Identification of Bacterial Pathogens of Poultry by Practicing Veterinarians

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SINCE DIAGNOSIS IS paramount to the solution of a poultry disease problem, the experienced practitioner realizes that he cannot rely entirely on a good case history, symptoms and lesions found at necropsy to establish a positive diagnosis in many instances. The alert veterinarian soon learns that various diseases may manifest themselves in a similar manner. In such cases a differential diagnosis is essential if subsequent treatment, control measures and eradications are to be effective.

Poultrymen in general, especially large poultry producers, consider it mandatory to establish an accurate diagnosis early in a disease outbreak to curb their losses. The identification of bacterial pathogens by veterinary practitioners has proved to be a valuable diagnostic aid. In years past this work was accomplished only at a few state-owned or commercial laboratories. A growing number of practitioners have found it very satisfactory to establish and operate small diagnostic laboratories of their own where postmortem and cultural work can be accomplished as needed. These men have demonstrated that such laboratory procedures are both feasible and practical. The cost of establishing a workable laboratory has not proved prohibitive; and the procedure, once a routine has been worked out, is not excessive in its demands on the time and energies of the veterinarian.

In doing his own culturing, he (1) saves the time and bother of mailing or taking specimens to a central laboratory, (2) he can examine more specimens than it would be feasible to ship, (3) he is usually able to establish a diagnosis on the basis of his findings, and (4) he has the satisfaction of using a greater portion of his training.

Equipment for the isolation and identification of bacterial pathogens need not be elaborate or expensive. A compound microscope with an oil immersion objective is a basic requirment. Growing numbers of veterinarians already own microscopes. A large pressure cooker will serve nicely as an autoclave. The kitchen range can supply the hot air oven. (Fig. C). An investment of $100 or less has proven ample to supply the additional minimal equipment and supplies (Fig B) according to the following estimate:

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1.	48 — 90 mm. petri dishes	$19.00
2.	Media, 7–¼ lb. assorted bottles	20.00
3.	Bunsen burner	1.50
4.	2 wire inoculating loops	2.50
5.	100 glass test tubes, 4 x ½ in.	3.00
6.	Test tube rack	3.00
7.	Balances	8.00
8.	Incubator (used refrigerator) plus thermostat and thermometer	22.50
9.	4 — 500 cc. Erlenmyer flasks	2.00
10.	1 — 100 cc. graduated cylinder	1.50
11.	Stains and containers (Gentian violet, Grams Iodine, acetone, alcohol, carbol fuchsin, Loeffler's methylene blue)	8.00
12.	Slides and cover slips	4.00
		$95.00

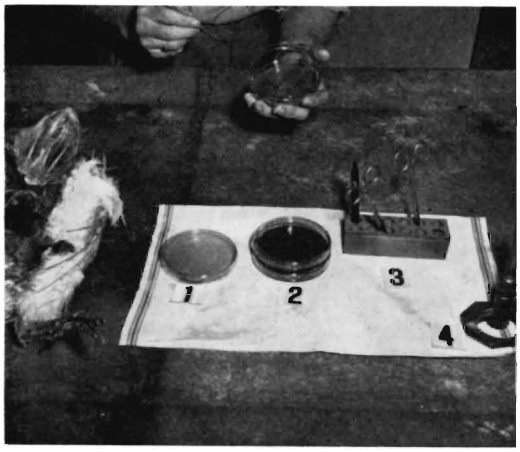

Fig. A: Tryptose agar (1), Brilliant green agar (2), Searing and incising instruments (3), Bunsen burner (4).

An incubator that will provide a uniform temperature of 37°C is indispensable for optimum growth of most bacterial pathogens. An economical and entirely satisfactory incubator can be made from a discarded electric refrigerator or icebox with well fitting doors (Fig. D). The refrigerating unit is removed, and in its place a thermostatic control unit is installed. The wafer type of thermostat commonly used in chick brooders may be purchased at most hardware stores and has proven very satisfactory. A 100 watt bulb placed in the circuit with the thermostat is the heating unit. As an added check, a thermomter is inserted through the top and left in place so that it can be read from the outside.

Media

Media for growing bacteria may be purchased in dehydrated form from various commercial sources such as Difco Laboratories, Detroit 1, Michigan. Dehydrated media is packaged in tightly sealed glass or metal containers and may simply be stored at room temperature.

For routine culturing of poultry viscera, three types of solid media, three carbohydrate broths, and one nutrient broth medium should suffice. Tryptose agar supports the growth of a wide variety of bacteria and is used when no selective action of growth is desired. Blood agar for detection of hemolysis may be prepared by adding sterile citrated ox blood in 5% concentration to the sterile melted and cooled (50°C) agar before pouring the plates.

