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QUALITY CONTROL METHOD - 3

Salmonella & Shigella Detection

Purpose:

This quality control procedure is designed to detect product or sample contamination with *Salmonella* and *Shigella* microorganisms. Since this procedure involves the handling of potentially pathogenic cultures, the trained laboratory technician employed to utilize this procedure should be supervised by the QC Manager or a company officer.

Equipment:

Incubator
Autoclave
Chemical balance (≤ 310 g capacity; 0.1 g precision)
Orbital shaker
Assorted laboratory glassware
Sterile spatulas
Assorted pipets
weighing dishes
Wash bottles
Ring stand with Ring
Glass measuring cylinder
Thermometer
Forceps
3 mm platinum loop (or blue 1 µl sterile disposable loop-needles)
Rapid-Flo double-gauze milk filter disks
Steri-Wrap II (green)
Covered sterile disposable petri dishes
BioMerieux API® 20E identification kits

Ingredients/ Solutions:

Standard Method Agar (QC-16) http://www.alken-murray.com/QC16.pdf
Lactose broth (QC-19) http://www.alken-murray.com/QC19.pdf
Tripticase Soy Agar (QC-22) http://www.alken-murray.com/QC22.pdf
BD CHROMagar Salmonella

Tetrathionate brilliant green broth (QC-23) http://www.alken-murray.com/QC23.pdf
PCA broth (QC-24) http://www.alken-murray.com/QC24.pdf
XLD plate (QC-18) http://www.alken-murray.com/QC19.pdf
TSI tube (QC-14) http://www.alken-murray.com/QC14.pdf
LIA tube (QC-13) http://www.alken-murray.com/QC13.pdf
Oxidase test (QC-7) http://www.alken-murray.com/QC7.pdf
Glucose/Dextrose (Difco 0155-17-4)
Saline solution
Standard BOD Phosphate Buffer (with Magnesium Sulfate) or*Weber DB Phosphate Dilution Buffer with magnesium sulfate # 3127-55)* individual bottles containing 99 mL of buffer solution
http://www.weberscientific.com
Salmonella antibody preparation (Difco 2264-48-2)
Bactrol Disks Set C (Difco 1656-32-7)
contains *Shigella* sample for quality control positive
Salmonella test positive control sample (Difco 1644-35-9)

1 SALMONELLA PROCEDURE

Note: All items associated with *Salmonella* testing must be autoclaved before disposal.

1.1 Pre-Enrichment

1.1.1 Keep liquid and dry samples separate.

1.1.2 Keep formulated samples and intermediate cultures separate.

1.1.3 Keep liquid preserved product types (e.g. Enz-Odor®, CF 5100, Nu-Bind, Treat-a-Loo®) separate from non-preserved Gram negative product types (CF 1200 base, 4100, all 7100 line liquids and nitrifier series).

1.1.4 Keep liquid nitrifier types separate from non-preserved Gram-negative product types (CF 1200 base, 4100, all 7100 line liquids).

1.1.5 Use asceptic technique (use sterile spatulas, pipets and weigh dishes) throughout this procedure.

1.1.6 Do not cross contaminate retained sample.

1.1.7 Use an estimated 25 grams of sample per 225 ml sterile lactose broth (prepared according to QC-19) for CF 7000 and CF 4000. Additional samples per lot may be used for increased sensitivity.

1.1.8 Use an estimated 1 gram of all other sample dry blends per 10 mls lactose broth.

1.1.9 Use 1 ml liquid sample per 10 mls lactose broth. Do not exceed the 1 part sample to 10 parts lactose broth ratio

1.1.10 Incubate at 35° C for 24 ± 2 hours.

1.2 Selective Enrichment

1.2.1 Transfer 1.0 ml of incubated suspension to 10 ml of freshly prepared iodine-activated, tetrathionate, Brilliant Green broth (QC-23). Gently shake mixture.

1.2.2 Incubate at 42 ± 0.5° C for 6-8 hours.

1.3 CHROMagar testing

1.3.1 Use a sterile 3 mm platinum loop to streak from the enrichment media in 1.2 to CHROMagar Salmonella to obtain presumptive positive isolated colonies. Avoid using the 10 µl size yellow disposable loops since well isolated colonies are difficult to obtain with that size disposable loop.. (Blue 1µl disposable loops are an acceptable alternative)

1.3.2 Incubate at 37° C for 24 ± 2 hours.

1.3.3 Save selective enrichment broth until culture is confirmed.

1.4 Interpretation of plates for typical *Salmonella* colonies.

1.4.1 Examine CHROMagar Salmonella plate at 24 ± 2 hours. Reincubate negative plates and re-examine the following day. If no typical colonies are observed, the sample(s) are assumed to be *Salmonella* free.

