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Isolation of *Salmonella* from Reptile and Environmental Samples

1. Purpose/Scope

The purpose of this Standard Operating Procedure (SOP) is to describe the procedures used for the isolation of *Salmonella* species other than *Salmonella* serotypes Pullorum/Gallinarum from samples including reptile, environmental, fecal, and cloacal. This protocol meets National Poultry Improvement plan guidelines for environmental testing and may be used for fecal or environmental samples from multiple species of animal.

2. Definitions – Not Applicable (N/A)

3. Safety Precautions – N/A

4. Equipment and Materials Required

4.1. Equipment/Instrumentation

- Pipetting device
- Vortex mixer
- Laboratory burner
- Forceps
- Surgical scissors or scalpels
- Inoculating loop
- Incubator, 37±2 C
- Pipetting device capable of delivering 100 µl, with sterile tips
- Laboratory balance
- Sterile craft sticks, tongue depressors, or plastic spoons
- Incubator, 42±2 C
- Inoculating needle
- Blender or stomacher
- Sterile stomacher bags or sterile Whirl-Pak bags
- Wide-mouth sterile serological pipettes, 10 ml
- Sterile swabs
- Pipetting device capable of delivering 2 ml
- Sterile cotton-plugged Pasteur pipettes

4.2. Reagents/Media/Supplies are stored and used according to SOP-BI-0057, *Procedure for Ordering and Evaluation of Bacteriological Media and Reagents*.

- Buffered Peptone Water (BPW) (National Veterinary Services Laboratories (NVSL) 10650) or equivalent
- Double strength Buffered Peptone Water (2X BPW) (NVSL 10650) or equivalent – request formulation with ½ the recipe amount of water added and labeled as 2X Buffered Peptone Water
- Hajna tetrathionate with 1.0% brilliant green (TET) (NVSL 10202) or equivalent
- Rappaport-Vassiliadis R-10 broth (RV) (NVSL 10467) or equivalent
- Iodine for tetrathionate (NVSL 30028) or equivalent
- Columbia agar with 5% sheep blood (blood agar) (NVSL 10229) or equivalent

- Brilliant green agar with novobiocin (BGN) (NVSL 10499) or equivalent
- Xylose-lysine-tergitol-4 agar (XLT-4) (NVSL 10516) or equivalent
- Modified Semisolid RV (MSRV) (NVSL 10384) or equivalent
- Nutrient agar slants (BD 20971) or equivalent
- Blood agar base slants (NVSL 10008) or equivalent
- 70% Ethanol

4.3. Control strains

- *Salmonella* Heidelberg (BI-17) or equivalent well characterized isolate of *Salmonella* species
- *E. coli* ATCC 25922 or equivalent well characterized *E. coli* isolate

5. Procedure

5.1. Control Procedure

Control cultures are stored and used according to SOP-BI-0057. A known *Salmonella* culture is used as a positive control. Typically *Salmonella* Heidelberg is used, however another well characterized isolate of *Salmonella* may be substituted at the discretion of the microbiologist. *E. coli* is used as a negative control. After the specimens are inoculated into BPW, inoculate 9 ml tubes of BPW with the positive and negative control. Controls are then processed in the same manner as the specimens, with the exception that they are not retracted to blood or tested by MALDI Biotyper. All work with controls should be performed only after all work with diagnostic samples is complete and surfaces must be decontaminated following work with controls. Controls are incubated on a separate shelf from samples but must be placed in the same incubator.

5.2. Specimen handling

- 5.2.1. All specimens should be collected and shipped overnight as soon as possible after collection, preferably to arrive at the lab within 2 days of collection. Most samples should be shipped on ice packs to maintain a temperature at or near 4°C. Dry fecal or dry environmental samples may be shipped at ambient temperature if no ice packs are available. Old, warm, or decomposed samples may still be tested at the discretion of the microbiologist, but a note must be added to the report stating the non-ideal condition of the samples.
- 5.2.2. Upon arrival, samples are stored at 4+/- 2°C until tested. Samples should be tested as soon as possible after arrival. Samples used for testing are consumed in the testing process and are not further stored. Following testing, any remaining unused sample is stored either frozen at -20+/- 2°C in the case of tissues, or refrigerated at 4+/- 2°C.

