Interim Proposal #3

NATIONAL POULTRY IMPROVEMENT PLAN PROGRAM STANDARDS

Subpart D – Molecular Examination Procedures

(3) Laboratory procedures recommended for the ~~conventional~~ polymerase chain reaction test for Salmonella Enteritidis.

~~Allows the use of a second primer set suitable for a real-time polymerase chain reaction (PCR) application that targets a region already approved for conventional PCR detection of Salmonella Enteritidis.~~

(a) Sample enrichment. Samples (drag swabs, chick paper swabs, etc.) are enriched in Tetrathionate enrichment for 18 to 24 hours at 37°C or 41.5~~2.0~~°C (see Section B(3)) and subcultured onto Modified Semi-solid Rappaport-Vassiliadis (MSRV) ~~E~~enrichment (see Section B(3)) in accordance with procedures set forth in these program standards.

(b) Quality control. A positive control~~;~~ (known Salmonella Enteritidis or ATCC strain) from BHI broth should be inoculated onto MSRV media, incubated for 18 to 24 hours, and 1 to 3 plugs harvested from the zone of white precipitate growth outside the initial inoculation site. ~~A negative control is h~~Harvested plugs from an area of uninoculated MSRV plate should be used as a negative control.

(c) DNA extraction. DNA is extracted from 1 to 3 plugs (~100-µl~~t~~) of MSRV agar in the zone of migration outside the initial inoculation site by boiling in 100µl PCR grade water for 10 minutes, or by another DNA extraction method. Samples are cooled to room temperature before PCR use, or stored at 2° to 8°C~~;~~ if PCR is not performed immediately. For the boiling method, the extracted samples are spun at 16,000 rcf for 3 minutes. The DNA is contained in the supernatant.

(d) Primer s~~S~~election. The SE-specific primers for conventional PCR are:
sdf I (forward) ~~–~~: 5'-_TGT_GTT_TTA_TCT_GAT_GCA_AGA_GG_-3'
sdf I (reverse) ~~=~~: 5'-_CGT_TCT_TCT_GGT_ACT_TAC_GAT_GAC_-3'.

The internal control primers are:
rpl I (forward) ~~–~~: 5'-_GGG_TGA_TCA_GGT_TAA_CGT_TAA_AG_-3'
rpl I (reverse) ~~=~~: 5'-_CTT_CGT_GTT_CGC_CAG_TGG_TAC_GC_-3'.

(e) Polymerase chain reaction. The following multiplex-PCR reaction (2 sets of primers per reaction tube) should be set up in a 200µl PCR tube or a 25-µl PCR tube, in a clean environment.

Reaction Mix	50-µl Volume	25-µl Volume	Final Concentration
10X PCR Gold Buffer	5µl	2.5µl	1X
MgCl2 (25mM)	5µl	2.5µl	2.5mM
10mM dNTP mix	3µl	1.5µl	150µM each
sdf I Forward Primer	4µl	2.0µl	0.4µM
sdf I Reverse Primer	4µl	2.0µl	0.4µM
rpl I Forward Primer	4µl	2.0µl	0.4µM
rpl I Reverse Primer	4µl	2.0µl	0.4µM
Amplitaq Gold Polym.	0.5µl	0.25µl	2.5U
Sterile PCR Grade Wa.	15.5µl	5.25µl	
DNA Template*	5.0µl	5.0µl	

*The DNA template is 5.0µl of the boiled MSRV solution or DNA from another DNA extraction method.

(f) PCR amplification program.:

Polymerase Activation Step	95°C for 10 minutes	1 cycle
Denaturation Step	95°C for 30 seconds	
Annealing Step	60°C for 30 seconds	35 cycles
Extension Step	72°C for 1 minute	
Final Extension	72°C for 7 minutes	1 cycle
4°C Hold	infinite hold until samples are refrigerated or frozen	

(g) Electrophoresis. After conventional PCR is completed, samples should be analyzed by DNA electrophoresis. A 3 percent RAGE gel, or a 1 percent to 3 percent conventional gel with a sample volume of 3.5µl, in addition to 1.5µl of loading dye, is recommended. The *sdf I* primers will yield a 293bp band only in the presence of Salmonella Enteritidis DNA, and the *rpl I* primers will yield a 343bp band in the presence of any bacterial DNA (the *rpl I* primers have worked well with every organism so far with the exception of *Proteus mirabilis*).

Reference:

1. Charlton BR, Walker RL, Kinde H, Bauer CR, Channing-Santiago SE, et al. (2005) Comparison of a Salmonella Enteritidis-Specific Polymerase Chain Reaction Assay to Delayed Secondary Enrichment Culture for the Detection of Salmonella Enteritidis in Environmental Drag Swab Samples. Avian Diseases: Vol. 49, No. 3 pp. 418–422.

(h) Real-time PCR. ~~Or alternatively in a real time SYBR based PCR assay the following primers may be utilized~~ -
The SE-specific primers for real-time PCR are:
F2 (forward): 5'- TTG ATG TGG TTG GTT CGT CAC T -3'--;
R2 reverse): 5'- TCC CTG AAT CTG AGA AAG AAA AAC TC -3'.

(~~h~~i) Alternative methods (equipment and reaction components). Alternative methods may be utilized by an approved laboratory as long as the ~~appropriate~~ PCR primers ~~as~~ listed in this Standard are utilized, and a Group DSalmonella proficiency test provided by the ~~s~~Service has been passed, ~~utilizing the method~~ indicating the laboratory is performing at equivalent or better detection levels with their desired PCR method. ~~—~~If using real-time PCR assays, ~~Q~~quantitative positive controls from a known Salmonella Enteritidis strain should be ~~made to~~ used with each run. ~~of this assay from a known Salmonella enteritidis Strain (as confirmed by NVSL), or ATCC Strain extracted using Qiagen DNeasy Tissue Kit Cat. No. 69506 or equivalent.)~~ Records shall be maintained to document ~~show~~ the origin, production ~~of the controls~~ and consistency of ~~the reactions of said~~ these controls ~~in this assay~~ over time ~~to the Official State Agency upon request~~.

Reason: Correction of *sdf I* reverse primer sequence to match reference publication, plus general formatting updates for consistency, flow and understanding. Since some of the changes are so small, they are highlighted in blue in addition to the traditional underlined designation.

Sponsors: Undine Taldo
Cobb-Vantress, LLC

Dr. Nikki Shariat
Poultry Diagnostic and Research Center, University of Georgia