine dinucleotide or coenzyme I) should be prepared as a 1 percent solution.¹⁹

(9) Cysteine hydrochloride, prepared as a 1 percent solution, is used to reduce the NAD for *M. synoviae* growth.

(10) A purified agar product such as Nobel (Special agar) is used in the MAM.²⁰

(11) Adjust the pH with NaOH.

(12) Sterilization may be accomplished by two methods:

(i) Filtration sterilization through a 0.20 micron filter is the recommended method. Aseptic techniques must be utilized.

(ii) Autoclave sterilization at 120°C, 15 pounds pressure (103 kPa), for 15 minutes may be used, if preferred, when following the procedure described in paragraph (e)(4) of this section.

¹⁷Thallium acetate may be obtained from Fischer Scientific Company.

¹⁸Mycoplasma Broth Base may be obtained from: (a) Product #M 33600, Gibco Diagnostics, 2801 Industrial Drive, Madison, WI 53711. (b) Product #3900-3212, Scott Laboratories, Inc., 8 Westchester Plaza, Elmsford, NY 10523.

¹⁹NAD Grade III may be obtained from: Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178.

²⁰Noble Agar may be obtained from: Difco Laboratories, Box 1058-A, Detroit, MI 48201.

(13) Phenol red, dextrose, and NAD may be omitted when culturing for *M. meleagridis* and *M. gallinarum*.

(14) When culturing for *M. meleagridis* from contaminated samples include 100 units/ml of Polymyxin B in MBM.

(f) Mycoplasma Broth Medium (Frey) is prepared as follows: To 850-880 ml of deionized distilled water;

Add:

Thallium acetate (ml)—2.5 (1:4000)

Potentially contaminated samples (ml)—5.0 (1:2000)

Mycoplasma Broth Base (g)—22.5

Aqueous penicillin (units)—500,000

Sterile serum (ml)—120 to 150.0

Phenol red plus (ml)—2.5

NAD (ml)—12.5

Cysteine hydrochloride (ml)—12.5

Dextrose (g)—1.0-1.5

Adjust pH to 7.8

Filter sterilize

(1) Broth may be stored at 4°C for at least 2 weeks or at -40°C for longer periods.

(g) Mycoplasma Agar Medium (Frey) is prepared as follows: To 850-880 ml of deionized distilled water;

Add:

Mycoplasma Broth Base (g)—22.5

Adjust pH to 7.8

Purified agar (g)—12.0

Autoclave and cool in 45°C water bath

Thallium acetate (ml)—2.0; (1:4000)

Sterile serum at 45°C (ml)—150.0

Aqueous penicillin (units)—400,000

NAD (ml)—12.5

Cysteine hydrochloride (ml)—12.5

(1) Rotate flask gently and pour about 15 ml of media into each petri dish.

(2) Stack petri dishes only 2-3 high in a 37°C incubator up to 2 hours to remove excess moisture.

(3) Wrap inverted plates in sealed bundles and store at 4°C for not more than 15 days.

(h) New component or media batches should be monitored to compensate for changes in formulation due to alterations of purity, concentration, preparation, etc. A known series of titrations from a single culture should be made on both new and old media. The media should be compared on the basis

of growth, colony size, and numbers of colonies which develop.²¹

[47 FR 21995, May 20, 1982, as amended at 57 FR 57343, Dec. 4, 1992; 59 FR 12805, Mar. 18, 1994; 61 FR 11524, Mar. 21, 1996]

§ 147.16 Procedure for the evaluation of mycoplasma reactors by in vivo bio-assay (enrichment).

This procedure has been shown to be sensitive enough to detect less than 100 mycoplasma organisms under proper conditions.²² Proper conditions are defined in this section.

(a) Obtain chickens or turkeys (test birds) which are at least 3 weeks of age and are free of *M. gallisepticum*, *M. synoviae*, and *M. meleagridis* and transport them in a manner to prevent their being contaminated by any infectious avian disease.

(1) Maintain test birds in an area that has been effectively cleaned and disinfected.

