(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. [19]

[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; 65 FR 8019, Feb. 17, 2000; 74 FR 14718, Apr. 1, 2009; 76 FR 15797, Mar. 22, 2011]

§ 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. [20] 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

[19] ''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.

[20] 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.

exudates, cervical, thoracic, and abdominal airsac tissues (including lesions), and portions of oviduct and 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 sero-converters.

(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; 65 FR 8019, Feb. 17, 2000; 74 FR 14718, Apr. 1, 2009; 76 FR 15797, Mar. 22, 2011]

§147.17 Laboratory procedure recommended for the bacteriological examination of cull chicks and poults 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 and poults from turkey flocks for salmonella.

(a) For cull chicks, 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. For poults, from a sample of 10 poults that died within 10 days after hatching, prepare organ pools, yolk pools, and intestinal pools as follows:

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

(2) *Yolk pool:* From each of five chicks or two poults, 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 or two poults, composite and mince approximately 0.5 cm^2 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) For cull chicks, repeat the steps in paragraphs (a) and (b) of this section for each 5-chick group until all 25 chicks have been examined, producing a total of 15 pools (5 organ, 5 yolk, and 5 intestinal). For poults, repeat the steps in paragraphs (a) and (b) of this section for each two-poult group until all the poults in the sample have been examined.

(d) Culture the 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