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Rapid Estimation of *Salmonella enterica* Contamination Level in Ground Beef – Application of the Time-to-Positivity Method Using a Combination of Molecular Detection and Direct Plating

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ABSTRACT (176 of 200 words)

Little progress has been made in decreasing the incidence rate of salmonellosis in the US over the past decade. Mitigating the contribution of contaminated raw meat to the salmonellosis incidence rate requires rapid methods for quantifying *Salmonella,* so that highly contaminated products can be removed before entering the food chain. Here we evaluated the use of Time-to-Positivity (TTP) as a rapid, semi-quantitative approach for estimating *Salmonella* contamination levels in ground beef. Growth rates of 14 *Salmonella* strains (inoculated at log 1 to -2 CFU/g) were characterized in lean ground beef mTSB enrichments and time-to-detection was determined using culture and molecular detection methods. Enrichments were sampled at five timepoints and results were used to construct a prediction model of estimated contamination level by TTP (superscript indicates time in hours) defined as TTP^4: ≥5 CFU/g; TTP^6: ≤5, ≥1 CFU/g; TTP^8: ≤1, ≥0.01 CFU/g; with samples negative at 8h estimated ≤0.01 CFU/g. Model performance measures showed high sensitivity (100%) and specificity (83% and 93% for two detection methods) for samples with a TTP^4, with false negative rates of 0%.

Highlights

- Time-to-Positivity sampling method can detect *Salmonella* levels ≥ 5 CFU/g in ground beef enrichments at 4h.
- Direct plating with Petrifilm™ EB and replica plating to XLD provides confirmation of counts and evidence of viability.
- Average DT of *Salmonella* in enrichments was 19.5 min, however some strains grew faster with DT of 15.5 min.
- At low inoculation levels certain *Salmonella* grew poorly and were outcompeted by ground beef microflora.

1. Introduction

Salmonella enterica is a leading cause of bacterial foodborne illness in the U.S. with an estimated incidence rate (IR) of 18.3 per 100,000 people and a projected 1.2 million cases each year (CDC, 2013; Painter et al., 2013; Scallan et al., 2011; Tack et al., 2019). A recent survey conducted by the Food Safety and Inspection Service (FSIS) showed that the majority of *Salmonella* outbreaks are broadly attributed to a number of commodity groups, with poultry, eggs, seeded vegetables and beef at the top of the list of food categories identified (17%, 12%, 12% and 9% respectively; (IFSAC, 2018)). Beef related outbreaks are generally limited in scope, with the number of illnesses (on average <30 cases/outbreak) tending to be a fraction of those caused by produce or poultry sources (on average >100 illnesses/outbreak)(Laufer et al., 2014). However, recent outbreaks in the U.S. attributed to the consumption of ground beef contaminated with *Salmonella* Newport have resulted in over 400 illnesses and the recall of more than 10 million pounds of ground beef (4.5×10^6 kg) (Marshall et al., 2018; Plumb et al., 2019). These developments have resulted in increased scrutiny on the presence of *Salmonella* in ground beef.

While *Salmonella* is not presently regulated as an adulterant in raw meat, FSIS has historically established performance standards for *Salmonella* contamination in raw meat products in order to monitor whether establishments have effective process controls in place to address *Salmonella* contamination (USDA-FSIS, 2015, 1996). Moreover, FSIS has recently proposed new performance standards making use of a 52-week moving window sampling approach in beef establishments that produce greater

than 50,000 pounds of raw ground beef per day (2.3 x 10^4 kg), with no more than two of 48 samples being positive for *Salmonella* within the moving window (USDA-FSIS, 2019a). However, in spite of these monitoring efforts and the implementation of numerous process controls in various food production industries, there has been little progress in decreasing the IR of salmonellosis over the past decade, which has in fact increased 9% from a level of 15 cases per 100,000 in 2010, to 18.3 cases per 100,000 in 2018 (McEntire et al., 2014; Sampedro et al., 2018; Tack et al., 2019). While many factors undoubtedly contribute to this outcome, a central issue is that the current approach to monitoring *Salmonella* contamination in foods is based only on prevalence testing (presence-absence or qualitative testing) and has no requirement for estimating the contamination levels present in the commodities being tested (McEntire et al., 2014; Sampedro et al., 2018). This is likely because enumeration methods that are currently recommended or approved for use are time consuming, expensive and not practical for high throughput analyses or use in large scale production settings (Kim et al., 2017; Owen et al., 2010). And yet, an understanding of the range of contamination levels encountered in various phases of any food production system is needed to identify the critical control points of that system (Crump et al., 2002; Koyuncu et al., 2010). The present lack of quantification data on *Salmonella* in products makes it almost impossible to determine the threshold values that lead to illnesses and outbreaks.

For outbreaks where it has been possible to estimate the *Salmonella* contamination level, it has been shown that a majority of these (83%) have resulted from consuming *Salmonella* contaminated products with doses >100 CFU (Blaser and Newman, 1982;

Teunis et al., 2010). Additionally, the results of a limited number of published human inoculation studies indicate that consuming products with higher levels of *Salmonella* is more likely to result in infection (Haas, 2002; Haas, 1983; McCullough and Eisele, 1951). Thus, what is needed to achieve meaningful gains in public health is 1) an understanding of the levels of contamination encountered in food production systems, and 2) the ability to rapidly detect products contaminated at higher levels so that they can be removed from the food chain.

The standard method for bacterial enumeration in foods has traditionally been the most probable number (MPN) method (Cochran, 1950; Sutton, 2010). While the MPN method makes use of enrichments of dilutions of a given sample to estimate initial contamination level, a different approach for estimating pathogen level that has not yet been extensively explored in the area of food safety is sampling enrichment cultures over time to examine Time-to-Positivity (TTP). The concept of TTP has been explored in the field of human medicine for the past 25 years, and increasingly so in the past decade (Lamy, 2019). With regard to blood cultures which are continuously monitored for growth, TTP provides indirect information on the contamination level and growth rate of bacteria present. In the area of food safety, Weidemaier et al., have described an approach for real-time monitoring of pathogen level during enrichment using Surface Enhanced Raman Scattering nanoparticles in combination with a pathogen specific immunoassay (Weidemaier et al., 2014). They found that continuous monitoring of pathogens in enrichment cultures decreased the time-to-results by taking advantage of variation in bacterial load, with more highly contaminated samples showing a decreased

TTP. While the authors concluded that the cost of reagents for large sample volumes and the stability of antibodies present in the enrichment cultures were limitations for the practical application of this method in food industry settings, the study never-the-less demonstrated the utility of real-time monitoring of pathogens in enrichments for identifying more highly contaminated samples.