(2) The area should be isolated from other birds or animals.

(3) Personnel caring for the test birds should take the necessary precautions (see § 147.26(b)) to prevent the mechanical transfer of infectious avian diseases from other sources.

(b) Test birds to be used for inoculation with contaminated tissues should be serologically negative by the serum plate agglutination test.

(1) Inoculated test birds should be isolated from non-inoculated control birds for the length of any experiment.

(c) Aseptically obtain tracheal, turbinate, and sinus mucosa, lung and sinus exudates, cervical, thoracic, and abdominal airsac tissues (including lesions), and portions of oviduct and

²¹ "Laboratory Procedures and Medium For The Isolation Of Mycoplasma From Clinical Materials." *Laboratory Diagnosis of Mycoplasma in Food Animals*, Proceedings of Nineteenth Annual Meeting, The American Association of Veterinary Laboratory Diagnosticians, 1976, pp. 106-115, AAVLD, 6101 Mineral Point Road, Madison, WI 53705.

²² Research results are described in the following two publications: (a) Bigland, C. H. and A. J. DaMassa, "A Bio-Assay for Mycoplasma Gallisepticum." In: United States Livestock Sanitary Association Proceedings, 67th, 1963, pp. 541-549. (b) McMartin, D. A., "Mycoplasma Gallisepticum in the Respiratory Tract of the Fowl." In: The Veterinary Record, September 23, 1967, pp. 317-320.

synovial fluid from at least four suspect, donor birds. In a sterile device, blend the tissues completely in four times their volume of Mycoplasma Broth Medium (Frey), (see § 147.15(f)). Suspensions may be made from tissue pools. Inoculate test birds within 30 minutes for preparation of suspensions.

(1) Inoculate at least four test birds for each suspension pool via the abdominal air sac and infraorbital sinus, with up to ½ ml of inoculum per site.

(2) Test birds should be bled every 7 days for 35 days to identify seroconverters.

(3) At 35 days, test birds should be sacrificed and bacteriologic isolation and identification of mycoplasma attempted (see § 147.15). Note especially the sites of inoculation for typical gross or microscopic mycoplasma lesions.

(d) Donor birds are considered infected when:

(1) Test birds have serum plate antibodies for the mycoplasma for which the donor birds were tested, regardless of HI test results, *and* control birds stay serologically negative; or

(2) Mycoplasma organisms are isolated from the test birds and serotyped positive for the mycoplasma for which the donor birds were tested, *and* control birds stay serologically and culturally negative.

(e) Laboratory findings may be verified by direct cultures of material from sick birds or by inoculating seronegative birds from the suspect flock and comparing serological findings with those from the test birds.

[47 FR 21996, May 20, 1982, as amended at 57 FR 57343, Dec. 4, 1992; 59 FR 12805, Mar. 18, 1994; 61 FR 11524, Mar. 21, 1996]

§ 147.17 Laboratory procedure recommended for the bacteriological examination of cull chicks for salmonella.

The laboratory procedure described in this section is recommended for the bacteriological examination of cull chicks from egg-type and meat-type chicken flocks and waterfowl, exhibition poultry, and game bird flocks for salmonella.

(a) From 25 randomly selected 1- to 5-day-old chicks that have not been placed in a brooding house, prepare 5

organ pools, 5 yolk pools, and 5 intestinal tissue pools as follows:

(1) *Organ pool*: From each of five chicks, composite and mince 1- to 2-gram samples of heart, lung, liver, and spleen tissues and the proximal wall of the bursa of Fabricius.

(2) *Yolk pool*: From each of five chicks, composite and mince 1- to 2-gram samples of the unabsorbed yolk sac or, if the yolk sac is essentially absent, the entire yolk stalk remnant.

(3) *Intestinal pool*: From each of five chicks, composite and mince approximately 0.5 cm² sections of the crop wall and 5-mm-long sections of the duodenum, cecum, and ileocecal junction.

(b) Transfer each pool to tetrathionate selective enrichment broth (Hajna or Mueller-Kauffmann) at a ratio of 1 part tissue pool to 10 parts broth.

