

TSP Study – Stage I

Objective:

1. To compare the quantity of total coliforms, the quantity and incidence of *E. coli* and the incidence of *Salmonella sp.* of whole bird carcasses on the production line at the following sites: pre-TSP, post-TSP (pH adjusted), and post chill.
2. To compare the quantity of total coliforms, the quantity and incidence of *E. coli* and the incidence of *Salmonella sp.* of pH adjusted post-TSP whole bird carcass rinses that have been split into two aliquots – one refrigerated/chilled and one frozen.

Experimental Design:

Material: Sterile shaker bags
Sterile gloves
Sterile specimen containers
Sterile disposable pipets
Permanent water resistant markers
Coolers
Wet Ice
Dry Ice
pH meter or pH paper strips
Chlorine kit: Total and Free
0.2N Hydrochloric Acid (HCl)
0.1N Sodium Hydroxide (NaOH)
400ml sterile Butterfield's Phosphate Buffer solution
3M *E. coli* petrifilm
Sterile dilution blanks
Salmonella: conventional and/or rapid method supplies
Salmonella Poly O:A-I & Vi

Sample #: 100 rinses Pre-TSP
100 rinses Post-TSP (post Inside Outside Bird Wash),
Split each rinse into two groups: 100 refrigerated and 100 frozen
100 rinses Post-Chill
Total Number of Rinses = 300
Total Number of Samples = 400

Sampling dates: Collect samples over four days of production or in such a manner that a test (15 total rinses: 5 pre-TSP, 5 post-TSP, 5 post-chill) is completed on the same day and contain birds from the same farm.

Methods:

- Whole bird carcass rinses will be performed at three designated sites along the production line.
 1. pre-TSP (post IOBW)
 2. post-TSP (drip time of ~ 5 minutes on line)
 3. post-chill

Note: If possible, allow approximately five minutes of on-line drip time after TSP application before collecting Post-TSP carcasses. If a five-minute on-line drip time is not possible remove carcasses from the line and place in a sanitized stationary shackle for approximately five-minutes (after TSP application). After necessary hang time, rinse bird with 400ml Butterfield's.

- Carcasses will be collected aseptically using the approved Mega-Reg collection method of an inverted sterile shaker bag or sterile gloves (change after each bird).
CUSTOM EXEMPT ESTABLISHMENT REVIEW PROCEDURES
(Includes Official Meat Establishments
shake for one minute in a one-foot arc. Aseptically transfer rinse back into the original Butterfield's container.
- Discard all but ~100ml of the Post-TSP rinse and adjust the pH to the level of 6.8 +/- 0.2 with 0.2N HCl or 0.1N NaOH.
- Carcass rinses are to be kept chilled on wet ice or refrigerated until transported to the plant lab or outside lab for setting. Carcass rinses that are to be frozen should follow the handling directions outlined in the flow charts.
- Pre-TSP, Post-TSP refrigerated, Post-TSP Frozen, and Post-Chill Rinses:
 1. Sixty of the one hundred rinses will be set on 3M *E. coli* petrifilm.
 2. All 100 rinses will be tested for the presence/absence of *Salmonella* using a conventional method or validated rapid screening method. Positive results on conventional and rapid screening methods must be confirmed biochemically and serologically.
 3. Set a media and positive controls for each day of sampling.
- At the start and end of sample collection, chlorine levels are to be checked at the beginning, middle and end of the chiller.

Reporting Results:

Submit the raw data (not log transformed) of the whole bird carcass rinses performed at the designated sites in the Excel spreadsheet format provided (see attached file) to spretanik@chickenusa.org.