5.3. Processing of Specimens

5.3.1. Whole reptiles or reptile tissues

At the discretion of the microbiologist, a surface swab may be taken from the animal by wetting a sterile swab in a 10ml tube of BPW or using a pre-moistened sterile collection swab and vigorously swabbing the skin of the animal, paying particular attention to cloacal or discolored areas. Place the swab back into the

tube of BPW or transport media and break the stick to maintain sterility. Place swabs into a 10ml tube or appropriate volume of BPW in a sterile specimen cup or bag.

Recommended tissue samples include an organ pool consisting of non-gastrointestinal organs such as liver, lung, heart, and kidney; a second pool of reproductive tissues if the animal is an adult female; and an intestinal pool. For submission of a single sample, a small intestinal or ileocecal sample is strongly recommended. Each set of tissues is inoculated at an approximately 1:10 ratio into BPW. Large tissues may be stomached in BPW if necessary.

5.3.2. **Reptile Eggs**

Eggs should be submitted individually packaged in so as to prevent damage. Using sterile forceps, sample the exterior of the egg by collecting packing material from around the egg. Alternatively, wet a sterile swab in a 10ml tube of BPW and swab the outside surface of the egg thoroughly. Place the swab back into the tube of BPW or transport media and break the stick to maintain sterility. Following surface testing, disinfect the surface of the shell by immersing for at least 10 seconds in egg dip solution (Appendix A). Remove from the dip and allow to dry on a sterile surface or rinse excess egg dip with sterile water. Cut or break egg shell with sterile scissors and place contents into BPW at an approximately 1:10 ratio.

5.3.3. **Cloacal, fecal, or environmental swabs**

Place small swabs into a 10ml tube of BPW and vortex. Place drag swabs or shoe cover swabs into appropriately sized sterile containers and add enough BPW to cover the samples, typically 50-500 ml.

5.3.4. **Substrate, Bedding, Feed, Fecal or other environmental samples**

Place loose dry or wet materials into an appropriate sterile container. Add BPW at an approximate 1:10 ratio. For very voluminous, dry, or fluffy material, additional BPW may be added to cover the entire sample.

5.3.5. **Water**

If volume is 50ml or greater and has minimal particulates, water may be filtered through an 0.22um filter and the filter folded with sterile forceps and placed in a 50ml centrifuge tube containing sufficient BPW to cover the filter. If volume is less than 50ml or water has significant particulates, inoculate into BPW at a 1:10 ratio or inoculate into 2X BPW at a 1:1 ratio.

5.3.6. **Other sample types**

Other sample types may be tested at the discretion of a microbiologist or Veterinary Medical Officer. When possible, samples should be inoculated into BPW at either a 1:10 ratio or an amount sufficient to cover the sample.

5.4. **Day 1: Pre-Enrichment**

5.4.1. BPW is added to each sample as described in section 5.3.

5.4.2. Incubate samples at 37±2 C for 18-24 hours.

5.5. Day 2: Enrichment

- 5.5.1. Iodine must be added to TET within 1 hour of use. Add 200 µl into 10 ml tube (1:50 ratio).
- 5.5.2. Mix each pre-enriched sample prior to transferring.
- 5.5.3. Transfer 1ml of pre-enriched sample into one tube of RV and incubate at 42±2 C for 18-24 hours.
- 5.5.4. Transfer 1ml of pre-enriched sample into one tube of TET and incubate at 37±2 C for 18-24 hours.
- 5.5.5. Discard pre-enrichments.

5.6. Day 3: Plating

- 5.6.1. Vortex each enrichment prior to plating.
- 5.6.2. Using a sterile swab, inoculate one plate each of XLT-4 and BGN from each enrichment. Streak for isolation. Discard enrichments.
- 5.6.3. Incubate all plates at 37±2 C for 18-24 hours.

5.7. Day 4: Reading

- 5.7.1. From each plated enrichment, pick up to 4 suspect colonies and streak for isolation on blood agar plates. Choose colonies preferentially from XLT-4 if suspect colonies are present, and from BGN if no suspects are present on XLT-4.
- 5.7.2. Typical *Salmonella* will produce red colonies on BGN, and red-orange or yellow colonies with black centers as a result of hydrogen sulfide (H₂S) production on XLT-4. Some *Salmonella* may not produce H₂S on XLT-4.
- 5.7.3. Re-incubate plates an additional 18-24 hours for a final reading on day 5.