(c) Repeat the steps in paragraphs (a) and (b) of this section for each five-chick group until all 25 chicks have been examined, producing a total of 15 pools (5 organ, 5 yolk, and 5 intestinal).

(d) Culture the 15 tetrathionate pools as outlined for selective enrichment in illustration 2 of §147.11. Incubate the organ and yolk pools for 24 hours at 37 °C and the intestinal pools at 41.5 °C. Plate as described in illustration 2 of §147.11 and examine after both 24 and 48 hours of incubation. Confirm suspect colonies as described. Further culture all salmonella-negative tetrathionate broths by delayed secondary enrichment procedures described for environmental, organ, and intestinal samples in illustration 2 of §147.11. A colony lift assay may also be utilized as a supplement to TSI and LI agar picks of suspect colonies.

[61 FR 11525, Mar. 21, 1996]

Subpart C—Sanitation Procedures

§ 147.21 Flock sanitation.

To aid in the maintenance of healthy flocks, the following procedures should be practiced:

(a) Baby poultry should be started in a clean brooder house and maintained in constant isolation from older birds and other animals. Personnel that are in contact with older birds and other animals should take precautions, including disinfection of footwear and

change of outer clothing, to prevent the introduction of infection through droppings that may adhere to the shoes, clothing, or hands. (See §147.24(a).)

(b) Range used for growing young stock should not have been used for poultry the preceding year. Where broods of different ages must be kept on the same farm, there should be complete depopulation of brooder houses and other premises following infection of such premises by any contagious disease.

(c) Poultry houses should be screened and proofed against free-flying birds. An active rodent eradication campaign is an essential part of the general sanitation program. The area adjacent to the poultry house should be kept free from accumulated manure, rubbish, and unnecessary equipment. Dogs, cats, sheep, cattle, horses, and swine should never have access to poultry operations. Visitors should not be admitted to poultry areas, and authorized personnel should take the necessary precautions to prevent the introduction of disease.

(d) Poultry houses and equipment should be thoroughly cleaned and disinfected prior to use for a new lot of birds. (See §147.24(a).) Feed and water containers should be situated where they cannot be contaminated by droppings and should be frequently cleaned and disinfected. Dropping boards or pits should be constructed so birds do not have access to the droppings.

(e) Replacement breeders shall be housed at the proper density consistent with the type of building and locality and which will allow the litter to be maintained in a dry condition. Frequent stirring of the litter may be necessary to reduce excess moisture and prevent surface accumulation of droppings. Slat or wire floors should be constructed so as to permit free passage of droppings and to prevent the birds from coming in contact with the droppings. Nesting areas should be kept clean and, where appropriate, filled with clean nesting material.

(f) When an outbreak of disease occurs in a flock, dead or sick birds should be taken, by private carrier, to a diagnostic laboratory for complete examination. All Salmonella cultures

isolated should be typed serologically, and complete records maintained by the laboratory as to types recovered from each flock within an area. Records on isolations and serological types should be made available to Official State Agencies or other animal disease control regulatory agencies in the respective States for followup of foci of infection. Such information is necessary for the development of an effective Salmonella control program.

(g) Introduction of started or mature birds should be avoided to reduce the possible hazard of introducing infectious diseases. If birds are to be introduced, the health status of both the flock and introduced birds should be evaluated.

(h) In rearing broiler or replacement stock, a sound and adequate immunization program should be adopted. Since different geographic areas may require certain specific recommendations, the program recommended by the State experiment station or other State agencies should be followed.

(i) Feed, pelleted by heat process, should be fed to all age groups. Proper feed pelleting procedures can destroy many disease producing organisms contaminating feedstuffs.

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[36 FR 23121, Dec. 3, 1971, as amended at 41 FR 14257, Apr. 2, 1976; 41 FR 48726, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 50 FR 19900, May 13, 1985; 59 FR 12805, Mar. 18, 1994]

§ 147.22 Hatching egg sanitation.