Here, we evaluated the use of the TTP approach for rapidly estimating *Salmonella* contamination levels in ground beef products. To accomplish this, we examined *Salmonella* growth rates and time to detection in inoculated lean ground beef samples using 1) two commercially available kits based on molecular detection of *Salmonella*, 2) immunomagnetic separation (IMS) with secondary enrichment in RVS followed by plating on selective medium, and 3) direct plating enumeration of viable *Salmonella.* The results describe the growth rates of various *Salmonella* strains and serotypes in ground beef enrichments (GBE); the efficacy of molecular detection methods for identifying *Salmonella* contamination in ground beef enrichments at given timepoints; and performance measures of a novel testing method for estimating *Salmonella* contamination levels in ground beef samples based on TTP.

2. Materials and methods

2.1 Strains and culture conditions

The *Salmonella* strains used in this study are listed in Table 1. All strains were revived from -80 °C glycerol stocks and cultured on Tryptic Soy Agar plates (TSA; Difco, Becton-Dickinson, Franklin Lakes, NJ, USA) at 37°C for 18 to 22 h. Prior to ground beef inoculation, each culture was prepared by inoculating a single colony from a fresh TSA plate into 5 ml of mTSB (modified Tryptic Soy Broth, Becton-Dickinson) that was incubated overnight without shaking at 37°C for 18 to 20 h. Each culture was diluted 1:100 into fresh mTSB medium and incubated without shaking at 37°C to an OD_{600} of ≈0.06 (approximately 10^7 CFU/ml). Bacterial cells were then harvested by centrifugation at 3000 x g for 10 min at 4°C. The mTSB supernatant was removed and the pellet resuspended in 10 ml of phosphate buffered saline (PBS; Amresco, LLC, Solon, OH, USA). The resulting bacterial suspension was serially diluted to10^{-5} (≈10^2 CFU/ml). The bacteria were then stressed by starvation in PBS maintained at room temperature (RT) for 24h. Prior to inoculation, the starved inoculum was diluted to 10^{-8} such that four inoculation levels were used for each strain (10^2/ml, 10^1/ml, 10^0/ml, and 10^{-1}/ml). A 1 ml portion of each dilution was plated on Petrifilm™ EB (3M, St. Paul, MN, USA) and incubated at 37°C for 18 to 20 h to estimate the concentration of each inoculum.

2.2 Ground beef inoculation

159 For each strain evaluated, ground beef in five-pound (2.3 kg) chubs, that originated from
160 the same lot (code and expiration date) and that had been maintained at 4°C in original
161 packaging prior to inoculation was used. Five portions of ground beef (325g; 93% lean:
162 7% fat) were placed into sterile filter bags (BagPage+ 3500, Interscience, Woburn, MA,
163 USA) and each was inoculated with 10 ml of *Salmonella* as prepared above, or with 10
164 ml of sterile PBS (non-inoculated control). The inoculated ground beef samples were
165 allowed to rest at RT for 5 min prior to the addition of 975 ml of prewarmed (42°C)
166 mTSB and were then stomached at 420 RPM for 1 min in a laboratory blender
167 (JumboMix 3500, Interscience, Woburn, MA, USA). The resulting ground beef
168 enrichments (GBE) were incubated statically at 42°C for 24 h. To measure the growth of
169 each strain at each inoculation level in the absence of ground beef and the resulting
170 microflora, parallel mTSB cultures (97 ml mTSB also pre-warmed at 42°C) were
171 inoculated with either 1ml each of the 10^2 or 10^1 CFU/ml inocula, or 10ml each of the
172 10^0, 10^{-1} CFU/ml or the sterile PBS control. These mTSB control enrichment samples
173 were also incubated statically at 42°C.
174
175 2.3 *Salmonella* prevalence and concentration estimates in GBE and mTSB enrichment
176 samples
177
178 At each timepoint (T in hours: T0, T4, T6, T8, and T24) an 8 ml portion from each GBE
179 and a 5 ml portion from each mTSB control, was removed to assess the presence and
180 level of *Salmonella*. The difference in the volume of samples collected from each
181 sample type was to ensure that 1) we had sufficient GBE sample for the subsequent

182 steps in the analysis which included IMS, glycerol stock storage for downstream
183 analysis and lysis for the *Salmonella* molecular detection assays used; and 2) that we
184 did not significantly deplete the control mTSB sample with repeated sampling over the
185 course of enrichment. GBE samples were briefly centrifuged at 76 x g for 30 sec to
186 pellet debris, then 1 ml was used for IMS with anti-*Salmonella* IMS beads (Applied
187 Biosystems, Foster City, CA, USA) as previously described (Bosilevac et al., 2009).
188 Recovered IMS beads were transferred to 3 ml of RVS medium and this secondary
189 enrichment was incubated at 42°C for 18 to 22h. Sterile cotton swabs were used to
190 subculture RVS enrichments (~50 ul) to XLD (Oxoid) agar plates, which were streaked
191 for isolation and then incubated at 37°C for 18 to 22h. *Salmonella* were identified on
192 XLD plates as black colonies with a clear outer ring. Any suspect colonies (including
193 those with abnormal colony morphology on XLD) were picked for confirmation using a
194 *Salmonella* specific PCR assay for the *invA* gene (Nucera et al., 2006; Rahn et al.,
195 1992).
196
197 *Salmonella* concentrations in both GBE and mTSB cultures were assessed at the
198 following timepoints by plating 1 ml each of enrichment or 10-fold dilution in PBS (**T0**:
199 10^{0}; **T4**: 10^{0}, 10^{-1}, 10^{-2}; **T6**: 10^{-2}, 10^{-3}, 10^{-4}; **T8**: 10^{-4}, 10^{-5}, 10^{-6}; and **T24**: 10^{-7}, 10^{-8} and
200 10^{-9}) onto Petrifilm™ EB then incubating at 37°C for 18 to 22h and counted manually or
201 with a 3M Petrifilm™ counter. *Salmonella* counts were estimated using a previously
202 described replica plating method (Webb et al., 2017) that involves carefully peeling back
203 and removing the plastic film lid of the EB Petrifilm™ plate, and replica plating it onto an
204 XLD agar plate (Figure S1). XLD plates were incubated at 37°C for 18 to 22h and