Day Test Log # Site Treatment Coliforms* E. coli* Salmonella**

*Units: cfu/ml=colony forming units per milliliter

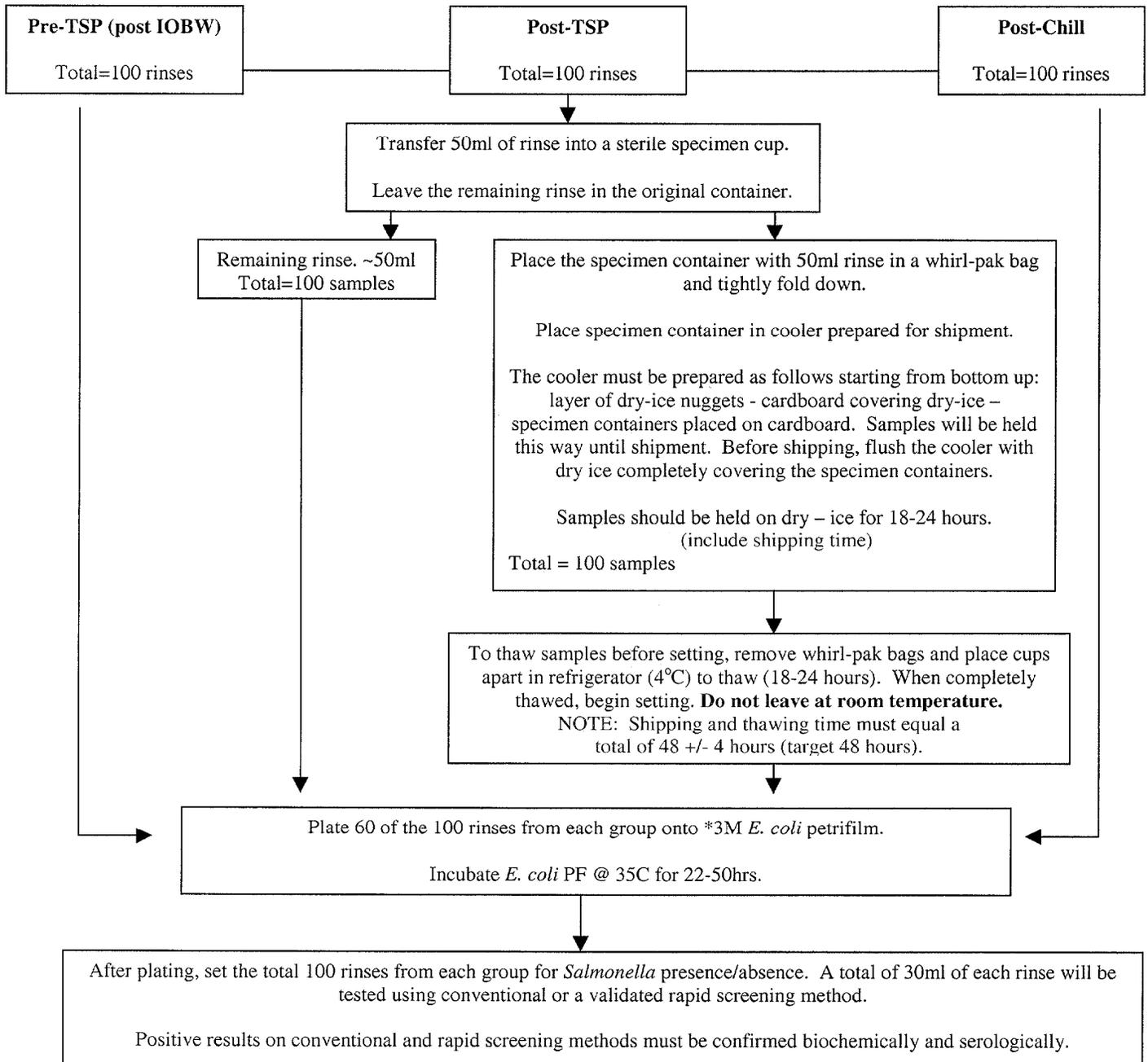
**Units: +/- per milliliter

NOTE: Data may be summarized with average cfu/ml Total Coliforms and *E. coli* in each sample type and the incidence level of *E. coli* and *Salmonella sp.* in percentage in each sample type.

TSP Study - Stage I: Comparison of Total Coliforms, *E. coli*, and *Salmonella* at the Following Production Line Sites: Pre-TSP(Post IOBW), Post-TSP, and Post-Chill.

From each designated sample site (pre-TSP, Post-TSP, and Post Chill) aseptically collect a whole carcass in a sterile shaker bag and rinse in 400ml of refrigerated Butterfield's Phosphate Buffer. Shake birds for 1 minute in a one-foot arc. Aseptically transfer rinse into the original Butterfield's container and place on wet ice or refrigerate to keep chilled until transported from plant floor.

Note: Discard all but ~100ml of Post-TSP rinse and aseptically adjust pH to 6.8 +/-0.2. Remember to keep rinses chilled at all times. Do not leave at room temperature.



*Recommended plating scheme: *E.coli* - 10⁰, 10⁻¹, 10⁻², 10⁻³

**Remember to check the total and free chlorine levels of the beginning, middle, and end of chill tank. Test at the start and end of sample collection.