Hatching eggs should be collected from the nests at frequent intervals and, to aid in the prevention of contamination with disease causing organisms, the following practices should be observed:

(a) Cleaned and disinfected containers should be used in collecting the eggs, and precautions taken to prevent contamination from organisms that may be present on the hands or clothing of the person making the collection.

(b) Dirty eggs should not be used for hatching purposes and should be collected in a separate container from hatching eggs. Slightly soiled eggs may

be dry cleaned by hand or motor driven buffer.

(c) The visibly clean eggs should be fumigated (see §147.25) or sanitized as soon as possible after collection. The sanitized eggs shall be stored in a cool place at temperatures which will prevent the eggs from sweating at any time.

(d) Egg handlers should thoroughly wash their hands with soap and water and change to clean outer garments prior to handling the sanitized eggs. Sanitized eggs should be immediately removed from the cleaning and grading area and preferably removed to a separate clean and sanitized room. A wall-installed fumigation cabinet (or authorized sanitizing equipment) through which eggs can be passed from the receiving and cleaning area to the sanitary packing and storage areas is a good practice.

(e) The sanitized eggs should be placed in new flats or sanitized reusable flats or racks. New or clean, fumigated, or otherwise sanitized used cases should be utilized for packing. Proper temperature and humidity in the egg cleaning, packing, and storage areas should be maintained. Eggs should be stored no longer than necessary before setting.

(f) The entire egg processing area should be cleaned and sanitized daily on a routine basis; dust, insects, feathers, and other airborne debris should be effectively controlled to prevent recontamination of sanitized eggs. Ink stamps and pads shall be maintained in a sterile condition.

(g) The egg processing building or area should be designed, located, and constructed of such materials as to assure that proper egg sanitation procedures can be carried out, and that the building itself can be easily, effectively, and routinely sanitized. The egg processing building or area should be considered part of a hatchery and the same construction details and physical and personnel sanitation requirements implemented.

[36 FR 23121, Dec. 3, 1971, as amended at 41 FR 14257, Apr. 2, 1976; 41 FR 48726, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 49 FR 19806, May 10, 1984; 57 FR 57343, Dec. 4, 1992; 63 FR 3, Jan. 2, 1998]

§ 147.23 Hatchery sanitation.

An effective program for the prevention and control of Salmonella and other infections should include the following measures:

(a) The hatchery building should be arranged so that separate rooms, with separate ventilation, are provided for each of the four operations: Egg receiving, incubation and hatching, holding of baby poultry, and disposal of offal and cleaning of trays. These rooms should be placed under isolation so that admission is granted only to specifically authorized personnel who have taken proper precautions to prevent introduction of disease.

(b) The hatchery rooms, and tables, racks, and other equipment in them should be thoroughly cleaned and disinfected frequently. All hatchery wastes and offal should be burned or otherwise properly disposed of, and the containers used to remove such materials should be cleaned and sterilized after each use.

(c) The hatching compartments of incubators, including the hatching trays, should be thoroughly cleaned and fumigated or otherwise sanitized after each hatch.

(d) Only clean eggs should be used for hatching purposes. All eggs set should be fumigated or otherwise sanitized prior to setting or as soon as possible (preferably within 12 hours) after they are placed in the incubator. They should also be fumigated or otherwise sanitized after transfer to a separate hatcher. (See §147.25.)

(e) Only new or clean, fumigated or otherwise sanitized egg cases should be used for transportation of hatching eggs. Soiled egg case fillers should be destroyed.

(f) Day-old chicks, poults, or other newly hatched poultry should be distributed in clean, new boxes. All crates and vehicles used for transporting started or adult birds should be cleaned and disinfected after each use.

[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 49 FR 19806, May 10, 1984; 57 FR 57343, Dec. 4, 1992]

§ 147.24 Cleaning and disinfecting.

The following procedures are recommended:

(a) In the poultry houses, hatchery rooms and delivery trucks:

(1) Settle dust by spraying lightly with the disinfectant to be used.

(2) Remove all litter and droppings to an isolated area where there is no opportunity for dissemination of any infectious disease organisms that may be present. Housing where poultry infected with a mycoplasmal disease were kept should remain closed for 7 days before removal of the litter.