205 *Salmonella* counts were determined by counting typical black colonies present on the
206 XLD medium. Questionable isolates on XLD were picked and confirmed using the *invA*
207 PCR assay described above. *Salmonella* counts from replica plating GBE samples at
208 T0, were used to calculate the observed starting concentration of inoculated
209 *Salmonella,* in combination with the theoretical T0 counts estimated by plating 1ml of
210 each inoculum (-5, -6, -7, -8) to EB Petrifilm™, and then multiplying by 10 to account for
211 the 10ml inoculum, resulting in starting CFU/g estimates for each inoculated GB
212 sample.
213
214 2.4 Growth curves and doubling time calculations in mTSB and GBE
215
216 Growth curves were constructed for each *Salmonella* strain evaluated at each
217 inoculation level, using log transformed viable cell number estimates (CFU/ml)
218 determined at each timepoint as described above. Linear regression analysis was
219 performed on the exponential growth phase for each curve, from T4 to T8, and the
220 resulting linear equations were used to calculate doubling time (DT) using three
221 concentrations (3.0, 3.3 and 3.6 log CFU/ml) as input. For each strain used, DT
222 estimates for each growth condition (mTSB or GBE) were averaged using the values
223 calculated from each inoculation level as a replicate (i.e. 10^{1}, 10^{0}, 10^{-1} and 10^{-2}) and are
224 reported along with the corresponding standard deviation (SD) in Table 1. DT in GBE
225 were ranked shortest to longest and then averaged among “fast” or “slow” growers
226 (Table 1). Changes in DT for growth in mTSB versus growth in GBE were assessed by
227 subtracting average DT in GBE from that in mTSB.

228

229 2.5 Molecular detection of *Salmonella* in GBE

230

231 Enrichment samples were assayed for the presence of *Salmonella* at each timepoint
232 using two molecular detection methods: the 3M Molecular Detection Assay 2 -
233 *Salmonella* (MDA2SAL96; 3M, St. Paul, MN, USA; here after referred to as 3M), and
234 the BAX® System Real-Time PCR Assay *Salmonella* (KIT2006; Hygiena, Camarillo,
235 CA, USA; here after referred to as BAX). For the 3M assay, samples were handled as
236 per manufacturer's instructions, with the exception that the enrichment medium used
237 was that indicated in FSIS MLG-4, (i.e. mTSB), as opposed to BPW (USDA-FSIS,
238 2019b). In short, at each timepoint, 20 ul of enrichment was added to 580 ul of lysis
239 buffer, incubated at 99 to 101 °C for 15 min, then cooled to 20 to 25 °C for 5 min. An
240 aliquot of 20 ul of this lysate was used in the molecular detection assay where the 3M
241 Molecular Detection Software (version 2.5.0.0) called positives and negatives. For the
242 BAX® assay, samples were analyzed as per manufacturer's instructions, with the
243 exception that novobiocin was not added to the mTSB enrichment medium. For this
244 assay, at each timepoint, 5 ul of enrichment was added to 200 ul of lysis-buffer that was
245 incubated at 37 °C for 20 minutes, then at 95 °C for 10 minutes, and finally cooled to 2
246 to 8 °C for a minimum of 5 minutes. A 30 ul aliquot of this lysate was used in the BAX
247 molecular detection assay and positive and negative samples were called on the BAX®
248 System Q7 instrument software (version 3.6.6005).

249

250 2.6 Analysis of the relationship between Time-to-Positivity (TTP) in GBE and starting
251 contamination level of *Salmonella*
252
253 The relationship between initial (T0) *Salmonella* contamination level and the Time-to-
254 Positivity (TTP) of an enrichment using the molecular detection methods, was examined
255 for each *Salmonella* strain at each inoculation level (n= 56 data points for 14 strains
256 inoculated at four different levels, 10^1, 10^0, 10^{-1} or 10^{-2} CFU/g). Sample starting
257 concentrations were grouped by timepoint when first detected as positive with either the
258 3M or BAX method, and then the mean starting (T0) concentration (log CFU/g), the 95%
259 confidence interval of this mean were calculated, and the minimum, and maximum
260 values for each category were identified (Figure 1). The distribution of the resulting data
261 points was further visualized by plotting starting contamination level (log CFU/g) by
262 timepoint first detected positive (T4, T6, T8 or T24), using a violin plot (displaying the
263 median and quartile contamination levels within each timepoint category; Figure 1).
264 Finally, direct plating estimates of *Salmonella* levels present in enrichments at each
265 timepoint evaluated (n=280 timepoints for 14 strains at 4 inoculation levels, sampled at
266 5 timepoints) allowed for an examination of the limits of detection (LOD) of each of the
267 molecular methods used and are summarized in Tables 2 and 3.
268
269 2.7 Statistical Analyses
270
271 Growth rates (DT) in GBE and mTSB for each *Salmonella* strain were calculated as
272 described in section 2.4 above. Significant differences between “fast” and “slow”

growing *Salmonella* in GBE and mTSB, and in changes in DT for growth in GBE or mTSB were analyzed using Prism 8.0 software (GraphPad, San Diego, CA) and a two-tailed unpaired t-test of significance, with $P \leq 0.05$ (Table 1). Violin plots also were constructed using Prism 8.0. To analyze the efficacy of TTP as an estimate of starting concentration, performance measures for the prediction model using BAX or 3M molecular detection methods were calculated, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), false positive rate (FPR) and false negative rate (FNR). To examine sensitivity and limits of detection for the *Salmonella* assays, the *Salmonella* concentration in enrichment samples were grouped either by T0 inoculation level (CFU/g, n=56; Table 2) or by average concentration at detection (log CFU/ml, n=236; Table 3). The mean *Salmonella* log CFU/ml, SD and percentage of samples in each log interval found positive by the IMS, BAX or 3M assays were calculated using Prism 8.0 and reported in Tables 2 and 3.