Brilliant green agar is a selective medium of practical value in differentiating *Salmonella spp.* from *E. coli* and other lactose-fermenting bacteria. It should be borne in mind from the outset that certain gram negative rod organisms besides *Salmonella spp.* and coliforms may grow on brilliant green agar. For example, *Proteus spp.* and *Pseudomonas aeruginosa* will grow on this medium and might be mistaken for *Salmonella.* However, colonies of *Salmonella spp.* present a characteristic appearance differing from the lactose or sucrose fermenting organisms which may grow on the medium. After incubation for about 18 hours, the Salmonella colonies appear as slightly pink-white opaque growths surrounded by a brilliant red medium. The colonies of lactose or sucrose fermenting organisms, if any are present, form a yellow-green colony surrounded by an intense yellow-green zone.

SS (Salmonella and Shigella) agar is another medium with about the same range of selectivity as brilliant green agar. On SS agar, however, coliform organisms (*Aerobacter spp.* and *E. coli*) form red colonies, while *Salmonella spp., Proteus spp.* and *Pseudomonas aeruginosa* colonies are colorless.

Agar plate media should be prepared as needed. Clean and dry petri dishes may be sterilized by placing them in the oven

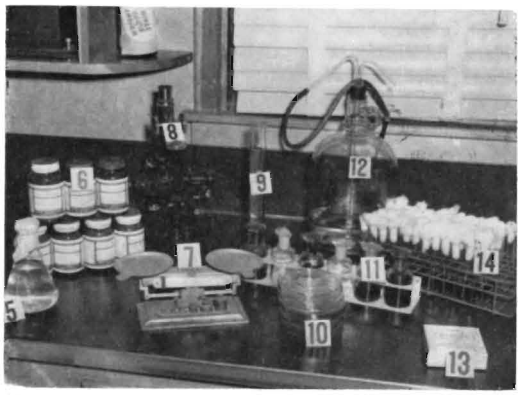

Fig. B: Erlenmyer Flasks (5), Dehydrated Media (6), Balances (7), Microscope (8), Graduate cylinder (9), Petri dishes (10), Stains and containers (11), Distilled water (12), Slides (13), Test tubes and rack (14).

at 370°F. for at least one hour. In the meantime, the necessary amount of dehydrated media can be weighed and placed in a flask containing the proper volume of distilled water. Solution of the material is accelerated by stirring and heating. The flask is stoppered with a cotton or gauze plug, capped with a paper wrap, and sterilized in the pressure cooker for 20 minutes at 15 lbs. of pressure. When the plates and flask of agar have cooled just enough that they may be comfortably held in the hand, the plates are poured. Strict aseptic techniques must be followed to avoid air-borne or contact contamination of the plates at this time. A dust-free room without air currents should be used. Sponge the surface of a bench or table and place the petri dishes on this surface. Light the bunsen burner, remove the hood and plug from the flask, flame the pouring lip and cautiously pour the desired quantity of the melted agar into each plate, taking care to raise the lid only enough to introduce the fluid. Two hundred and fifty ml. of melted agar should pour 20 to 25 plates of satisfactory thickness for a cost of less than one cent per plate. After the agar has solidified, the plates should be inverted and refrigerated until used.

SIM medium is a solid agar medium that should be reconstituted and dispensed in test tubes prior to autoclaving. It is intended for detection of indol formation, H_2S production and motility all in a single tube.

Sterile liquid media may be prepared by reconstituting a weighed portion of the dehydrated preparation and tubing it in about 3 ml. amounts prior to autoclaving. The tubes are stoppered with cotton, placed in racks, and sterilized for 15 minutes at 15 lbs. of pressure. Sterile tubed media should be refrigerated until used. It is emphasized that the directions of the manufacturer should be followed explicitly.

Selection of Cases

Sound judgment should be used in selection of cases for culturing, and it cannot be overemphasized that birds submitted for a necropsy examination should be accompanied with a complete case history. The correct diagnosis may be established in many instances without resorting to culturing as in obvious cases of external or internal parasitisms. Specimens showing characteristic lesions of tuberculosis or fowl leukosis need not be cultured, but an acid-fast stain should be made to establish the absence or presence of acid-fast organisms Microscopic examination of intestinal scrapings should be carried out as a matter of routine. Be

Fig. C: Pressure cooker (15), oven used for sterilizing Petri dishes (16).

sure to determine whether sulfonamids or antibiotics have been administered as they may inhibit growth on cultures even when bacterial pathogens are present in the tissues. Two to three birds should ordinarily be necropsied to see if a similar set of lesions prevail in each in order to evaluate subsequent bacteriological findings. The reader is invited to read an excellent article by C. L. Nelson, D.V.M., of Jewell, Iowa on *Diagnosing of Poultry Diseases* which appeared in Vol. XIII, No. 3, 1951 of this publication.

Culturing Sites

Heart's blood, liver, spleen and kidney are good sites for culturing in case of systemic diseases. Lung and intestinal cultures may yield *S. pullorum* in affected young chicks. Gall bladder and intestinal lumen may occasionally yield *Salmonella spp.* when other sites are negative. Three to five cultures can be streaked on a single plate. Localized lesions in any part of the body may be cultured.

Culturing equipment including a Bunsen burner, a wire inoculating loop, a small scalpel, a small spatula and the sterile agar plates should be at hand (Fig. A).