(3) Scrub the walls, floors, and equipment with a hot soapy water solution. Rinse to remove soap.

(4) Spray with a disinfectant which is registered by the Environmental Protection Agency as germicidal, fungicidal, pseudomonocidal, and tuberculocidal, in accordance with the specifications for use, as shown on the label of such disinfectant.

(b) In the hatcher:

(1) Remove trays and all controls and fans for separate cleaning. The ceiling, walls, and floors should be thoroughly wetted with a stream of water; then scrubbed with a hard bristle brush. Rinse until there is no longer any deposit on the walls, particularly near the fan opening.

(2) Replace the cleaned fans and controls. Replace the trays, preferably still wet from cleaning, and bring the incubator to normal operating temperature.

(3) The hatcher should be fumigated (see §147.25) or otherwise sanitized prior to the transfer of the eggs.

(c) If the same machine is used for incubating and hatching, the entire machine should be cleaned after each hatch. A vacuum cleaner should be used to remove dust and down from the egg trays; then the entire machine should be vacuumed, mopped, and fumigated (see §147.25) or otherwise sanitized.

[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 49 FR 19806, May 10, 1984; 57 FR 57343, Dec. 4, 1992; 63 FR 3, Jan. 2, 1998]

§ 147.25 Fumigation.

Fumigation may be used for sanitizing eggs and hatchery equipment as an essential part of a sanitation program. APHIS disclaims any liability in the use of formaldehyde for failure on

the part of the user to adhere to the Occupational Safety and Health Administration (OSHA) standards for formaldehyde fumigation, published in the Dec. 4, 1987, FEDERAL REGISTER (52 FR 46168, Docket Nos. H-225, 225A, and 225B).

[36 FR 23121, Dec. 3, 1971, as amended at 41 FR 14257, Apr. 2, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 49 FR 19807, May 10, 1984; 54 FR 23958, June 5, 1989; 57 FR 57343, Dec. 4, 1992]

§ 147.26 Procedures for establishing isolation and maintaining sanitation and good management practices for the control of Salmonella and Mycoplasma infections.

(a) The following procedures are required for participation under the U.S. Sanitation Monitored, U.S. M. Gallisepticum Clean, U.S. M. Synoviae Clean, U.S. S. Enteritidis Monitored, and U.S. S. Enteritidis Clean classifications:

- (1) Allow no visitors except under controlled conditions which insure sanitation. Such conditions shall be approved by the Official State Agency and the Service;
- (2) Maintain breeder flocks on farms free from market birds, or follow proper isolation procedures as approved by the Official State Agency;
- (3) Eliminate other domesticated fowl from breeder farm;
- (4) Dispose of all dead birds by burning, deep burial, or by putting them into special disposal pits.
- (b) Recommended procedures:
 - (1) Avoid the introduction of Salmonella, Mycoplasma gallisepticum, or Mycoplasma synoviae infected poultry;
 - (2) Prevent indirect transmission from outside sources through contaminated equipment, footwear, clothing, vehicles, or other mechanical means;
 - (3) Provide adequate isolation of breeder flocks to avoid airborne transmission from infected flocks;
 - (4) Minimize contact of breeder flocks with free-flying birds;
 - (5) Keep the rodent population and other pests under control;
 - (6) Tailor vaccination programs to needs of farm and area;
 - (7) Clean and disinfect equipment after each use;
 - (8) Provide clean footwear and provide an adequate security program;

- (9) Clean and disinfect houses before introducing a new flock;
- (10) Use well-drained range;
- (11) Use clean, dry litter free of mold;
- (12) Keep accurate records of death losses;
- (13) Seek services of veterinary diagnostician if unaccountable mortality or signs of disease occur;
- (14) Adopt and maintain a clean-egg program.
- (15) Use only crates and vehicles that have been cleaned and disinfected in accordance with the provisions of § 147.24(a) to haul live poultry to and from the premises.

