Poultry Enterprise Environmental Sample Microbiological Testing for Salmonella spp.

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Abstract

The microbiological testing method for Salmonella spp. as used for testing primary envrionmental samples collected from poultry environments

The method is described as detailed in accordance with the Australian Standard 5013.10-2009 Horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD).

This method is a modification of the ISO 6579:2002 Horizontal method for the detection of *Salmonella* spp.. The difference in methodology is due to the use of a different control isolate.

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Materials

- Buffered Peptone Water CM0509 by Oxoid Microbiology Products - Thermo Fischer
- Modified Semi-solid Rappaport Vassiliadis medium base CM0910 by Oxoid Microbiology Products - Thermo Fischer
- Xylose Lysine Desoxycholate Agar CM0469 by Oxoid Microbiology Products - Thermo Fischer
- Cystine Lactose Electrolyte Deficient Agar CM0301 by Oxoid Microbiology Products - Thermo Fischer
- Brilliant Green Agar (Kauffmann Medium) CM0263 by Oxoid Microbiology Products - Thermo Fischer
- Triple Sugar Iron Agar CM0277 by Oxoid Microbiology Products - Thermo Fischer

Lysine Iron Agar CM0381 by Oxoid Microbiology Products - Thermo Fischer

o-nitrophenyl-p-D-galactosidase (ONPG) broth 2085 by Contributed by users

Tryptone Soya Broth CM0129 by Oxoid Microbiology Products - Thermo Fischer

Ammonium Salt Sugar Mannitol 2344 by Contributed by users

SyberSafe DNA Gel Stain S33101 by Invitrogen - Thermo Fisher

Hyperladder 1kb BIO33025 by Bioline

Protocol

Standard

Step 1.

Microbiological testing was conducted in accordance with:
Australian Standard : 5013.10-2009: Food Microbiological Method 10: Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD)

Specific details for each method can be obtained from the standard.

Any modifications made to the standard (Step 4, 5) are described below. Additions to the standard are detailed in Step 6, 8 and 9.

Primary Sample Processing

Step 2.

Primary sample processing is described in the Protocol: Poultry Enterprise Environmental Sample Handling and Processing for Salmonella spp. Detection

All samples were collected and processed for microbiological testing on the same day.

Primary Sample Pre-Enrichment

Step 3.

Each primary sample had BPW added and was prepared for incubation as described elsewhere (Step 2)
Samples were handled with minimal mixing, sufficient to ensure samples were wet and covered with pre-enrichment media prior to incubation

Incubation Conditions

Aerobic static incubation

Incubation Time and Temperature

18-24 hours at 37°C

TEMPERATURE

37 °C Additional info:

Primary Selective Media - Modifed Semi-solid Rappaport Vassiliadis (MSRV)

Step 4.

Primary Culture Sub-sampling onto MSRV

After incubation of the primary sample
Three aliquots (each 33 µL: total 0.1 mL) were taken from the primary sample and inoculated onto Modifed Semi-solid Rappaport Vassiliadis (MSRV) agar plates.
Aliquots were collected from three different locations in the primary sample, as close to the sample container and surface liquid interface as possible

Incubation conditions

Aerobic static incubation

Incubation Time and Temperature

41.5°C and visually examined at 12, 24 and 48 hours post inoculation.

MSRV Colony/Growth Characteristics

Salmonella suspect positive MSRV plates have evidence of swarming growth
Swarming growth in MSRV media is characterised by a grey-white turbid zone extending from the inoculation point with a clearly defined edge

The principal of selection with the semi-solid media MSRV is that *Salmonella* spp. will swim faster than other *Enterobacteriaceae*.
Collecting samples from the furtherest edge of the turbid zone will enable better detection of *Salmonella* spp, and reduce secondary contamination of the secondary selection media
If the original samples are heavily contaminated with non-target organisms such as *E. Coli* then early inspection of plates is warranted.
Sometimes *Salmonella* spp, in MSRV, particularly when the number of organisms is low, may not appear as a grey-white turbid zone
In this case zones of growth may appear as a darker 'shadowed' area within the MSRV that may be missed if the plate is not inspected against a dark background

Overgrowth by *E.Coli* will occur in these samples if they are not inspected at 12 hours.

AMOUNT

33 µl Additional info: x 3
Aliquots from primary
incubated sample
inoculated onto MSRV

TEMPERATURE

41.5 °C Additional info:

Secondary Selective Media - XLD, BGA, CLED

Step 5.