First day

1. Heat the spatula until it glows red and use it to sear the surface of the organ to eliminate surface contamination.
2. Heat the scalpel and incise the capsule in the seared area.
3. Flame the inoculating loop, thrust it through the incised opening and rotate it to gather a small portion of material from the interior of the organ.
4. Raise the lid of the petri dish just enough to admit the wire loop which should be brushed gently back and forth in parallel lines over the surface of the agar.
5. Close the lid, sterilize the loop and identify the plate with a wax pencil.
6. *Invert* the inoculated plates and place them in the 37°C. incubator.

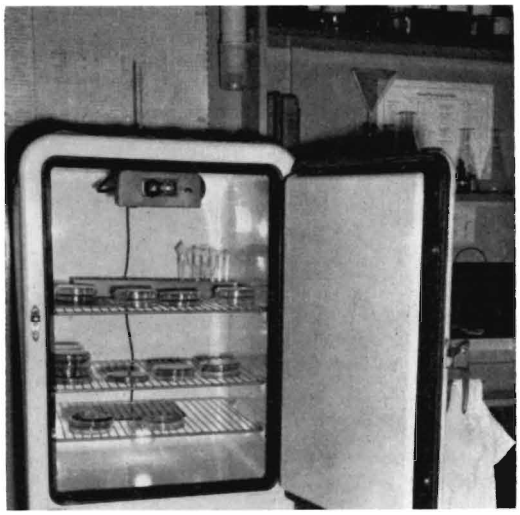

Fig. D: Incubator (converted refrigerator).

Second day

7. Plates may be checked for bacterial colonies after 18 to 24 hours of incubation. The nature of the colony growth can suggest much to the experienced observer. This is particularly true in evaluating growth on the selective media where the range is narrowed and where color changes in the colonies and/ or medium may enable the observer to make a shrewd guess at the identity of the organism. Further evidence may be obtained in case of suspected Salmonella colonies by a rapid agglutination test prepared by mixing a loopful of colony growth and a drop of commercially available polyvalent anti-salmonella serum. If a positive agglutination resulted, one could be reasonably sure at that early date (within 24 hours after the plates were streaked) that the organism was one of the Salmonella group. With some experience one can identify *E. rhusiopathiae* growing on tryptose or blood agar on the basis of colony morphology and a gram stain of the cells.
8. Colonies requiring further identification should be transferred to nutrient broth. A gram stain should be made.

Fig. E Some useful selective and differential media.

Bacteria	Brilliant[1] Green Agar	SS Agar[1]	Polyvalent anti-Salmonella serum	Lac.	Suc.	Dex.	Indol.	H_2S	Motility
E. coli	yellow-green agar	Red Colony	−	AG	−[3]	AG	+	−[3]	±
Proteus spp	red agar	Discrete colonies (No swarming) ± black centers	−	−	±	AG	−	+	+
Pseudomonas aernginosa	red agar	viscid or tough colonies	−	−	−	−	−	−	+
Salmonella	red agar	Discrete buttery colony ± black centers	+	−	−	AG[2]	−	±	±
Pasteurella	no growth	no growth	−	=	A	A	+	−	−

Key: A = acid, G = gas, − = negative, ± = variable, + = positive, AG = Acid & Gas.
[1] It is suggested that either Brilliant Green agar or SS agar (not both) be used.
[2] *Sal. gallinarum* produces only acid in dextrose.
[3] Some strains positive.

9. The broth culture (8), after several hours' incubation, should be used to inoculate lactose, sucrose and dextrose sugar broths and the SIM agar medium (stab a loopful of inoculum to the bottom of the tube).

Third day

10. Check the incubated sugar broth series for evidence of growth (turbdity). Reinoculate tubes showing no growth. Acid production and consequent lowering of pH in a tube of sugar broth can be detected by a color change in the medium. Gas production may be detected by simply observing small bubbles of gas rising in the broth or by including a small inverted glass vial in each tube to trap any gas formed beneath it. H_2S production in SIM medium may be detected by a brown or black discoloration of the agar. A sharply defined growth confined to the stab line is considered due to a non-motile organism. A diffuse growth radiating from the stab line indicates motility (a more reliable motility check can be made by microscopic examination of a hanging-drop preparation of a young broth culture.) A positive indol test is indicated by the formation of a pink to cherry red ring on addition of a few drops each of choloroform and Kovac's reagent.
11. The identity of the organism may be apparent as the result of the sugar reactions. For convenience, a chart (see Fig. E) showing the reactions of certain bacteria may be prepared and posted in the laboratory for quick reference.

Veterinarians in general practice also use the above techniques and procedure in identification of bacterial pathogens from other animal viscera and in the diagnosis of mastitis.

Summary

Suggestions for 1) minimal supplies and equipment, 2) techniques and procedures, and 3) interpretation of findings have been offered in connection with the establishment and use of a laboratory by the practicing veterinarian for the identification of bacterial pathogens of poultry.