1.4.1.1 *Salmonella* will have mauve colonies, while other strains will be blue, colorless or inhibited.

1.4.2 For those colonies that are positive on CHROMagar Salmonella, select a minimum of two colonies (whenever possible) for oxidase. (procedure as in **QC-7)**

1.4.2.1 If colonies are oxidase (+) they are not *Salmonella.*

1.4.2.2 If colonies are oxidase (-) proceed to inoculate TSI and LIA slants.

1.4.3 If suspect colonies are not well isolated, place a loopful of growth from the lactose enrichment flask into tetrathionate broth and incubate overnight at 35° C. Restreak onto CHROMagar Salmonella. Incubate at 37° C overnight and observe for mauve colored colonies.

1.5 Biochemical Screening (Use of TSI: QC-14 and LIA: QC-13 slants)

1.5.1 Whenever possible, select two isolated suspect colonies from each plate for a total of 6 isolates per sample. Incubate overnight at 35°C. Avoid touching the agar surface where unseen organisms suppressed by the media may be viable.

1.5.2 Inoculate TSI and LIA slants, according to procedure in QC-11, with a sterile platinum inoculating needle. Touch the center of the colony once and then stab the butt and streak the TSI slant. Without going back to the colony, double

stab the butt and streak the LIA slant. Finally without going back to the colony streak a nonselective plate, such as Tryptic Soy Agar (QC-22), Nutrient Agar (QC-35) or Standard Methods Agar (QC-16) to check for purity, and for serology and biochemical testing if needed.

1.5.3 Incubate slants at approximately 35° C with their **caps loose** (to prevent excess H_2S production) for 18-24 hours.

1.6 Biochemical Screening (TSI and LIA Interpretation)

1.6.1 Examine TSI and LIA reactions as a set. See table to interpret reactions.

1.6.2 Yellow is an acid reaction and red or purple is an alkaline reaction in both TSI and LIA.

1.6.3 Acid/acid (slant/butt) at 18-24 hours in TSI indicates either lactose, sucrose or both have been fermented. Do not read reactions early since at 8 to 12 hours non-lactose fermenters may utilize the 0.1% glucose causing both the deep and the slant to be yellow. However, with additional incubation, the glucose supply will be exhausted, and the non-lactose fermenters release of amines soon counteract the small quantities of acid present in the slant. By 18-24 hours the entire slant reverts to an alkaline (purple) pH.

1.6.4 In LIA, *Salmonella* typically produce an alkaline butt and slant (purple reaction). Consider only distinct yellow in the LIA butt as an acidic reaction. Do not eliminate cultures that produce discoloration in the butt of tube solely on this basis.

1.6.5 A brick-red (not purple) slant on LIA is not *Salmonella* and is indicative of deamination typical of *Proteus* and *Providencia* species.

1.6.6 Positive H_2S reaction results in blackening of the TSI and LIA medium. Most *Salmonella* cultures produce H_2S in LIA.

1.6.7 A black deep and alkaline slant in LIA are highly characteristic of *Salmonella*.

1.6.8 Discard all tubes not matching these sets of reactions otherwise proceed to additional identification steps.

1.7 Serological screening

1.7.1 Follow manufacturer's instructions for reconstitution, mixing, dilution and storage of antisera. Pretest antisera with known test cultures to ensure reliability of results with unknown cultures.

1.7.2 Using a wax pencil, mark off 2 sections on a clean glass microscope slide.

1.7.3 Place 1 drop of 0.85% sterile saline directly in one section of slide.

1.7.4 Place 1 drop of *Salmonella* O antiserum Polyvalent A-I, factors 1-16, 19, 22-25, 34 and Vi (Difco #2264-47-2), or equivalent in other section of slide.

1.7.5 Transfer and emulsify culture from TSI or non-selective plate in the saline and

then Polyvalent antisera drops (do not reverse the order of this process).

1.7.6 Tilt slide back and forth and watch for agglutination for up to one minute. Read against a dark background. Do not read agglutination tests with a hand lens.

1.7.7 If there is agglutination with the saline alone, discard the slide and identify culture by biochemical reactions using Biolog identification, biomerieux API strips or equivalent. Refer to " Identification of Enterobacteriaceae," Edwards and Ewing, 4th Edition for additional information.