3. Results

3.1 *Salmonella* growth rates in mTSB ground beef enrichments

In this study, the growth rates of 14 *Salmonella* strains in mTSB alone and mTSB ground beef enrichments (GBE) at 42°C were determined. Growth rates in mTSB alone were found to be on average 20.4 min but in GBE were observed to be about three minutes faster, (17.6 min; Table 1). Ranking strains by shortest to longest average DT in mTSB or GBE revealed that some strains were able to grow faster than others in both

296 growth conditions. Fast growing strains had an average DT of 15.5 min or 19.4 min, as
297 opposed to slow growing strains with a DT of 19.5 min or 21.4 min in GBE or mTSB
298 respectively. Moreover, strains that grew faster in mTSB and GBE also were observed
299 to have a greater decrease in DT for growth in GBE as compared with mTSB (on
300 average 3.9 min shorter), while those that grew more slowly demonstrated an average
301 1.8 min decrease in DT between GBE and mTSB (Table 1). For the six serotypes
302 evaluated, both fast and slow growing strains were found in serotypes Newport,
303 Enteritidis, Typhimurium and Montevideo, suggesting that clade specific differences
304 within serotype may impact growth rates in enrichment. Conversely, both Anatum
305 strains were found to be fast growers (average DT of 15.8 min and 18.9 min in GBE and
306 mTSB respectively), while both Dublin strains were slow growers (average DT of 19.3
307 min and 21.1 min in GBE and mTSB, respectively). However, a greater number of
308 strains would need to be evaluated to confirm if these are consistent growth phenotypes
309 for these serotypes.
310
311 3.2 Determination of detection limits for *Salmonella* immunocapture and molecular
312 detection methods
313
314 Molecular detection assays for *Salmonella* from inoculated and uninoculated GBE
315 resulted in the analysis of 350 GBE samples. Of these, 70 were negative control
316 samples, that would only have been positive for *Salmonella* contamination if the ground
317 beef used was already contaminated with *Salmonella*, and this was not observed to be
318 the case in this study. The remaining 280 samples were obtained from GBE inoculated

with *Salmonella* at the levels described. Of these, 84.3% were found to be culture positive by direct plating on EB Petrifilm™ and replica plating to XLD, 81.1% were found positive by IMS, 61.4% by BAX and 58.2% by 3M assays. The data collected provided the opportunity to examine the detection limits of the assays used. Samples were grouped by inoculation level (average log CFU/g = 0.95 (n=13); -0.02 (n=14); -1.02 (n=14); -2.07 (n=15)) and the average level of *Salmonella* (log CFU/ml) in enrichments for each of these groups by timepoint was determined (Table 2). This analysis revealed the average *Salmonella* concentration by inoculation level and timepoint, where each detection assay used was found to have the highest accuracy for detecting *Salmonella*. As shown in Table 2, for samples inoculated at 0.95 log CFU/g this was T4 (average *Salmonella* concentration was 3.14 log CFU/ml), for -0.02 log CFU/g this was T6 (average concentration 4.28 log CFU/ml), for -1.02 log CFU/g this was T8 (average concentration 5.57 log CFU/ml) and for -2.07 log CFU/g this was T24 (average concentration 7.55 log CFU/ml).

Detection limits of the tests employed also were examined by grouping all samples found positive for *Salmonella* (n=236) by the observed log CFU/ml concentration in enrichment, by log intervals (0, 1, 2, etc.). For each group of values, the n, average log CFU/ml, SD and % of samples found positive by either the IMS, BAX or 3M tests were calculated and are summarized in Table 3. This analysis showed that the IMS method consistently detected *Salmonella* contamination in enrichments once the average concentration reached 2.44 log CFU/ml, while BAX and 3M demonstrated consistent *Salmonella* detection once levels in enrichment reached 4.43 to 5.51 log CFU/ml (Table

342 3). This analysis also demonstrated the difference in sensitivity between the 3M and
343 BAX detection methods, as BAX identified more samples as positive for *Salmonella*
344 contamination at lower concentrations in enrichment culture.
345
346 3.3 Examining the relationship between *Salmonella* starting concentration and Time-to-
347 Positivity (TTP) in enrichment
348
349 Examination of the relationship between starting contamination level and TTP using the
350 3M and BAX methods revealed that testing at defined timepoints could be used to
351 reliably estimate *Salmonella* starting concentration. This phenomenon was first explored
352 by examining the frequency distribution of starting concentration (log CFU/g) as a
353 function of timepoint first detected positive. For this analysis, 55 data points were
354 examined. The BAX assay included: T4, n=20; T6, n=24; T8, n=9; T24, n=2; and the 3M
355 assay included: T4, n=16; T6, n=22; T8, n=16; T24, n=1 (Figure 1). Grouping T0 log
356 CFU/g values by TTP allowed for the calculation of mean T0 CFU/g and 95%
357 confidence intervals, as well as minimum and maximum values within each group, by
358 detection assay. This analysis showed that samples with an average starting
359 concentration of 0.58 log CFU/g (95% CI 0.33, 0.83) for BAX and 0.76 log CFU/g (95%
360 CI 0.54, 0.99) for 3M assays, could be detected by T4 (Figure 1). Samples that were
361 negative for *Salmonella* detection at T4 but positive at T6 were found to have an
362 average starting concentration of -0.90 log CFU/g (95% CI -1.22, -0.58) for BAX and -
363 0.61 log CFU/g (95% CI -0.90, -0.31) for 3M. Accordingly, a matrix of average starting
364 concentrations and TTP was constructed. From this matrix, sensitivity, specificity and