Sub-culture after incubation

MSRV plates showing evidence of swarming growth were sub-cultured using a loop
The loop is carefully inserted into the semi-solid MSRV media and removed without taking excessive media from the plate
Samples should be obtained from the leading edge of the swarming growth and plated onto the secondary media

All positive inoculation points (if more than one) on the MSRV plate were sub-cultured onto secondary media

No further sub-culturing from MSRV is conducted when swarming growth is observed in the first 24 hours of incubation.

Secondary Selective Media

Suspect positive growth is plated by conventional streaking onto at least two different secondary selective media:
Xylose–Lysine–Desoxycholate agar (XLD), and either
Brilliant Green agar (BGA) or Cystine Lactose Electrolyte Deficient agar (CLED)

Incubation Conditions

Aerobic static incubation

Incubation Time and Temperature

24 hours at 37°C

Secondary Selective Media Colony Characteristics

Suspect *Salmonella* spp. colonies on each of the media have the following growth characteristics:

Test Media	Colony Morphology	Agar Colour Reaction	Other Growth Characteristics
XLD (CM0469)	Red with black centre	Red	Non H_2S fermenters are red only
CLED (CM0301)	Flat blue colonies	No Colour change	-
BGA (CM0263)	Red-Pink-white opaque colonies	Red	Lactose fermenters pink colonies

TEMPERATURE

37 °C Additional info:
Incubation

Salmonella Confirmation - Biochemical Testing

Step 6.

For each plate of test media containing suspect *Salmonella* colonies
Three morpohologically distinct colonies from each plate were selected for biochemical testing.

Biochemical test media

Triple Sugar Iron agar (TSI)
Lysine Iron agar (LIA)

Test Method

Using a sterile inoculating needle lightly touching the surface of the colony to be sampled
Using the inoculating needle streaked the surface and then stab into the butt of the media

Incubation Conditions

Aerobic static incubation

Incubation Time and Temperature

24 hours at 37°C

Test reaction for Salmonella positive biochemical positive reactions

Test media	Slant	Butt	Gas	H_2S Production
TSI	Red or no change	Yellow	Yes	Black
LIA	Purple	Purple	No	Black

Elimination of *Salmonella* Sofia isolates

Salmonella Sofia isolates were identified from broiler and processing samples to eliminate these samples for *Salmonella* serotyping at the reference laboratory.

Each TSI/LIA *Salmonella* positive isolate were tested using

Biochemical test media

ONPG Broth
Mannitol broth

Test Method

Using a sterile inoculating needle touch the surface of the test colony

Inoculate the ONPG broth with the inoculating needle and mix well
Repeat for the mannitol broth and mix well

Incubation Conditions
Aerobic static incubation

Incubation conditions
24 hours at 37°C

Test Media	Start Colour	*Salmonella* Sofia Positive	*Salmonella* Spp.
ONPG Broth	Colourless	Yellow	No Change
Mannitol Broth	Blue-Green	Yellow	No Change

Gan E, Baird FJ, Coloe PJ, Smooker PM. Phenotypic and molecular characterization of *Salmonella enterica* serovar Sofia, an avirulent species in Australian poultry. Microbiology-Sgm. 2011;157:1056-65.

Isolate Storage

Step 7.
All positive samples were sub-cultured into
Tryptone Soya broth (Oxoid CM0129) containing 30% glycerol, and stored at -80°C, and
Salmonella maintenance media for long-term storage at room temperature

Serotyping by RT-PCR

Step 8.
RT-PCR Methods
Methods for *Salmonella* confirmation and serotyping via RT-PCR were validated and used in accordance with published methods:

Shanmugasundaram M, Radhika M, Muralia HS, Batra HV. Detection of Salmonella enterica serovar Typhimurium by selective amplification of fliC, fliB, iroB, rfbJ, STM2755, STM4497 genes by polymerase chain reaction in a monoplex and multiplex format. World Journal of Microbiology and Biotechnology. 2009;25(8):1385-94.

Kardos, G., et al., Novel PCR assay for identi cation of Salmonella enterica serovar Infantis. Letters in Applied Microbiology, 2007. 45(4): p. 421- 425.

Malorny, B., et al., Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an International Standard. Applied and Environmental Microbiology, 2003. 69(1): p. 290-296.

