

plating media (XLT4, BGN, etc.), delayed secondary enrichment procedures, and colony lift assays detailed in 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; 65 FR 8019, Feb. 17, 2000]

**§ 147.15 Laboratory procedure recommended for the bacteriological examination of mycoplasma reactors.<sup>15</sup>**

(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 CO<sub>2</sub> 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:<sup>16</sup>

(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

<sup>15</sup>Yoder, 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.

<sup>16</sup>Trade 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.

in tap water and twice in deionized distilled water.<sup>17</sup>

(3) Thallium acetate in a 10 percent solution is added to an approximate final concentration of 1:4000; however, highly contaminated specimens may require a final concentration of 1:2000.<sup>18</sup> 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.<sup>19</sup> 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.<sup>20</sup>

(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.<sup>21</sup>

(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.

(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

<sup>17</sup> Alcojet is available from: Alconox, Inc., New York, NY 10003.

<sup>18</sup> Thallium acetate may be obtained from Fischer Scientific Company.

<sup>19</sup> Mycoplasma 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.

<sup>20</sup> NAD Grade III may be obtained from: Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178.

<sup>21</sup> Noble Agar may be obtained from: Difco Laboratories, Box 1058-A, Detroit, MI 48201.

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.<sup>22</sup>

[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]

**§ 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.<sup>23</sup> 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.

<sup>22</sup> "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.

<sup>23</sup> 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.

(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 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.

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