1.7.8 If the sample culture does not agglutinate with saline and the polyvalent antiserum does, then consider the culture to be potentially a species of *Salmonella*. Positive serological results must be confirmed biochemically since cross reactions with other Enterobacteriaceae can occur.

1.7.9 If the sample culture does not agglutinate with the polyvalent antiserum, a glass test tube containing a very heavy suspension (in sterile saline) may be placed into boiling water for 30 minutes, cooled to room temperature, and the suspension retested with the antiserum. Boiling destroys the surface Vi capsular antigen that can block agglutination. If the boiled sample culture does not agglutinate with saline and the polyvalent antiserum does, then consider the culture to be potentially a species of *Salmonella*. Positive serological results must be confirmed biochemically since cross reactions with other Enterobacteriaceae can occur.

1.7.10 If the sample does not agglutinate, the sample is probably not *Salmonella*. Per the Difco™ & BBL™ Manual of Microbiological Culture Media, 98% of the strains isolated are serologically in somatic groups A to E and the Difco antisera includes a broader range of groups A-I. Negative serology results should be confirmed biochemically.

1.7.11 If polyvalent serum agglutinates, further *Salmonella* O grouping and flagellar H agglutination tests may be used to group and type the isolate.

1.8 Biochemical Confirmation and Identification

1.8.1 Biochemical identification can be completed using Biolog, bioMerieux API 20E strips, Enterotubes or equivalent kits. Follow manufacturer's instructions for each kit. For bioMerieux API 20E, enter results into their website program to confirm species identified using the API 20E test protocol.

1.9 Quality Control

1.9.1 Once a week run a positive *Salmonella typhimurium* control, using

ATCC 14028 (Difco 1644-35-9)

1.9.2 From a cryovial, inoculate one test tube containing sterile Plate Count Broth and incubate overnight at 42°C until turbidity develops.

1.9.3 Prepare a 1:10 dilution in phosphate buffer and measure the OD at 580 nm.

1.9.4 Calculate the estimated cell concentration using undiluted OD constant of 0.66

= 4.8 x 10^8 cfu/ml.

1.9.5 Dilute accordingly in sterile phosphate buffer to obtain a dilution containing 1-10 *Salmonella* cells/ml.

1.9.6 Add an estimated 25 grams of a known *Salmonella* negative dry product to 225 ml of sterile lactose broth.

1.9.7 Asceptically inoculate with 1 ml of the diluted *Salmonella* to achieve a starting concentration of 1-10 *Salmonella* per 25 grams (flask).

1.9.8 Perform plate count on SMA, QC-16 (final dilutions usually 10^6 and 10^7) to determine the actual starting *Salmonella* concentration.

1.9.9 Complete the rest of the Salmonella test.

2 SHIGELLA PROCEDURE

2.1 Enrichment

2.1.1 Inoculate pH 8 nutrient broth, prepared according to QC-20 (high pH used to limit coliform growth). Use the *Salmonella* pre-enrichment criteria with the following changes. Compositing is allowed for dry products and only 1 gram instead of 25 grams should be used for dry intermediate lot.

2.1.2 Incubate for 6-18 hours at 35° C.

2.2 Selective isolation

2.2.1 From *Shigella* nutrient broth flasks <18 hours old, streak XLD plates, QC-18, to obtain isolated colonies.

2.2.2 Incubate at approximately 35° C for 22-24 hours.

2.2.3 Save nutrient broth flasks until isolates are confirmed.

2.3 Interpretation of XLD plates for typical *Shigella* colonies.

2.3.1 Examine XLD plates at 24 ± 2 hours for translucent colonies (non of the carbohydrates are utilized). Reincubate negative plates and re-examine the following day. If no typical colonies are observed, the sample(s) are assumed to be *Shigella* free. If suspect colonies are not well isolated, place a loopful of growth from the original enrichment flask into a tube of pH 8.0 nutrient broth and incubate overnight at approximately 35° C. Restreak onto XLD agar. Incubate at approximately 35° C overnight and observe for typical colonies.

2.4 Identification

2.4.1 If typical non-lactose fermenting, but non H_2S producing colonies are present,

test a minimum of two colonies (whenever possible) for oxidase. If colonies are oxidase (+) they are not *Shigella.*

2.4.2 Observed oxidase (-) colonies microscopically (using phase-contrast) for motility. If motile, this organism is NOT *Shigella*.