365 other metrics of precision were determined for both assays, for the ability to estimate
366 starting concentration (Table 4). These analyses showed that both detection methods
367 had high sensitivity for identifying samples contaminated at or above the defined levels
368 (TTP superscripts indicate time in h): $TTP^4 \geq 5.0$ CFU/g; $TTP^6 \leq 5.0, \geq 1.0$ CFU/g; and
369 $TTP^8 \leq 1.0, \geq 0.01$ CFU/g), although specificity, precision and accuracy for the 3M
370 assay were all greater than those observed for the BAX assay, at each of the defined
371 levels. Negative predictive values and false negative rates were however the same for
372 the BAX and 3M assays (Table 4).
373
374 **4. Discussion**
375
376 Here we explored the utility of TTP as a way of rapidly estimating *Salmonella*
377 contamination level in ground beef samples. To accomplish this, we characterized the
378 growth dynamics of *Salmonella* in ground beef enrichments by determining growth rates
379 of 14 strains of *Salmonella* inoculated at concentrations ranging from log 1 to -2 CFU/g.
380 Prior to inoculation, all *Salmonella* strains used were starved in PBS in order to simulate
381 potential environmental stress encountered by the organism that might impact the
382 length of the lag phase. We examined the limits of detection of two molecular detection
383 methods by testing five enrichment timepoints. At each timepoint two culture methods
384 also were used to detect *Salmonella*. This combination of culture and molecular
385 detection at various timepoints, allowed for an examination of the relationship between
386 initial contamination level and TTP. The data collected support the idea that enrichment

analysis at select timepoints, as opposed to the canonical end-point sampling, can be used to estimate the starting contamination level of the sample being tested.

It should be noted that while the testing method described here was developed using lean ground beef enrichments, the resulting workflow for characterizing the relationship between starting concentration and TTP, could readily be applied to any enrichment type (poultry, produce, etc.) with an understanding of the growth rate of *Salmonella* in a given enrichment/microflora matrix, and the limits of detection of the test method employed. Use of this approach can facilitate the identification of highly contaminated product before it enters the food chain. This testing method would also enable food producers to investigate the root causes of practices that result in higher levels of contamination, ultimately decreasing the incidence of exposure to levels of *Salmonella* that cause illness, and positively impacting human health (McEntire et al., 2014).

The utility of this concept was investigated by plotting the distribution of estimated initial *Salmonella* concentration by timepoint first detected positive using either molecular detection method (Figure 1). This analysis showed that the majority of samples positive at T4 had initial concentrations of *Salmonella* greater than 5 CFU/g (log 0.69), with a smaller fraction actually contaminated at lower concentrations (i.e. false positive, in that they were misclassified as having a greater starting concentration than that experimentally measured; illustrated in Figure 1). Those positive at T6 had a much broader range of initial concentrations, ranging from 0.01 to 2.3 CFU/g (log -2 to 0.36) with an average of 0.4 CFU/g (log -0.39). Samples that were negative at T4 and T6 but

410 positive at T8 had initial concentrations ranging from 0.003 to 0.2 CFU/g (log -2.5 to -
411 0.69). These data were used to construct a prediction model of estimated contamination
412 level by TTP (Table 4) defined as (with TTP superscript indicating time in h) TTP^4: ≥5
413 CFU/g; TTP^6: ≤5, ≥1 CFU/g; TTP^8: ≤1, ≥0.01 CFU/g; with samples found negative at
414 T8 estimated to have levels ≤0.01 CFU/g. Performance measures evaluating the ability
415 of the model to predict true positives and true negatives showed the model to be highly
416 sensitive (100%) for identifying samples with concentrations equal to or greater than the
417 predicted levels. Further, false negative rates were 0% and negative predictive values
418 were 100%. However, more variability was observed with respect to specificity, positive
419 predictive value and accuracy performance measures (Table 4). For samples
420 contaminated at higher levels (log CFU/g 0.95 with a TTP of 4 h), BAX and 3M assays
421 demonstrated false positive rates (as defined above) of 17% and 7%, respectively. It
422 was also noted that samples inoculated with higher levels of *Salmonella* appeared to
423 have an increased competitive advantage over the background flora, resulting in a
424 decreased time to detection, possibly due to the production of siderophores or microcins
425 (Guillier et al., 2013; Sassone-Corsi et al., 2016).
426
427 Samples inoculated at lower starting concentrations (≤ 1 CFU/g) had greater false
428 positive rates (as defined above) and were more likely to be misclassified as having a
429 greater starting concentration than that experimentally measured. The likelihood of this
430 was greater for samples tested with BAX than with the 3M assay, with false positive
431 rates of 61% and 43%, respectively (Table 4 and Figure 1). This is likely the result of
432 differences in sensitivity between the two assays. While both assays target DNA, each

433 uses a different chemistry. The BAX assay is qPCR based while the 3M uses isothermal
434 amplification. Also, each assay evaluates slightly different enrichment sample volumes
435 with different lysis buffer systems and molecular targets. As such, we found that for
436 enrichment samples where the *Salmonella* concentration had reached an average log
437 CFU/ml of 2.44 (n=31) or 3.46 (n=31), BAX was able to detect it in 51.6% and 83.9% of
438 samples, while 3M detected 32.3% and 74.2%, respectively (Table 3). By the time
439 *Salmonella* levels in enrichments reached an average log CFU/ml of 4.43 (n=28) or 5.51
440 (n=18) the difference in sensitivity was negligible however, as both BAX and 3M
441 detected *Salmonella* in 96.4% and 100% of enrichments with these levels, respectively.
442 As a result of its increased sensitivity, the BAX assay identified samples contaminated
443 with lower starting concentrations at earlier timepoints, with concomitantly shorter TTP
444 values. In the present study, samples were collected from enrichments every two hours
445 during the exponential growth phase (T4 to T8). Future work examining TTP with more
446 narrow sampling time periods may result in an improved model of initial contamination
447 level and TTP. The data presented however, demonstrate the impact of method
448 sensitivity on TTP and the need for validation of each detection platform used for this
449 purpose.
450
451 Finally, differences in *Salmonella* growth rates in mTSB and GBE were observed in this
452 study (Table 1) and appeared to contribute to greater variation in specificity and
453 accuracy of the TTP estimation method for identifying samples contaminated in the
454 mean 0.43 to 0.03 CFU/g (log -0.37 to -1.5) range (TTP^6 and TTP^8). Specifically, faster
455 growing strains demonstrated shorter TTP and were identified by the prediction model