Primers for RT-PCR for *Salmonella* spp., *Salmonella* Typhimuirum and *Salmonella* Infantis

Gene	Primer ID	Primer Sequence (5'-3')	Product Size (Base Pairs)
STM (S. Typhimurium)	STM4497F	GGAATCAATGCCCGCCAATG	523
	STM4479R	CGTGCTTGAATACCGCCTGTC	
FliB (S. Infantis)	878F	TTGCTTCAGCAGATGCTAAG	413
	1275R	CCACCTGCGCCAACGCT	
InvA (Salmonella spp.)	139-141F	ACAGTGCTCGTTTACGACCTGAAT	244
	139-141R	AGACGATGGTACTGATCGATAAT	

RT-PCR Method

Each suspect colony was suspended in sterile water (200 µL), and incubated at 100°C for 2 minutes
Once cooled, suspensions were centrifuged at 16000x g for 5 minutes
The supernatent was removed from the pellet and stored at -20°C
The supernatant was used as template

PCR Reagents

RT-PCR was performed in a final volume of 20 µL containing 1 µL template, dNTPs (1.25 mM), oligonucleotide primers (10 mM), MgCl2 (25 mM), 5 x GoTaq® buffer, water and 1 x GoTaq® enzyme (Promega).

Reaction Cycling Conditions

Initial denaturation step 95°C for 5 minutes, then 35 cycles of denaturation at 95°C for 1 minute, primer annealing at 62°C for 30 seconds and extension at 72°C for 1 minute, followed by the nal extension step at 72°C for 10 minutes.

Gel Electrophoresis

PCR products were electrophoresed on a 1.5% agarose gel, prepared in 0.4x TBE with SyberSafe® DNA gel stain (Invitrogen) at 80 V/cm for 20-30 minutes.
Hyperladder-IV (Bioline) was used as a DNA molecular weight marker.

Reference Laboratory Serotyping, Phage Typing and MLVA profiling

Step 9.

Serotyping, phage typing and multiple-locus variable number tandem-repeats analysis (MLVA) were conducted at the Victorian *Salmonella* Reference Laboratory (Microbiological Diagnostic Unit, University of Melbourne, Melbourne, Australia), in accordance with internationally recognised standard methods. Methods used by the diagnostic reference laboratory are described for reference purposes only.

Serotyping

All samples were initially screened using the H antigen (flagella).
Putative *Salmonella* Typhimurium isolates (H = i) were fully serotyped, phage typed and multi-locus variable tandem-repeat analysis (MLVA) sequence typed.

For each sample that was not H=i, one sample per shed per submission was fully serotyped.

Isolates were serotyped using the Kaufman–White–Le Minor scheme:
Grimont, P.A.D. and F.-X. Weill, eds. Antigenic formulae of the Salmonella serovars, 9th Edition. 2007, WHO Collaborating Center for Reference and Research on Salmonella, Institute Pasteur: Paris, France. 166.

Phage Typing

Phage typing was conducted using the Anderson phage typing scheme:
Anderson, E.S., et al., Bacterio- phage-typing designations of *Salmonella* Typhimurium. Journal of Hygiene, Cambridge, 1977. 78: p. 297.

MLVA Profiling

All *Salmonella* Typhimurium confirmed isolates were MLVA profiled in accordance with the European MLVA protocols:.

Lindstedt, B.-A., et al., Multiple-locus variable-number tandem-repeats analysis of *Salmonella enterica* subsp. *enterica* serovar Typhimurium using PCR multiplexing and multicolor capillary electrophoresis. Journal of Microbiological Methods, 2004. 59: p. 163-172.

Results were reported using the Australian MLVA naming convention.

Conversion from AU to EU naming convention

It is possible to convert the Australian MLVA profile into the equivalent EU profile by using the following conversion:

Heuzenroeder, M. W., I. L. Ross, H. Hocking, D. Davos, C. C. Young and G. Morgan (2013). An integrated typing service for the surveillance of *Salmonella* in chickens. Molecular and classical typing of *Salmonella* populations in chickens. Project No. PRJ-003419. ACT, Australia, Rural Industries Research and Development Corporation. **13/025:** 1-65.

The profile number is equal to p = n+1, p is the profile number, n is the number of tandem repeats within the fragment.

A zero at the profile locus means a single copy of the repeat fragment is present with no repeats

Locus	STTR-9	STTR-5	STTR-6	STTR-10	STTR-3
Repeat Fragment Length (bp)	9	6	6	6	33
Fragments to remove (bp) Primers	135	169	258	305	-
Example Fragment Length (bp)	162	313	324	371	523
Example Australian Code	03	24	11	11	523