***Before plating rinses, shake vigorously.

****USE ASEPTIC TECHNIQUE IN ALL PROCEDURES!

TSP Study – Stage I

Whole Bird Carcass Rinse: *Salmonella* Testing

From each designated sample site (pre-TSP, Post-TSP, and Post Chill) aseptically collect a whole carcass in a sterile shaker bag and rinse in 400ml of refrigerated Butterfield's Phosphate Buffer. Shake birds for 1 minute in a one-foot arc. Aseptically transfer rinse into the original Butterfield's container and place on wet ice or refrigerate to keep chilled until transported from plant floor.

Note: Discard all but ~100ml of Post-TSP rinse and aseptically adjust pH to 6.8 +/-0.2. Remember to keep rinses chilled at all times. Do not leave at room temperature.

Pour 30ml of rinse water into a sterile specimen container.

Add 30ml of 2X Buffered Peptone to the 30ml of rinse in the specimen container. Mix well.

Incubate 20-24 hours @ 35°C.

Follow *Salmonella* testing method of choice.

TSP Study - Stage II

Objective:

1. To compare the quantity of total coliforms, the quantity and incidence of *E. coli* and the incidence of *Salmonella sp.* of whole bird carcasses on the production line at the following sites and types: Visually Clean / No TSP, Visually Contaminated / with TSP (pH adjusted), and Visually Contaminated / Off Line Reprocessed / No TSP.
2. To compare the quantity of total coliforms, the quantity and incidence of *E. coli* and incidence of *Salmonella sp.* of pH adjusted post-TSP whole bird carcass rinses that have been split into two aliquots - one refrigerated/chilled and one frozen.

Experimental Design:

Materials:

Sterile shaker bags
Sterile gloves
Sterile specimen containers
Sterile disposable pipets
Permanent water resistant markers
Coolers
Wet Ice
Dry Ice
pH meter or pH paper strips
Chlorine kit: Total and Free
0.2N Hydrochloric Acid (HCl)
0.1N Sodium Hydroxide (NaOH)
400ml sterile Butterfield's Phosphate Buffer solution
3M *E. coli* petrifilm
Sterile dilution blanks
Salmonella: conventional and/or rapid method supplies
Salmonella Poly O:A-I & Vi
Sterile rubber bands or other marking device for carcasses on-line

Sample #:

100 Rinses Visually Clean / No TSP
100 Rinses Visually Contaminated / with TSP
Split each rinse into two groups: 100 refrigerated and 100 frozen
100 Rinses Visually Contaminated / Off Line Reprocessed / No TSP
Total Number of Rinses = 300
Total Number of Samples = 400

Sample Types:

- **"Visually Clean / No TSP"**: 100 Whole bird carcass rinses from birds determined to be contamination free at the USDA inspection station will be tagged and returned to the line. The tagged bird will be removed from the line prior to marked point A for collection site.
- **"Visually Contaminated / with TSP"**: 100 Whole bird carcass rinses from birds determined to be contaminated at the USDA inspection station will be tagged and returned to the line. The tagged bird will be removed from the line after TSP application. See line diagram marked point B for sample collection site.

the first bird wash. See line diagram site

removed from the line prior to marked point A for collection

- **"Visually Contaminated / Off Line Reprocessed / No TSP":** 100 Whole bird carcass rinses from birds determined to be contaminated will be tagged, removed from the line and reprocessed off-line. (This will be in the manner that reprocessing was accomplished before the installation of TSP). The tagged birds will be collected after reprocessing chlorine rinse step. (No TSP treatment.) See line diagram marked point C for sample collection site.

Sampling Dates: Collect samples over four days of production or in such a manner that a test (15 total rinses: 5 no contamination/no TSP, 5 contamination/with TSP, 5 contamination/off-line reprocessed/no TSP) is completed on the same day and contain birds from the same farm.

Methods:

- Whole bird carcass rinses will be performed on three sample types at designated sites along the production line.
 1. Visually Clean / No TSP collected prior to the first bird wash.
 2. Visually Contaminated / with TSP collected after TSP treatment.
 3. Visually Contaminated / Off Line Reprocessed / No TSP collected after chlorine rinse step.

Note: If possible, allow approximately five minutes of drip time on line after TSP application before collecting Post-TSP carcasses. If a five-minute on-line drip time is not possible remove carcasses from the line and place in a sanitized stationary shackle for approximately five-minutes (after TSP application). After necessary hang time, rinse bird with 400ml Butterfield's.

