Historic, archived document

Do not assume content reflects current scientific knowledge, policies, or practices.

A 41.9
R 315 Ar
No. 36

**recommended
procedure
for the isolation of**

SALMONELLA **ORGANISMS**

**from animal feeds
and meat byproducts**

**ARS 91-36
October 1962**

**Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE**

This brochure has been prepared by the Animal Disease Eradication Division in cooperation with a committee of nationally recognized authorities in the area of Salmonella diagnosis. The techniques outlined in the brochure are recommended as a guide in examining animal feeds and meat byproducts for Salmonella organisms.

Grateful acknowledgment is made to the members of this committee: *Cochairmen* - E. M. Ellis, Discipline Leader, and E. E. Wedman, Chief, Diagnostic Services, National Animal Disease Laboratory, Ames, Iowa; *Other Committee members* - Mildred Galton, Chief, Veterinary Public Health, Department of Health, Education, and Welfare, Atlanta, Ga.; L. C. Grumbles, In Charge, Diagnostic Laboratories, Texas A. and M. College, College Station; Wilson Henderson, Alternate -In -Charge, Department of Veterinary Science, Purdue University, Lafayette, Ind.; C. F. Niven, Jr., Associate Director, American Institute Foundation, University of Chicago, Chicago, Ill.; B. S. Pomeroy, Chairman, Division of Bacteriology and Public Health, College of Veterinary Medicine, St. Paul, Minn.; and H. Van Roekel, Research Professor, Department of Veterinary Science, University of Massachusetts, Amherst, Mass.

CONTENTS

	Page
Test Procedure	1
Serologic Identification	3
Biochemical Confirmation	3
Appendix	5

RECOMMENDED PROCEDURE FOR THE ISOLATION OF SALMONELLA ORGANISMS FROM ANIMAL FEEDS AND MEAT BYPRODUCTS

Identification of Salmonella organisms involves biochemic study and serologic typing. Complete biochemic study is time consuming, whereas serologic typing is impractical except in laboratories with a large volume of specimens. Therefore, screening procedures should be applied.

According to Kauffmann,¹ "In the Salmonella group are included various types of gram-negative bacteria whose serologic homogeneity is expressed by the Kauffmann-White schema and which are characterized by definite cultural, biochemic and serologic characteristics."

A more specific definition is made by Wilson and Miles² as follows: Gram-negative, nonsporing rods, usually 1 to 3 microns long and 0.5 to 0.7 microns broad. Primarily intestinal parasites, widely distributed in man, mammals, and birds. With few exceptions all species are motile, by peritrichate flagella; easily cultivatable on ordinary media; aerobic and facultatively anaerobic. Apart from a few serotypes that form acid only, acid and gas are produced from glucose, mannitol, dulcitol, and sorbitol. Lactose, sucrose, adonitol, and except rarely, salicin are not fermented. Indol and acetyl-methylcarbinol are not formed. Gelatin is seldom liquefied. H₂S production is usual. The serotypes are closely related to each other by somatic and flagellar antigens; most serotypes are diphasic. Pathogenic for man, animals, birds, or all three, they give rise to food poisoning, enteritis, or typhoidlike infections.

The following procedure is recommended for the isolation of Salmonella spp. from feeds and animal byproducts:

TEST PROCEDURE

1. Weigh out the sample of feed or animal byproduct (30 grams). Culture of more than one sample will increase the possibility of making an isolation from materials which contain few organisms.

¹Kauffmann, Fritz. Enterobacteriaceae. Collected studies on Salmonella . . . 2 ed. 382 pp. Munksgaard. 1954.

²Topley, Wm. W. C., and Wilson, G. S. Principles of bacteriology and immunity. Rev. by G. S. Wilson and A. A. Miles. 4th ed. in 2 vol. 1955.

Samples of feed or animal byproducts should be received in amounts of at least 100 grams. These may be packaged in paper bags, plastic bags, or ice cream containers. Such containers should be new and clean, and similar to the containers intended for the packaging of human food. (See appendix, par. 1.)