(Approved by the Office of Management and Budget under control number 0579-0007)

[36 FR 23121, Dec. 3, 1971, as amended at 40 FR 1504, Jan. 8, 1975; 41 FR 48727, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979; 47 FR 746, Jan. 7, 1982; 47 FR 21996, May 20, 1982; 48 FR 57473, Dec. 30, 1983; 61 FR 11525, Mar. 21, 1996]

§ 147.27 Procedures recommended to prevent the spread of disease by artificial insemination of turkeys.

- (a) The vehicle transporting the insemination crew should be left as far as practical from the turkey pens.
- (b) The personnel of the insemination crew should observe personal cleanliness, including the following sanitary procedures:
 - (1) Outer clothing should be changed between visits to different premises so that clean clothing is worn upon entering each premises. The used apparel should be kept separate until laundered. This also applies to gloves worn while handling turkeys;
 - (2) Boots or footwear should be cleaned and disinfected between visits to different premises;
 - (3) Disposable caps should be provided and discarded after use on each premises.
 - (c) The use of individual straw or similar technique is highly recommended. Insemination equipment which is to be reused should be cleaned and disinfected before reusing. Equipment used for the convenience of the workers should not be moved from premises to premises.
 - (d) No obviously diseased flock should be inseminated. If evidence of

active disease is noted after insemination is begun, operations should be stopped and the hatchery notified.

(e) Care should be taken during the collection of semen to prevent fecal contamination. If fecal material is present, it should be removed before the semen is collected. Likewise, care should be taken not to introduce fecal material into the oviduct of the hen.

Subpart D [Reserved]

Subpart E—Procedure for Changing National Poultry Improvement Plan

§ 147.41 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

Department. The U.S. Department of Agriculture.

Egg type chickens. Chickens bred for the primary purpose of producing eggs for human consumption.

Exhibition Poultry. Domesticated fowl which are bred for the combined purposes of meat or egg production and competitive showing.

Game birds. Domesticated fowl, such as pheasants, partridge, quail, grouse, and guineas, but not doves and pigeons.

Meat type chickens. Chickens bred for the primary purpose of producing meat.

Plan Conference. A meeting convened for the purpose of recommending changes in the provisions of the Plan.

Plan or NPIP. The National Poultry Improvement Plan.

Service. The Animal and Plant Health Inspection Service, Veterinary Services, of the Department.

State. Any State, the District of Columbia, or Puerto Rico.

Waterfowl. Domesticated fowl that normally swim, such as ducks and geese.

[36 FR 23121, Dec. 3, 1971, as amended at 38 FR 3038, Feb. 1, 1973. Redesignated at 44 FR 61586, Oct. 26, 1979; 59 FR 12805, Mar. 18, 1994]

§ 147.42 General.

Changes in this subchapter shall be made in accordance with the procedure described in this subpart: *Provided,*

That the Department reserves the right to make changes in this subchapter without observance of such procedure when such action is deemed necessary in the public interest.

§ 147.43 General Conference Committee.

(a) The General Conference Committee Chairperson and the Vice Chairperson shall be elected by the members of the General Conference Committee. A representative of the Animal and Plant Health Inspection Service will serve as Executive Secretary and will provide the necessary staff support for the General Conference Committee. The General Conference Committee shall consist of one member-at-large who is a participant in the National Poultry Improvement Plan and one member to be elected, as provided in paragraph (b) of this section, from each of the following regions:

(1) North Atlantic: Maine, New Hampshire, Vermont, Massachusetts, Rhode Island, Connecticut, New York, New Jersey, and Pennsylvania.

(2) East North Central: Ohio, Indiana, Illinois, Michigan, and Wisconsin.

(3) West North Central: Minnesota, Iowa, Missouri, North Dakota, South Dakota, Nebraska, and Kansas.

(4) South Atlantic: Delaware, District of Columbia, Maryland, Virginia, West Virginia, North Carolina, South Carolina, Georgia, Florida, and Puerto Rico.

(5) South Central: Kentucky, Tennessee, Alabama, Mississippi, Arkansas, Louisiana, Oklahoma, and Texas.