- Carcasses will be collected aseptically using the approved Mega-Reg collection method of an inverted sterile shaker bag or sterile gloves (change after each bird).
- 400ml of refrigerated Butterfield's Phosphate Buffer solution will be aseptically poured inside and outside the carcass and rinsed. Close bag to prevent leakage and shake for one minute in a one-foot arc. Aseptically transfer rinse back into the original Butterfield's container.
- Discard all but ~100mls of the Post-TSP rinse and adjust the pH to the level of 6.8 +/- 0.2 with 0.2N HCl or 0.1N NaOH.
- Carcass rinses are to be kept chilled on wet ice or refrigerated until transported to the plant lab or outside lab for setting. Carcass rinses that are to be frozen should follow the handling directions outlined in the flow charts.
- Visually Clean / No TSP; Visually Contaminated / with TSP; and Visually Contaminated / Off Line Reprocessed / No TSP:
 1. Sixty of the one hundred rinses will be set on 3M *E. coli* Petrifilm.
 2. All 100 rinses will be tested for the presence/absence of Salmonella using a conventional method or validated rapid screening method. Positive results on conventional and rapid screening methods must be confirmed biochemically and serologically.
 3. Set media and positive controls for each day of sampling.
- At the start and end of sample collection, chlorine levels are to be checked at the beginning, middle and end of the chiller.

Reporting Results:

Submit the raw data (not log transformed) of the whole bird carcass rinses performed at the designated sites in the Excel spreadsheet format provided (see attached file) to spretanik@chickenusa.org.