as having greater starting concentrations than those measured experimentally. Conversely, slower growing strains appeared to have a diminished capacity to compete with background flora, especially when inoculated at lower levels. It has been observed previously that competition with background flora may exert a marked effect on the growth of *Salmonella*, inhibiting outgrowth in an enrichment medium and leading to failure to detect *Salmonella* when they were actually present (Daiquigan et al., 2016; Litchfield and Insalata, 1973). An example of this phenomenon was observed with both serovar Dublin strains used in this study. When inoculated at the -2 log CFU/g level, the Dublin were found to have reached 6.5 log CFU/ml by T24, while more competitive *Salmonella* strains inoculated at the same level were 100-fold higher with an average 8.18 log CFU/ml. These data suggest that for some *Salmonella* strains detection might be improved by sampling at earlier timepoints (likely T8 to T12) where they have reached the level of detection but have not yet been outcompeted by background microflora. These data also suggest that IMS may be an important tool for detecting *Salmonella* that are poor competitors in GBE. Further research on this phenomenon is needed to examine the distribution of these growth phenotypes within and among *Salmonella* serotypes, and to determine their impact on TTP in different enrichment matrices.

5. Conclusions

We evaluated the use of TTP for estimating *Salmonella* contamination levels in ground beef. The data presented lay the foundation for a rapid, semi-quantitative test making use of a combination of culture and molecular detection methods (Figure 2). Two

assumptions should be met for the results of the test to be valid. The first is that *Salmonella* present in a sample are viable and able to grow in the enrichment conditions being used (medium, incubation temperature, etc.). The second is that the background microflora in a sample are not present at abnormally high concentrations (not exceeding 10^5 to 10^6 CFU/g (Doerscher et al., 2015)), so as to inhibit either the growth of, or the ability to detect, *Salmonella*. With these assumptions met, we found that *Salmonella* contamination levels greater than 5 CFU/g can be detected in GBE at 4h with 100% sensitivity (i.e. the molecular tests did not fail to detect any samples contaminated at this level) and 83% (BAX) to 93% (3M) specificity (i.e. the ability of the molecular tests to accurately identify a sample as being contaminated at the 5CFU/g level at the indicated timepoint, with some samples actually contaminated at lower levels being misclassified as more highly contaminated). The use of direct plating of enrichment samples as described provides confirmation of estimated of counts, evidence of pathogen viability (by comparing results at T0 to later timepoints), and the opportunity to further characterize *Salmonella* isolates. Additional research is needed to determine the value of the TTP method for estimating *Salmonella* levels in naturally contaminated products, and to more thoroughly examine the impact of variation in growth rate of different *Salmonella* strains on time to detection. However, this rapid, semi-quantitative approach has the potential to identify highly contaminated products before they enter the food chain. Use of this approach could positively impact human health, by decreasing exposure to higher *Salmonella* levels more likely to cause disease.

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641

642 **Table and figure legends**
643

644 **Table 1**

645 *Salmonella* strains used and average growth rates observed in GBE and mTSB at 42°C.
646 DT is doubling time in min; Delta (∆) DT is the difference in DT between mTSB and
647 GBE.

648

649 **Table 2**

650 *Salmonella* Mean Log initial CFU/g and corresponding CFU/ml in GBE at the given
651 timepoints, along with percent of samples found positive with the detection methods
652 indicated. Values in bold indicate concentration and timepoint where all methods
653 detected *Salmonella* present.

654

655 **Table 3**

656 Summary of detection limits of the IMS, BAX and 3M tests used to detect *Salmonella* in
657 GBE. A total of 236 enrichment samples where *Salmonella* was detected by any of the
658 three methods used, were grouped by *Salmonella* concentration (log CFU/ml) as
659 determined by the culture methods described. The number of samples in each group
660 (n), mean log CFU/ml and standard deviation (SD) are reported, as well as the percent
661 of samples in each category, found positive by the detection method indicated. The heat
662 map highlights the *Salmonella* log ranges where the detection methods used
663 demonstrated greater variability in performance (dark grey). Bold text indicates the log

range where all detection methods used consistently identified the presence of *Salmonella*.

Table 4

Performance measures for the time-to-positivity (TTP) model to predict estimated *Salmonella* CFU/g in ground beef. Presence of *Salmonella* was assayed using two molecular tests (BAX and 3M) and the timepoints (T in hours) evaluated are listed.

Figure 1

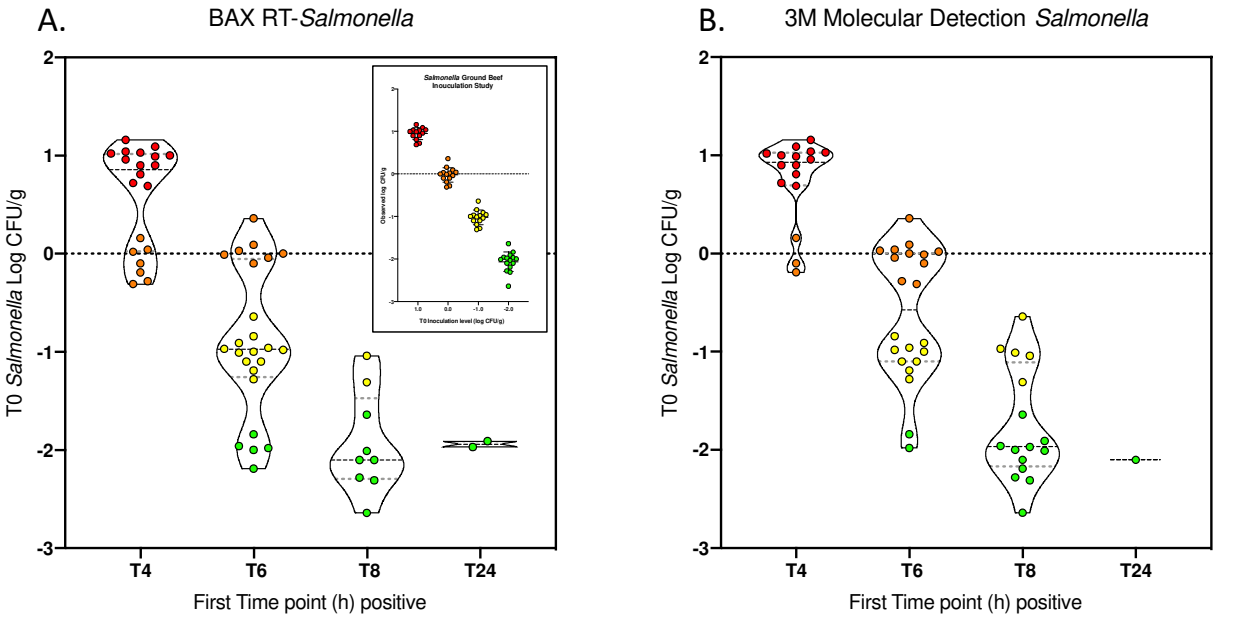
Violin plot depicting distribution of initial *Salmonella* contamination level (log CFU/g) in ground beef, by timepoint first detected positive with either the BAX (1A) or the 3M (1B) detection assays. Inset of 1A. shows the observed log CFU/g starting concentrations for the 56 inoculated ground beef samples, grouped and colored by log range, with red log 1.0 (n=13), orange log 0.0 (n=14), yellow log -1.0 (n=14), and green log -2.0 (n=15). The tables below each graph summarize the initial mean log CFU/g, 95% confidence interval, min and max values within each timepoint positive category.