Samples should be weighed out using methods that assure an uncontaminated specimen.

2. Place 30-gram amount in a jar containing 100 ml. of tetrathionate broth (See appendix, par. 2), and add 6 ml. of a 10-percent solution of Tergitol No. 7. About the cheapest and most convenient container for tetrathionate or other enrichment are the standard one-pint wide-mouth refrigerator jars. Each has a metal cap with rubber seal, and the jars with the caps may be autoclaved repeatedly. Allow to stand 1 hour at room temperature and then shake vigorously.

3. Incubate 18 to 24 hours at 37° C.

4. Plate to brilliant green agar containing sodium sulfadiazine, using a 5- to 6-millimeter loop. (See appendix, par. 3)

Streak plate in such a manner that isolated colonies are obtained. A 1:100 and 1:1000 dilution of tetrathionate culture may be made in dilution bottles (sterile water) and inoculated to additional brilliant green agar plates, streaking to obtain isolated colonies. Colonies on brilliant green agar usually appear transparent pink to deep fuchsia. Occasionally they will be brownish with little change in color of the medium. If the plate is heavily contaminated with coliform organisms, the Salmonella may be washed out and appear as transparent green colonies. Close observation by transmitted light will reveal the brownish appearance of the colonies.

5. Triple sugar iron agar is the recommended medium for inoculation. (See appendix, par. 4.) Suspicious colonies are picked to triple sugar iron agar, stabbing the butt and streaking the slant. White, opaque colonies against a bright green-colored medium are generally not Salmonella. A minimum of three colonies should be selected. The inoculated tubes are incubated 18 to 24 hours. Typical reactions on triple sugar iron agar slants are found in table 1, appendix. The medium indicates the ability of an organism to ferment lactose, sucrose, and dextrose with formation of acid and gas, and also its ability to produce hydrogen sulfide.

Triple sugar slants showing an acid butt (yellow) with gas, the production of hydrogen sulfide (blackening of medium), and an alkaline slant (red), should be screened with polyvalent Salmonella serum.

SEROLOGIC IDENTIFICATION

Identification by serologic means is most important; however, a word of caution is required. The agglutination test with polyvalent Salmonella antiserum will give infrequent, but occasional "false positive results." The antiserum detects not only Salmonella, but organisms with antigens related to the Salmonella antigens. Some paracolon strains will react with Salmonella antiserum; therefore, positive reactions should be confirmed by biochemical tests. Clumping in saline is an indication of rough somatic antigens and invalidates a reaction in serum. Therefore, a control saline suspension is always made.

The test is performed by emulsifying a small amount of growth in a drop of physiological saline on one end of a glass slide. Organisms should be added to form a milky suspension. A drop of saline and organisms should be used as a control.

A drop of Salmonella polyvalent serum is dropped on each bacteria-saline mixture and thoroughly mixed with an applicator stick. The slide should be rocked and observed for agglutination. Clumping occurring after 1 minute should be regarded with caution. If positive, the culture should be confirmed with biochemicals, and if the biochemical screening is not typical of Salmonella, the slant should be streaked out to insure it is a pure culture.

The laboratory should have available commercial polyvalent serum and the following grouping serums, Groups B, C¹, C², D, and E. Spicer-Edwards pooled "H" antisera may be used for additional preliminary information and may be quite helpful.

BIOCHEMICAL CONFIRMATION

Biochemical confirmation consists of inoculating the following mediums:

- Tryptone Broth (See appendix, par. 7)
- Urea Agar (See appendix, par. 9)
- Simmons' Citrate Agar (See appendix, par. 8)

MR-VP Broth (See appendix, par. 5)

KCN Broth (See appendix, par. 10)

Lysine-decarboxylase Broth (See appendix, par. 11)

The following carbohydrate broths should be included (See appendix, par. 12):

Glucose

Lactose

Sucrose

Salicin

After preliminary screening dulcitol and maltose should be inoculated for differentiation of Salmonella pullorum and S. gallinarum. Trehalose and sorbitol are used to differentiate S. cholerae-suis.