Day Test Log # Site* Treatment Coliforms *E. coli*** *Salmonella******

***Site = A:** Clean/No TSP,

B: Contaminated/with TSP

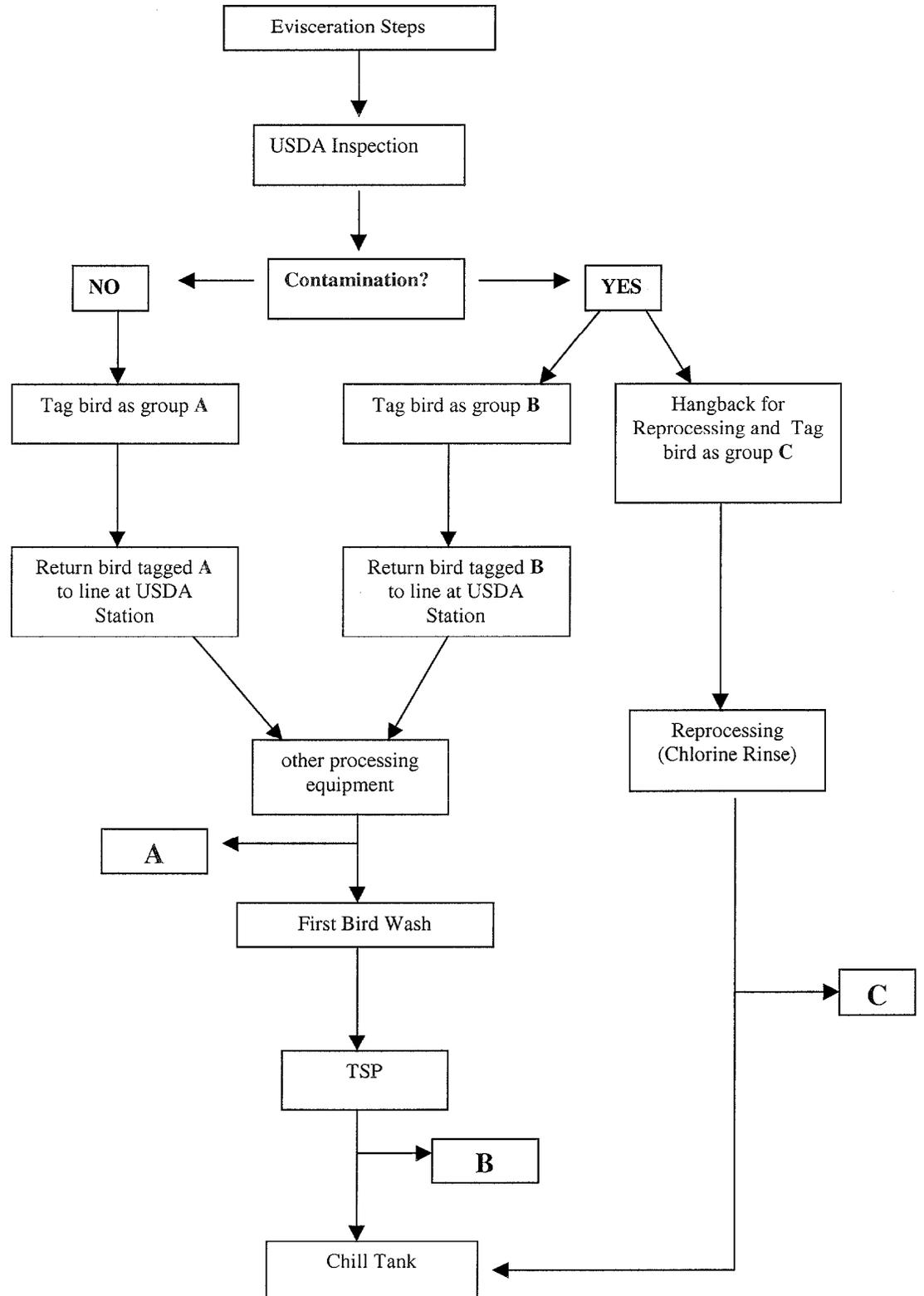
C: Contaminated/Reprocess/No TSP

****Units:** cfu/ml=colony forming units per milliliter

*****Units:** +/- per milliliter

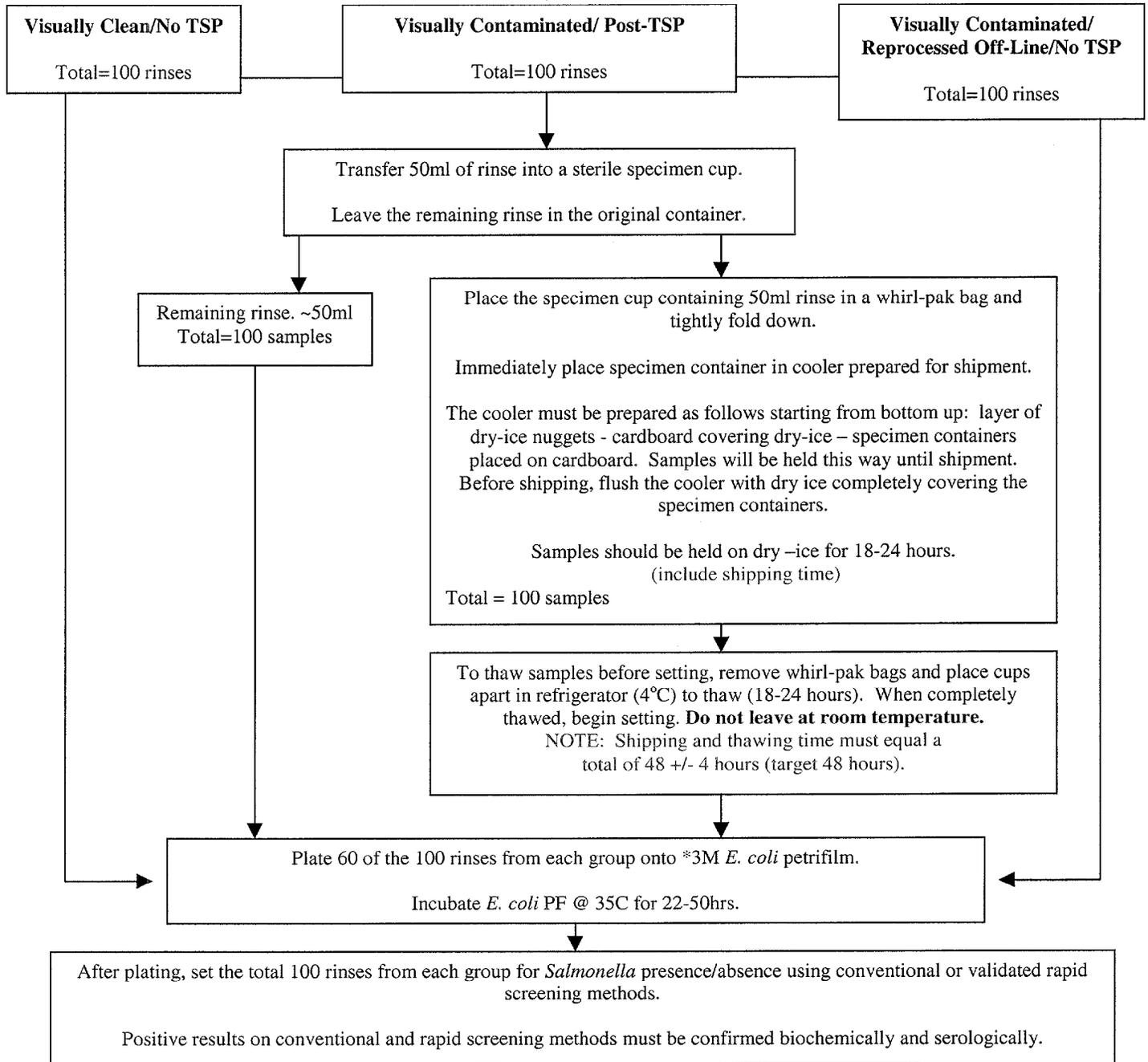
NOTE: Data may be summarized with average cfu/ml for total coliforms and *E. coli* in each sample type and the incidence level of *E. coli* and *Salmonella sp.* in percentage in each sample type.