Figure 2

Summary of time-to-positivity (TTP) method for estimating *Salmonella* contamination level in ground beef. Estimated level of *Salmonella* can be determined within 10 to 12 h. Culture confirmation of estimated counts using EB Petrifilm™ replicaplated to XLD can be completed within 48 to 72h.

687 **Figure S1.** Depiction of the EB Petrifilm™ replicaplate method for the estimation of
688 *Salmonella* counts using XLD medium. The XLD plates in panels 4A. and 4B. show the
689 results post replica plating and incubation, with 4A. showing a majority of non-
690 *Salmonella* colonies (yellow) and 4B. showing a majority of *Salmonella* colonies (black)
691 on the agar plate.
692

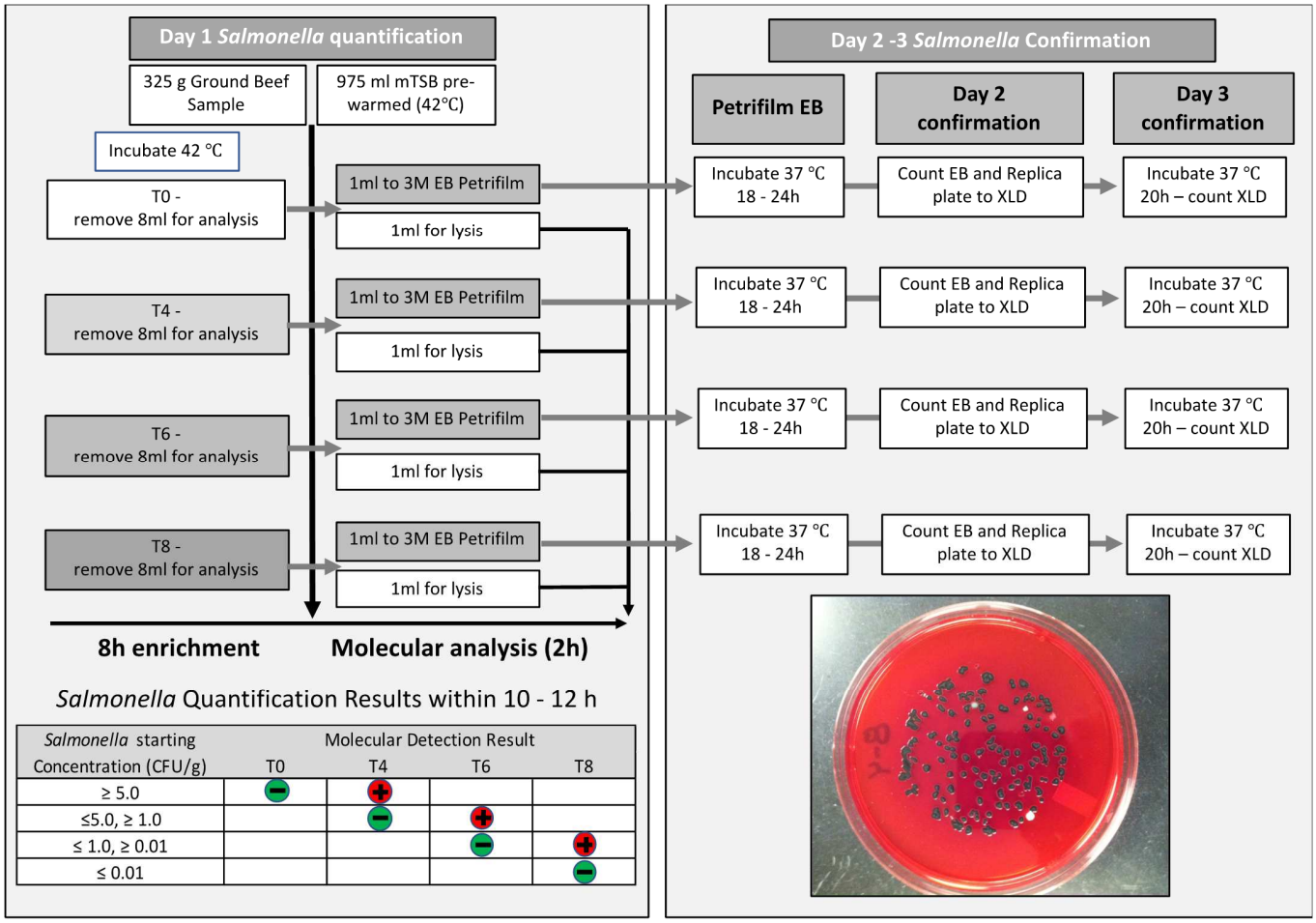
Figure 1.