(6) Western: Montana, Idaho, Wyoming, Colorado, New Mexico, Arizona, Utah, Nevada, Washington, Oregon, California, Alaska, and Hawaii.

(b) The regional committee members and their alternates will be elected by the official delegates of the respective regions and the member-at-large will be elected by all official delegates. There shall be at least two nominees for each position, and the voting shall be by secret ballot.

(c) Three regional members shall be elected at each Plan Conference. All members shall serve for a period of 4 years, subject to the continuation of

the Committee by the Secretary of Agriculture, and may not succeed themselves: *Provided*, That an alternate member who assumed a Committee member vacancy following mid-term would be eligible for re-election to a full term. When there is a vacancy for the member-at-large position, the General Conference Committee shall make an interim appointment and the appointee shall serve until the next Plan Conference at which time an election will be held. If a vacancy occurs due to both a regional member and alternate being unable to serve, the vacant position will be filled by an election at the earliest regularly scheduled national or regional Plan Conference, where members of the affected region have assembled.

(d) The duties and functions of the General Conference Committee shall be as follows:

(1) Assist the Department in planning, organizing, and conducting the biennial National Poultry Improvement Plan Conference.

(2) Recommended whether new proposals (i.e., proposals that have not been submitted as provided in §147.44) should be considered by the delegates to the Plan Conference.

(3) During the interim between Plan Conferences, represent the cooperating States in:

(i) Advising the Department with respect to administrative procedures and interpretations of the Plan provisions as contained in 9 CFR.

(ii) Assisting the Department in evaluating comments received from interested persons concerning proposed amendments to the Plan provisions.

(iii) Recommending to the Secretary of Agriculture any changes in the provisions of the Plan as may be necessitated by unforeseen conditions when postponement until the next Plan Conference would seriously impair the operation of the program. Such recommendations shall remain in effect only until confirmed or rejected by the next Plan Conference, or until rescinded by the committee.

(4) Serve as a forum for the study of problems relating to poultry health and as the need arises, to make specific recommendations to the Secretary of Agriculture concerning ways in which

the Department may assist the industry in solving these problems.

[36 FR 23121, Dec. 3, 1971, as amended at 40 FR 1505, Jan. 8, 1975. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 45 FR 10316, Feb. 15, 1980; 47 FR 21996, May 20, 1982; 50 FR 19900, May 13, 1985; 59 FR 12805, Mar. 18, 1994; 61 FR 11525, Mar. 21, 1996]

§ 147.44 Submitting, compiling, and distributing proposed changes.

(a) Changes in this subchapter may be proposed by any participant, Official State Agency, the Department, or other interested person or industry organization.

(b) Except as provided in §147.43(d)(2), proposed changes shall be submitted in writing so as to reach the Service not later than 150 days prior to the opening date of the Plan Conference, and participants in the Plan shall submit their proposed changes through their Official State Agency.

(c) The name of the proponent shall be indicated on each proposed change when submitted. Each proposal should be accompanied by a brief supporting statement.

(d) The Service will notify all persons on the NPIP mailing lists concerning the dates and general procedure of the conference. Hatchery and dealer participants will be reminded of their privilege to submit proposed changes and to request copies of all the published proposed changes.

(e) The proposed changes, together with the names of the proponents and supporting statements, will be compiled by the Service and issued in processed form. When two or more similar changes are submitted, the Service will endeavor to unify them into one proposal acceptable to each proponent. Copies will be distributed to officials of the Official State Agencies cooperating in the NPIP. Additional copies will be made available for meeting individual requests.

[36 FR 23121, Dec. 3, 1971. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 49 FR 19807, May 10, 1984]

§ 147.45 Official delegates.

Each cooperating State shall be entitled to one official delegate for each of the programs prescribed in subparts B, C, D, and E of part 145 of this chapter

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in which it has one or more participants at the time of the Conference. The official delegates shall be elected by a representative group of participating industry members and be certified by the Official State Agency. It is recommended but not required that the official delegates be Plan participants. Each official delegate shall endeavor to obtain, prior to the Conference, the recommendations of industry members of his State with respect to each proposed change.