5.8. Day 5: Identification and Final Reading

- 5.8.1. Day 4 suspect isolates are identified by MALDI Biotyper according to SOP-BI-0115, Performing MALDI Biotyper Bacterial Identification or by biochemical identification as described in Appendix B: Biochemical Reactions.
- 5.8.2. If suspect colonies are present, pick additional suspect colonies from plates from which no *Salmonella* were isolated at 24 hours. Discard plated enrichments.

5.9. Day 6: Identification

- 5.9.1. Day 5 suspect isolates are identified by MALDI Biotyper according to SOP-BI-0115, Performing MALDI Biotyper Bacterial Identification or by biochemical identification as described in Appendix B.

5.10. Serotyping

- 5.10.1. Streak *Salmonella* isolates to nutrient agar or blood agar base slants, incubate overnight at 37±2 C and submit to the *Salmonella* serotyping laboratory for serotyping.

5.11. Alternative method: MSRV

- 5.11.1. MSRV may be used in place of RV. All other aspects of the testing remain the same.
- 5.11.2. On Day 2, instead of inoculating 1ml of BPW to RV, inoculated 200ul of BPW into the agar in the center of a MSRV plate. Caution – this media is very soft and prone to breakage. Do not turn plate upside down! Incubate plate at 42±2 C overnight.
- 5.11.3. On Day 3, if a zone of clear, pale blue or white agar has appeared in the plate, sample plate at multiple areas at the edge of the zone with a swab. Streak to BGN and XLT-4, incubate plates overnight at 37±2 C. Proceed with Days 4-6.
- 5.11.4. If there is no zone of clear, pale blue or white agar on Day 3, re-incubate the MSRV at 42±2 C overnight. On Day 4, sample either from the edge of the white zone as described in 5.11.3, or if no white zone is present, sample from the edge of the inoculation zone. Streak to BGN and XLT-4 and incubate plates overnight at 37±2 C. Proceed with instructions for Days 4-6.

5.12. Reporting

- 5.12.1. Samples from which *Salmonella* was not isolated are reported as “No Isolation Made” or “No *Salmonella* Isolated.”
- 5.12.2. Samples from which *Salmonella* was isolated are reported as “*Salmonella* Isolated.” Serotyping results are either reported under the Serotyping test, or as Bacterial Identification with the appropriate nomenclature according to the Kaufmann-White scheme.

6. Associated NVSL Quality Documents/References

- 9 CFR 147, Subpart B: §147.12 *Procedures for collection, isolation, and identification of Salmonella from environmental samples, cloacal swabs, chick box papers, and meconium samples.*
- *Bacteriological Analytical Manual, Chapter 5, Salmonella.* Version May 2014. Part C-2-A: Shell Eggs. Food and Drug Administration. Accessed online on 10/22/2014 at <http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm>
- *Antigenic Formulae of the Salmonella Serovars*, 9th ed. Patrick A.D. Grimont & François-Xavier Weill, World Health Organization accessed at <http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089>
- Difco Manual. 10th edition. Difco Laboratories, Detroit, MI. 1984.
- Edwards and Ewing’s Identification of Enterobacteriaceae. 4th edition. Ewing WH. Elsevier, NY. 1986.
- SOP-BI-0115, Performing MALDI Biotyper Bacterial Identification
- SOP-BI-0099, *Salmonella* Isolation and Identification
- SOP-BI-0057, Procedure for Ordering and Evaluation of Bacteriological Media and Reagents

7. Revision History

SOP-BI-0119.01 is a new document.

8. Appendices

Appendix A: Egg Dip Solution

To make 100ml of egg dip combine the following ingredients in a tightly sealed container and protect from light. Store at ambient temperature. Solution expiration is set by the expiration date of the iodine for tetrathionate used.

- 75ml 70% ethanol
- 12.5ml iodine for tetrathionate
- 12.5ml sterile water

Appendix B: Biochemical reactions

Typical <i>Salmonella</i> Biochemical Reactions		
Medium	Slant/Butt	Appearance
TSI	K/A + H ₂ S w/ or w/o gas	A = acid = yellow K = alkaline = red or no change H ₂ S = black
LIA	K/N = H ₂ S or K/K + H ₂ S w/ or w/o gas	A = acid = yellow K = alkaline = purple or no change H ₂ S = black [R = lysine deaminase = deep red (seen with <i>Proteus</i>)]
Urea	Negative	No change or yellow

Signature Manifest

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SOP-BI-0119 Isolation of Salmonella

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