TSP Study – Stage II EVISCERATION LINE DIAGRAM



TSP Study - Stage II: Comparison of Total Coliforms, *E. coli*, and *Salmonella* Under the Following Conditions: Visually Clean/No TSP, Visually Contaminated/Post-TSP, and Visually Contaminated/Reprocessed Off-Line/No TSP.

From each designated sample site (visually clean/no TSP, visually contaminated/with TSP, and visually contaminated/reprocessed off-line/no TSP) aseptically collect a whole carcass in a sterile shaker bag and rinse in 400ml of refrigerated sterile Butterfield's Phosphate Buffer. Shake birds for 1 minute in a one-foot arc. Aseptically transfer rinse into the original Butterfield's container and place on wet ice or refrigerate to keep chilled until transported from plant floor.
*Note: Discard all but ~100ml of TSP treated rinse and aseptically adjust pH to 6.8 +/-0.2.
Remember to keep rinses chilled at all times. Do not leave at room temperature.*



*Recommended plating scheme: *E. coli* - 10^0 , 10^{-1} , 10^{-2} , 10^{-3}

**Remember to check the total and free chlorine levels of the beginning, middle, and end of chill tank. Test at the start and end of sample collection.

***Before plating rinses, shake vigorously.

****USE ASEPTIC TECHNIQUE IN ALL PROCEDURES!

TSP Study – Stage II

Whole Bird Carcass Rinse: *Salmonella* Testing

From each designated sample site (visually clean/no TSP, visually contaminated/with TSP, and visually contaminated/reprocessed off-line/no TSP) aseptically collect a whole carcass in a sterile shaker bag and rinse in 400ml of refrigerated sterile Butterfield's Phosphate Buffer. Shake birds for 1 minute in a one-foot arc. Aseptically transfer rinse into the original Butterfield's container and place on wet ice or refrigerate to keep chilled until transported from plant floor.

*Note: Discard all but ~100ml of TSP treated rinse and aseptically adjust pH to 6.8 +/-0.2.
Remember to keep rinses chilled at all times. Do not leave at room temperature.*

Pour 30ml of rinse water into a sterile specimen container.

Add 30ml of 2X Buffered Peptone to the 30ml rinse in the specimen container. Mix well.

Incubate 20-24 hours @ 35°C.

Follow *Salmonella* testing method of choice.

TSP Study – Stage III

Objective: To determine if the Rhodia methodology affects the recovery of *Salmonella* and *E. coli* in carcasses rinsed with trisodium phosphate (TSP).

Experimental Design:

Materials:

- ◆ Sterile shaker bags
- ◆ Standard Methods agar (SMA)
- ◆ 400 ml Butterfield's
- ◆ Violet Red Bile agar w/MUG (VRB w/MUG)
- ◆ Sterile specimen cups
- ◆ EC broth w/MUG
- ◆ Sterile stomacher bags
- ◆ Eosin Methylene Blue agar (EMB)
- ◆ 25.0 ml pipets
- ◆ Gram stain supplies
- ◆ 2.0 ml pipets
- ◆ Lactose broth
- ◆ Petri dishes
- ◆ 10.0 ml Tetrathionate broth tubes (TB)
- ◆ 1% Brilliant Green Dye solution
- ◆ Potassium Iodine solution
- ◆ 0.2N Hydrochloric acid
- ◆ 10.0 ml Rappaport broth tubes (RV)
- ◆ pH strips
- ◆ Vitek GNI + cards
- ◆ 99 ml Butterfield's dilution blanks
- ◆ 9.0 ml Butterfield's dilution blanks
- ◆ 10 ml GN broth tubes
- ◆ Gene-Trak *Salmonella* kits
- ◆ Bismuth Sulfite agar plates (BS)
- ◆ Xylose Lysine Desoxycholate plates (XLD)
- ◆ Hektoen Enteric plates (HE)
- ◆ Poly "O" Antisera
- ◆ Triple Sugar Iron agar slants (TSI)
- ◆ Lysine Iron agar slants (LIA)
- ◆ Vitek GNI + Cards
- ◆ Oxidase test method