[41 FR 48727, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979, and amended at 45 FR 10317, Feb. 15, 1980]

§ 147.46 Committee consideration of proposed changes.

(a) The following four committees shall be established to give preliminary consideration to the proposed changes falling in their respective fields:

- (1) Egg-type chickens.
- (2) Meat-type chickens.
- (3) Turkeys.
- (4) Waterfowl, exhibition poultry, and game birds.

(b) Each official delegate shall be appointed a voting member in one of the committees specified in paragraph (a) of this section.

(c) Since several of the proposals may be interrelated, the committees shall consider them as they may relate to others, and feel free to discuss related proposals with other committees.

(d) The committees shall make recommendations to the conference as a whole concerning each proposal. The committee report shall show any proposed change in wording and the record of the vote on each proposal, and suggest an effective date for each proposal recommended for adoption. The individual committee reports shall be submitted to the chairman of the conference, who will combine them into one report showing, in numerical sequence, the committee recommendations on each proposal.

(e) The committee meetings shall be open to any interested person. Advocates for or against any proposal should feel free to appear before the ap-

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propriate committee and present their views.

[36 FR 23121, Dec. 3, 1971, as amended at 41 FR 48727, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979]

§ 147.47 Conference consideration of proposed changes.

(a) The chairman of the conference shall be a representative of the Department.

(b) At the time designated for voting on proposed changes by the official delegates, the chairman of the General Conference Committee and the four committee chairmen shall sit at the speaker's table and assist the chairman of the conference.

(c) Each committee chairman shall present the proposals which his committee approves or recommends for adoption as follows: "Mr. Chairman. The committee for Egg-type chickens recommends the adoption of Proposal No. ———, for the following reasons (stating the reasons): I move the adoption of Proposal No. ———." A second will then be called for. If the recommendation is seconded, discussion and a formal vote will follow.

(d) Each committee chairman shall present the proposals which his committee does not approve as follows: "Mr. Chairman. The Committee for Egg-type chickens does not approve Proposal No. ———." The chairman will then ask if any official delegate wishes to move for the adoption of the proposal. If moved and seconded, the proposal is subject to discussion and voted. If there is no motion for approval, or if moved but not seconded, there can be no discussion or vote.

(e) Discussion on any motion must be withheld until the motion has been properly seconded, except that the delegate making the motion is privileged, if he desires, to give reasons for his motion at the time of making it. To gain the floor for a motion or for discussion on a motion, the official delegate in the case of a motion, or anyone in case of discussion on a motion, shall rise, address the chair, give his name and State, and be recognized by the chair before proceeding further. While it is proper to accept motions only from official delegates and to limit voting only to such delegates, it is, however,

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equally proper to accept discussion from anyone interested. To conserve time, discussion should be pointed and limited to the pertinent features of the motion.

(f) Proposals that have not been submitted in accordance with §147.44 will be considered by the conference only with the unanimous consent of the General Conference Committee. Any such proposals must be referred to the appropriate committee for consideration before being presented for action by the conference.

(g) Voting will be by States, and each official delegate, as determined by §147.45, will be allowed one vote on each proposal pertaining to the program prescribed by the subpart which he represents.

(h) A roll call of States for a recorded vote will be used when requested by a delegate or at the discretion of the chairman.

(i) All motions on proposed changes shall be for adoption.

(j) Proposed changes shall be adopted by a majority vote of the official delegates present and voting.

(k) The conference shall be open to any interested person.

[36 FR 23121, Dec. 3, 1971, as amended at 41 FR 48727, Nov. 5, 1976. Redesignated at 44 FR 61586, Oct. 26, 1979]

§ 147.48 Approval of conference recommendations by the Department.

Proposals adopted by the official delegates will be recommended to the Department for incorporation into the provisions of the NPIP. The Department reserves the right to approve or disapprove the recommendations of the conference as an integral part of its sponsorship of the National Poultry Improvement Plan.