S. pullorum does not attack dulcitol. Maltose is usually not fermented. It is nonmotile.

S. gallinarum produces acid, but no gas from maltose and dulcitol.

S. cholerae-suis does not produce H₂S while the more common variety Kuzendorf, does. (See table 1.) It produces acid and gas from sorbitol, but does not attack trehalose.

The Salmonella in general ferment glucose with acid and gas produced. They do not ferment lactose, sucrose, and except rarely, salicin.

If the above carbohydrate reactions are characteristic and the organism reacts biochemically as follows, then it may be considered to be confirmed biochemically.

Indol - Negative

Citrate - Positive

Methyl red - Positive

Lysine-decarboxylation - Positive

Urease - Negative

KCN - No Growth

VP - Negative

If the organism groups properly, it may be assumed to be a Salmonella. It may be typed by submitting the culture to a Salmonella Typing Center.

APPENDIX

1. It is recommended that where several lots or bags of material are available, a sample should be taken from each and treated as separate or composite samples. The possibility of making an isolation increases with additional samples. Therefore, at least three areas of a given lot of feed or animal byproduct should be sampled. A suitable instrument for this is a core sampler used for obtaining soil samples. The instrument would need to be sterilized between samplings of different materials.

2. Tetrathionate broth is recommended as an enrichment medium and is available from Difco or Baltimore Biological Laboratories.³ Dehydrated medium should be reconstituted according to directions supplied by the manufacturer. In addition, 1 ml. of a 1:1000 solution of brilliant green should be added to each 100 ml. of prepared medium. Although essential for the culturing of all feeds and meat byproducts containing fat, Tergitol No. 7 should be added to the medium used for all samples. The medium should not be heated after the addition of the iodine solution (see instructions on the label). An alternative enrichment broth not used in this technique is selenite broth. Selenite broth is used widely and with success in isolating Salmonella organisms. Tergitol No. 7, used very successfully to break down fat particles and aid in the hydration of dry feeds not containing fat, can be purchased from: Union Carbide Chemical Co., Division of Union Carbide Corp., 2770 Leonis Blvd., Los Angeles 50, Calif.

3. Brilliant green agar is satisfactory and is recommended for plating Salmonella enrichment broth. It is advantageous to add 8 mg. sodium

³The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

sulfadiazine per 100 ml. of medium for the suppression of Pseudo-monas spp. Sodium sulfadiazine, U.S.P. is added to brilliant green agar medium, 8 mg. per 100 ml. of medium. Prepare sulfadiazine solution by adding 800 mg. of sodium sulfadiazine to 100 ml. of distilled water, pH 7. Sterilize by steam for 15 minutes at 121° C. Add 1 cc of this sterile solution to each 100 ml. of medium.

Brilliant green agar can be purchased in dehydrated form from Difco or Baltimore Biologics Laboratories, and used in Salmonella isolations where S. typhosa is not a factor. Care should be taken in preparation of the media so that a period of sterilization of 15 minutes at 121° C. is not exceeded. Longer sterilization will tend to decrease the selectivity of the medium.

4. Triple sugar iron agar may be purchased in dehydrated form from Difco or Baltimore Biologics Laboratories. No alternative media are recommended. Medium should be tubed and slanted in such a manner that a generous butt is obtained. Table 1 gives typical reactions of several organisms.

TABLE 1.--Typical reactions of several organisms
on triple sugar iron agar¹

Organism	Butt	Slant	H ₂ S
<u>Salmonella</u> spp.	YG	NC	² +
<u>Aerobacter</u> spp.	YG	Y	-
<u>Escherichia coli</u>	YG	Y	-
<u>Proteus</u> spp.	YG	Y or NC	+ or -
<u>Klebsiella pneumonia</u>	Y or YG	NC	--
<u>Pseudomonas aeruginosa</u> . . .	NC	NC	-
<u>Alcaligenes fecalis</u>	NC	NC	-
<u>Paracolon</u> spp. ³	YG	Y or 18 hr. = NC	-

¹NC = No change or alkaline reaction; Y = yellow acid formation; YG = acid and gas formation; + = positive for H₂S; - = negative.