Equipment:

- ◆ 35C incubator
- ◆ 43C incubator
- ◆ 44.5C incubator
- ◆ Long wave ultra-violet light

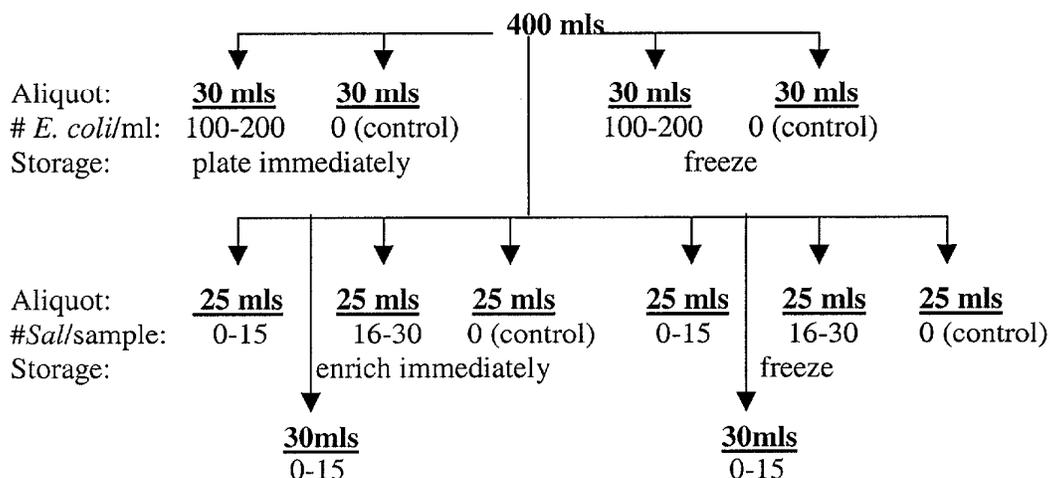
Sample Number (whole bird carcasses rinsed with 400 ml Butterfield's):

- ◆ 20 Post TSP rinses x 2 treatments x 7 inoculation schemes = 280
- ◆ 20 Post Chill rinses x 2 treatments x 5 inoculation schemes = 200

Inoculation scheme

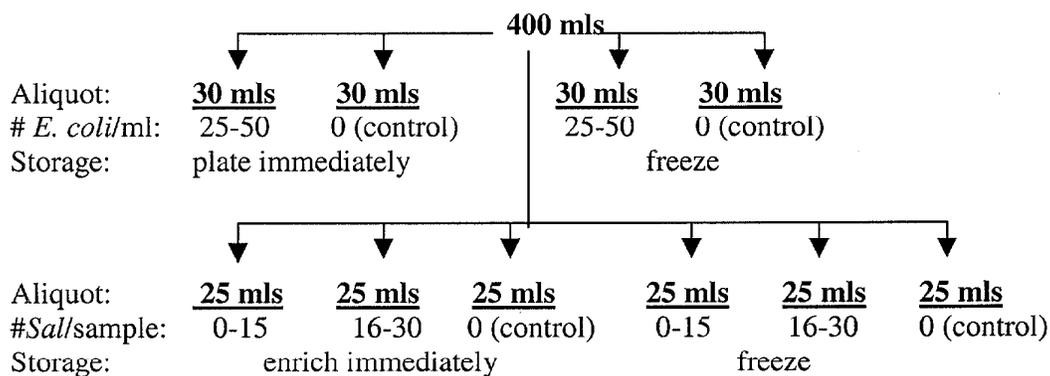
Post-TSP

Each 400 ml rinse will have the pH adjusted to 6.5-7.0 using pH paper strips and 0.2N hydrochloric acid, then aliquoted and inoculated as follows:



Post-Chill

Each 400 ml rinse will be aliquoted and inoculated as follows:



*Freeze = Pack on dry ice to freeze, store/ship overnight, thaw at refrigeration temp overnight and enrich next day (freezing and thawing time must equal 48 +/- 4 hours...target 48 hours).

Plating:

Each sample that is inoculated with *E. coli* (and controls) will be plated on SMA and VRB w/MUG.

Samples that are *Post-TSP* and are not inoculated (controls) will also be plated on Petrifilm.