2.4.3 If non-motile, the isolate should be identified biochemically using bioMerieux API 20E strips, BIOLOG, Enterotubes or equivalent identification systems. If identified as *Shigella*, the isolate should be sent to an outside laboratory to confirm serologically.

2.4.4 Once a month. Run a positive test using *Shigella flexneri* ATCC 12022 or *Shigella sonnei* 25931 from Bactrol Disks Set C (Difco 1656-32-7)

3. INTERPRETATION OF RESULTS AND RECORD KEEPING

3.1 If *Salmonella* or *Shigella* is not detected in the first test, the lot passes. Record the result in the appropriate QC database log.

3.2 If *Salmonella* or *Shigella* is detected in the first test, record the result in the QC database log but do not fail. Repeat the test using a new well mixed sample composited from five new samples from the same lot number. Test at the concentration (1 or 25 grams or mls).

3.3 If the second sample tests positive for *Salmonella* or *Shigella*, record the result in the appropriate QC database log, FAIL THE LOT and fill out a nonconformance form.

3.4 If the second sample tests negative for *Salmonella* or *Shigella*, record the result in the appropriate QC database log. Notify Claudia Velasco, who in turn will request disposition from the plant manager or from Kenneth J. Edwards, Jr. When disposition is obtained it should be recorded in the appropriate QC database log. Accompanying documentation shall be attached to the run sheets.

3.5 The description "Not Detected" should be used on Official Manufacturing Certificates, reports to USDA and AGCANADA, etc. if the results of the first or second tests are negative

SOURCES

1. Microbiology Laboratory Guidebook, as recommended by the USDA. Food and Safety Inspection Service
2. AOAC Official Methods of Analysis and BioControl Systems, Including instructions.
3. Manual of Clinical Microbiology, 6th edition, American Society of Microbiology, Washington, D.C.
4. Difco™ & BBL™ Manual of Microbiological Culture Media, copyright 2003, Becton,Dickinson and Company, Sparks, Maryland.
5. *Salmonellae* in Foods and Feeds, January 1981, US Department of Health and Human Services.
6. *Shigella* method adapted from "Standard Methods for the Examination of Water and Wastewater," copyright 2003, 21st ed., APHA, AWWA & WEF, Washington, D.C.

Salmonella and *Shigella* REACTIONS ON TSI AND LIA SLANTS (USDA guidelines)

	TSI SLANT	TSI BUTT	TSI H_2S	LIA BUTT	LIA H_2S
Salmonella	K	A	+	K	+
Salmonella	K	A	-	K	-
Salmonella	K	A	-	A	-
Salmonella	A	A	+	K	+
Shigella	K	A	-	A	-

(from "*Salmonellae* in Foods and Feeds")

	TSI REACTIONS						
LIA REACTIONS	K/A	K/Ag	K/A H2S+	K/Ag H2S+	A/A	A/Ag	A/Ag H2S+
K/K or N	*Serratia* *(S. typhi)* *(Hafnia alvei)*	*Hafnia alvei* *Klebsiella* *(Serratia)*	***(Salmonella)***		*Serratia*	*Klebsiella* *E. aerogenes/ liquifaciens* *E. coli*	
K/K or N H2S+	*(S. typhi)*	***(Salmonella)*** *(Arizona)*	*S. typhi (H2S+)* ***(Salmonella)*** *(Arizona)* *(Edwardsiella)*	***Salmonella*** *Arizona* *Edwardsiella*			*Arizona* ***(Salmonella)***
K/A	*E. coli (A-D)* ***Shigella*** *Morganella morganii* *E. agglomerans* *Y. pseudotuberculosis*	*E. agglomerans* *E. coli* *Morganella morganii* *Paratyphi A* *(S. flexneri 6)* *C. diversus*			*E. coli* *E. agglomerans* *Y. enterocolitica*	*E. sakazakii* *E. agglomerans* *C. diversus* *(E. coli)* *(Citrobacter)*	
K/A H2S+				*Citrobacter*			*Citrobacter*
R/A	*Providencia rettgeri* *Providencia* *Morganella morganii*	*Providencia* *Morganella morganii*		*Proteus mirabilis* *(Proteus vulgaris)*	*Providencia rettgeri*		*Proteus vulgaris* *(Proteus mirabilis)*

Key:
( ) = not the most common reaction
R = red, oxidation deamination of lysine
K = alkaline slant /K - alkaline butt
A = acid slant /A = acid butt
Ag = acid and gas H_2S = hydrogen sulfide production