Title: Protocol for identifying Highly Pathogenic *Salmonella* using the HPS Multiplex PCR assay

Abstract: This protocol describes how to perform a multiplex PCR assay for the identification of Highly Pathogenic *Salmonella* (HPS). The assay targets eight virulence genes including: *ssek2* (HPS-6), *sseK3* (HPS-1), *avrA* (HPS-3), *lpfB* (HPS-5), *spvD* (HPS-4), *sspH2* (HPS-7), *gtgA* (HPS-2) and *invA* (i). Comparative genomic research using 23 complete closed *Salmonella* genome sequences (1-23) revealed these genes to be shared among *Salmonella* serotypes noted for being invasive and/or causing most cases of salmonellosis (i.e. consistently on the CDC’s list of top 20 serotypes attributed to human illness) but to varing degrees absent among serotypes that are less frequently associated with human illness. Validation of this assay with 1303 *Salmonella* of 69 different serotypes confirmed the utility of this assay for identifying HPS (Harhay et. al., in preparation). *Salmonella* samples that result in the amplification of five or more targets are identified as HPS, and the markers amplified are reported as an HPS index (HPSi). The assay described is intended for use with DNA lysates of *Salmonella* isolates, not lysates of enrichment cultures, where more than one *Salmonella* serotype may be present. Notably, the HPS assay is not serotype specific (i.e. the results do not indicate the presence of a particular serotype) rather it provides an indication of the potential pathogenicity of the *Salmonella* being detected. This protocol includes information on the generation of template DNA, primer sequences needed, construction of the PCR master mix, thermal cycler program used for amplification, parameters for DNA gel electrophoresis, and amplicon visualization and scoring. Further evidence of the utility of the HPS gene targets is their recent addition to the AMRFinderPlus Reference Gene Catalog.

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**Bacterial Lysis (BAX lysis) Procedure to prepare DNA template**

**Equipment**:

Thermal cycler (BIO-RAD T100)

Lysis Program (see below)

PCR plates & Microseal B (BIO-RAD; #MSB-1001)
Multi-Channel Pipettors

**Buffers and reagents**:

BAX Lysis Buffer (Hygiena; item #ASY2011; ref. #D14403062)

BAX Protease Vial (Hygiena; item #ASY2012; ref. #D11134081)

Control strain (*S. Typhimurium* ATCC - 14028)

Overnight Culture of samples in question

**Protocol**:

BAX Lysis Buffer:

Add 150 µL Protease solution into 12 mL Lysis Buffer

Note: this solution is stable at 4C for two weeks.

Reaction:

Add 100 µL Protease/Lysis Buffer per well in the PCR plate

Add 2.5 µL Sample (overnight culture) per well

Lysis Thermal Cycler Program:

37 C – 20 min

95 C – 10 min

Cool 4 C – 5 min

Note: make sure lid of thermal cycler is set to 96C

In a PCR plate, dispense 100 µL of buffer per well, add 2.5 µL of overnight culture, seal with Microseal B, and place in thermal cycler. Run Lysis Protocol listed above (35 minutes).

Move on to PCR or freeze lysates

**HPS Multiplex PCR Reaction**

Primer list:

See Table 1.

Master Mix:

See Table 2.

**Equipment**:

Thermal cycler (BIO-RAD T100)

Program (see Table 3)

PCR plates & Microseal B (BIO-RAD; #MSB-1001)

Multi-Channel Pipettors (10ul and 20ul 12 channel preferrable)

**Buffers and reagents**:

Bullseye HS Taq Pol (MidSci; BE225108)

Deoxynucleotide (dNTP) Solution Mix (New England Biolabs; N0447L)

**Protocol**:

Make up HPS master mix (see Table 2). Store at -20 °C.

Thaw master mix, vortex, aliquot 20 µL per well in PCR plate.

Add 5 µL of lysate (if frozen, thaw and gently pipette to resuspend) per well. Final reaction volume is 25 µL.

Seal plate with Microseal B and place in thermocycler.

Run program (Table 3).

Once complete, begin gel or store PCR products at -20 °C.

**Agarose gel electrophoresis and staining, to visualize DNA amplicons**

**Equipment**:

4x26-Well Combs, Fixed Height, 1.5 mm Thickness, Multichannel Pipet Compatible (BIO-RAD; #1704457)

Sub-Cell GT Horizontal Electrophoresis System, 15 x 25 cm tray, with gel caster (BIO-RAD; #1704484)

0.5 L Erlenmeyer flask

**Buffers and reagents**:

HPS Marker generated using Control strain (*S. Typhimurium* ATCC - 14028)

10x TBE (Fisher Scientific; #BP1333) (note: It is important to use TBE; buffers such as SB do not perform as well i.e. results in poor band separation)

Bullseye General Purpose Agarose GP2 (MidSci; #BE-A500)

6x loading dye (purchased or homemade)

**Protocol**:

Make a 2% agarose gel (in 0.5 L flask, dissolve with heat 5 g agarose in 250 mL of 1 x TBE) and cast in 15 x 25 cm tray.

After gel is setup, transfer to buffer tank containing enough 1X TBE to cover gel and remove combs. To completed HPS PCR reaction plate, gently remove the Microseal B cover tape, add 6 µL of 6x loading dye to the reaction. Pipette up and down to mix, then with same tip, transfer 6 µL of mixture to the gel and dispense in an appropriate well.

Flank with control strain *S. Typhimurium* ATCC – 14028 on either side of reaction wells.

Run gel at 170 volts for approximately 60 minutes.

*Staining*
After electrophoresis, stain the gel with Ethidium Bromide (EtBr) (use caution and appropriate PPE with EtBr (carcinogen) (~1 μg/mL [from a 1% stock solution, add 10 μL EtBr per 100 mL dH2O) for 30 min. Use a BioRad staining tray with a lid and place this on a rocker to gently agitate the stain with the gel in the tray.

Carefully pour off the stain (using gloves and appropriate PPE)

Add 100 ml of water to the tray, to de-stain the gel (again using a rocker on low setting) for 15-20 minutes. Dispose of the de-stain water appropriately).

Image with a UV imager

**Tables**

Table 1. HPS primer list



Table 2. HPS Master Mix 

Table 3. Thermal cycler program for HPS assay – modified short EHEC from (Paton & Paton (1998) Journal of Clinical Microbiology 36:598-602)