Dilute the inoculated aliquot, which is the 10^0 dilution, using 1 ml to 99 ml, then subsequently 1 ml to 9 ml to a 10^{-4}

From each of these dilutions, place 1.0 ml into 2 empty sterile petri dishes (1 labeled "SMA" and the dilution factor and 1 labeled "VRB w/MUG" and the dilution factor. Plate any necessary *E. coli* Petrifilm at this time, also. One inoculated aliquot should yield 5 SMA plates and 4 VRB w/MUG when all dilutions are plated.

Pour approximately 15-20 ml of prepared SMA agar (must be cooled to 44-46C) into plates labeled "SMA" and swirl to mix. Allow agar to set up and place inverted in the 35C incubator for 48 hours.

Count plates. Countable range of 25-250.

Pour approximately 10 ml of VRB w/MUG (cooled to 47-48) into plates labeled "VRB w/MUG". Allow agar to set up, then overlay plates with approximately 10 ml of VRB w/MUG. Allow the overlay to set up and place plates inverted in the 35C incubator 18-24 hours.

Count purple/red colonies surrounded by a bile precipitate. Choose 5 typical colonies from the reportable dilution and inoculate each one into an EC w/MUG tube. Should more than one dilution be in the countable range, choose the lowest dilution to confirm. Incubate tubes 24-48 hours in the 44.5C incubator.

VRB w/MUG (cont'd)

If tubes are positive (gas, turbidity + fluorescence) after 24 hours, continue with confirmation. If any are negative, reincubate them for an additional 24 hours and re-read.



Positive samples will be confirmed by streaking all positive tubes from one sample onto a divided EMB agar plate. Incubate plates 24-48 hours at 35C. Twenty-five to thirty random positive EMB plates will also be confirmed using a biochemical identification system (Vitek GNI +).



Cells/ml can be obtained by multiplying the original colony count by the percentage of EC w/MUG tubes that come up positive.

One 25 ml aliquot inoculated with 0-15 Salmonella, one with 16-30 and one control from each rinse will be set using the Gene-Trak rapid method.

One 30 ml aliquot inoculated with 0-15 Salmonella from each Post-TSP rinse will be set using an adapted Mega-Reg protocol.

Gene-Trak Protocol

Combine 25 mls rinsate and 225 mls sterile Lactose Broth in a sterile stomacher bag. Mix well and adjust pH to 6.8 +/-2 with 1N sodium hydroxide or 1N hydrochloric acid. At this time, a media control and a positive control should also be set to check media in each step.



Close the bag by rolling down the top of the bag (leaving some air in the bag) and incubate at 35C for 22-26 hours.



Gently shake the incubated sample. Transfer 1.0 ml sample to 10 ml Tetrathionate Broth (TB)(with 0.2 ml potassium iodine and 0.1 ml of 1% brilliant green dye solution) and 1.0 ml sample to 10 ml Rappaport-Vassiliadis (RV) broth. Incubate TB 22-26 hours at 43C and RV 22-26 hours at 35C.



After incubation, vortex incubated selective enrichments (TB/RV) and transfer 1ml of each into corresponding 10 ml GN Broth tubes.

After performing transfer to GN, re-vortex tubes and streak 3mm loopfuls to XLD, HE, and BS plates.

Incubate all plates 22-26 hours at 35C.

Incubate GN broth tubes at 35C for 6 hours, then run Gene-Trak according to kit insert. If any samples are Gene-Trak positive, streak GN broths to XLD, HE, and BS.



After incubation, typical colonies are as follows:

XLD – Pink or clear colonies w/ or w/o black centers

HE – Blue/green or clear colonies w/ or w/o black centers

BS- Brown, gray or black colonies

Pick 2 or more typical colonies from each positive plate to TSI/LIA tubes. Incubate tubes 22-26 hours at 35C.

If BS plates do not have typical growth after the initial incubation period, they must be re-incubated an additional 24 hours and re-examined for typical growth before they can be considered negative.



After incubation, typical TSI/LIA tubes are as follows:

TSI-alkaline (red) slant with an acid (yellow) butt w/ or w/o
H₂S blackening

LIA-alkaline (purple) slant w/ or w/o H₂S blackening



Poly “O” the TSI from all positive sets of TSI/LIA. If both sets of tubes from a plate are positive or if all sets of tubes for a sample are positive, choose one of the Poly “O” positive typical TSI and streak this tube for isolation to a MacConkey (MAC) agar plates. Incubate MAC plates 20-24 hours at 35C and inoculate biochemical identification system (Vitek GNI +) according to kit insert.