²S. cholerae-suis does not produce H₂S and S. gallinarum may or may not. S. cholerae-suis var. Kuzendorf is H₂S positive.

³Slow lactose fermentation.

5. MR-VP Medium (Difco and Baltimore Biologics Laboratories)

MR-VP Medium is used in the differentiation of bacteria by means of the methyl red and Voges-Proskauer reactions. The VP reaction indicates the ability of an organism to form acetyl-methyl-carbinol. To conduct the test 1 gram of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ is dissolved in 40 ml. of concentrated NH_4OH and the solution added to 960 ml. of 10 percent KOH. Five ml. of this chemical, containing cupri-ammonium sulfate, are added to 5 ml. of culture. A positive reaction, indicating the presence of acetyl-methyl-carbinol, is evidenced by the appearance of a cherry-red color, in about 20 minutes. Salmonella spp. are VP negative.

6. Methyl Red Test--Five drops of indicator solution added to a 5-day broth culture will produce a red color if positive. Negative reaction is yellow. Salmonella are usually MR positive. Methyl-red indicator solution is prepared by dissolving 0.1 gram methyl red in 300 ml. of 95 percent alcohol. The solution is diluted to 500 ml. with distilled water.

7. Indol--a recommended medium is Tryptone (Difco)

Indol production is dependent upon the presence of the tryptophane group in the medium. Media having this group in sufficient amounts are suitable. The test may be performed as soon as heavy growth occurs.

The test for indol is that of Kovacs. This test is performed by adding 0.2 to 0.3 ml. of Kovacs' reagent to 5 ml. of a 24-hour culture of the organism under investigation. A dark red color in the surface layer constitutes a positive test; the original yellow color of the solution is a negative test when this reagent is used for indol detection. Kovacs' reagent is made by dissolving 5 grams of p-dimethyl-amino-benzaldehyde in 75 ml. of amyl alcohol and adding 25 ml. of concentrated hydrochloric acid.

8. Citrate Medium--(Baltimore Biologics Laboratories, Difco)

Simmons Citrate agar is inoculated to test the ability of the organism to utilize citrate as the sole source of carbon. A test is positive if growth occurs with a change in color from greenish to a distinct blue color. Salmonella are citrate positive.

9. Urea Agar--(Baltimore Biologics Laboratories, Difco)

Urea Agar indicates urease production by organisms and indicates the decomposition of urea. Cultures showing increased alkaline reaction (cerise color) are not Salmonella and may be Proteus or members of the paracolon-aerobacter group.

10. KCN--(Difco)--Potassium cyanide medium is inoculated and observed for growth. Care should be taken in making up the medium as potassium cyanide is extremely toxic.

The medium differentiates between the Salmonella, Bethesda-Ballerup Klebsiella, Escherichia, and Proteus groups. Salmonella spp. do not grow in this medium.

11. Lysine decarboxylase broth--(Difco)

Lysine decarboxylase broth contains 1-lysine for use in distinguishing the Salmonella and Arizona from the Bethesda-Ballerup group of Enterobacteriaceae. Its action depends upon the ability of certain bacteria to decarboxylate the amino acid 1-lysine. Salmonella organisms of animal origin give a positive reaction (purple color) in the medium. A negative reaction is indicated by a yellow color (acid reaction).

12. Carbohydrate broth

Brom cresol purple is recommended in the preparation of carbohydrate broth. It is best made up in standard 15 X 100 mm. tubes containing inverted Durham vials. Dispense 6 ml. of broth per tube. The Durham vials will fill with media and the air will be expelled during autoclaving.

Sugar solutions may be made up in 10 percent solution, tubed, and sterilized at 121° C. for 15 minutes. For use, aseptically dispense 0.6 ml. to each tube of brom cresol purple broth when needed. Be sure the pH indicated on the dehydrated media bottle is maintained after final sterilization.

Growth Through Agricultural Progress