BAX	T4	T6	T8	T24
n	20	24	9	2
Mean T0 Log CFU/g	0.58	-0.90	-1.94	-1.94
95% CI (lower, upper)	0.33 , 0.83	-1.22 , -0.58	-2.33 , -1.54	-2.32 , -1.56
min Log CFU/g	-0.31	-2.19	-2.64	-1.97
max Log CFU/g	1.16	0.36	-1.04	-1.91

3M	T4	T6	T8	T24
n	16	22	16	1
Mean T0 Log CFU/g	0.76	-0.61	-1.75	-2.10
95% CI (lower, upper)	0.54 , 0.99	-0.90 , -0.31	-2.06 , -1.44	-
min Log CFU/g	-0.19	-1.98	-2.64	-2.10
max Log CFU/g	1.16	0.36	-0.64	-2.10

Figure 2.



Salmonella Quantification Results within 10 - 12 h

Salmonella starting Concentration (CFU/g)	Molecular Detection Result T0	T4	T6	T8
≥ 5.0	−	+		
≤5.0, ≥ 1.0		−	+	
≤ 1.0, ≥ 0.01			−	+
≤ 0.01				−

703

704

Table 1.

	Serotype	Strain	Isolation Source	DT (min) at 42ºC[a] GBE (SD)	DT (min) at 42ºC[a] mTSB (SD)	ΔDT
FAST	Newport	N39	Bovine	13.4 (1.04)	17.9 (0.53)	4.5
	Enteritidis	95-14327	Human	14.2 (2.01)	19.8 (0.25)	5.6
	Anatum	A29	Bovine	15.1 (0.99)	18.9 (0.53)	3.8
	Typhimurium (1,4,[5],12:i:-)	3-H79	Bovine	16.2 (0.88)	20.2 (1.18)	4.0
	Typhimurium[b]	T36	Bovine	16.5 (1.17)	20.3 (1.18)	3.8
	Anatum	08-1092	Human	16.6 (0.23)	19.0 (0.56)	2.4
	Montevideo	2012K-1544	Human	16.8 (0.87)	19.8 (0.52)	3.0
	Average			15.5[ce] (1.58)	19.4[cf] (1.06)	3.9[g]
SLOW	Newport	2010K-2159	Human	17.2 (1.31)	19.6 (0.72)	2.4
	Enteritidis	95-2876	Human	18.5 (1.34)	22.4 (2.21)	3.9
	Dublin	SM73-2	Bovine	19.3 (1.00)	21.4 (0.58)	2.1
	Dublin	5-75-E	Bovine	19.3 (0.98)	20.9 (1.34)	1.6
	Newport	N17	Bovine	19.9 (1.09)	19.0 (0.42)	-0.9
	Montevideo[b]	H06	Human	20.6 (1.27)	20.6 (1.85)	0.0
	Typhimurium	14028S	Human	22.9 (2.08)	26.9 (1.96)	4.0
	Average			19.5[de] (1.95)	21.4[df] (2.68)	1.8[g]

[a] Average T0 inoculum was 0.89 CFU/g (95% CI = 0.69 - 1.08)

[b] Salmples incubated at 37ºC not 42ºC

Two-tailed, unpaired t-test of statistical significance with $P \leq 0.05$ defined as significantly different. Common superscript indicates values evaluated and outcome as follows: [c] Yes, $P < 0.0001$; [d] Yes, $P = 0.0358$; [e] Yes, $P = 0.0004$; [f] No, $P = 0.0632$; [g] Yes, $P = 0.0272$

Table 2.

Contamination level	Mean initial level	Enrichment Timepoint	Mean log *Salmonella* cfu/ml	SD	% IMS positive	% BAX positive	% 3M positive
n = 13		T0	0.47	0.14	76.9	0	0
Mean T0 CFU/g	9.25	**T4**	**3.14**	**0.42**	**100**	**100**	**100**
Mean T0 log CFU/g	0.95	T6	5.25	0.67	100	100	100
lower 95%CI of mean log	0.86	T8	7.51	0.96	100	100	100
Upper 95%CI of mean log	1.03	T24	8.60	0.66	100	100	100
n = 14		T0	-0.49	0.19	7.14	0	0
Mean T0 CFU/g	1.04	T4	2.41	0.33	100	50.0	21.4
Mean T0 log CFU/g	-0.02	**T6**	**4.28**	**0.55**	**100**	**100**	**100**
lower 95%CI of mean log	-0.12	T8	6.54	0.94	100	100	100
Upper 95%CI of mean log	0.08	T24	8.49	0.77	100	100	100
n = 14		T0	-1.49	0.16	0	0	0
Mean T0 CFU/g	0.10	T4	1.47	0.36	92.9	0.0	0
Mean T0 log CFU/g	-1.02	T6	3.42	0.41	100	85.7	64.3
lower 95%CI of mean log	-1.12	**T8**	**5.57**	**0.80**	**100**	**100**	**100**
Upper 95%CI of mean log	-0.92	T24	8.12	1.21	100	100	100
n = 15		T0	-2.54	0.23	0	0	0
Mean T0 CFU/g	0.01	T4	0.61	0.43	66.7	0	0
Mean T0 log CFU/g	-2.07	T6	2.44	0.47	100	33.3	13.3
lower 95%CI of mean log	-2.19	T8	4.42	0.73	100	86.7	86.7
Upper 95%CI of mean log	-1.94	**T24**	**7.55**	**1.30**	**100**	**100**	**100**

Table 3.

Average Observed *Salmonella* log CFU/ml	SD	n	IMS +	3M +	BAX +
0.45	0.24	24	70.8	0.0	0.0
1.39	0.30	19	89.5	0.0	0.0
2.44	0.29	31	100	32.3	51.6
3.46	0.30	31	100	74.2	83.9
4.43	0.34	28	100	96.4	96.4
5.51	0.27	18	100	100	100
6.44	0.25	20	100	100	100
7.52	0.32	25	100	100	100
8.48	0.26	29	100	100	100
9.48	0.20	11	100	100	100

Table 4.

Assay	BAX			3M		
Estimated CFU/g	≥ 5.0	≤ 5.0, ≥ 1.0	≤ 1.0, ≥ 0.01	≥ 5.0	≤ 5.0, ≥ 1.0	≤ 1.0, ≥ 0.01
TTP	T4	T6	T8	T4	T6	T8
n	20	35	11	16	39	17
Sensitivity	100	100	100	100	100	100
NPV	100	100	100	100	100	100
False Negative Rate	0	0	0	0	0	0
Specificity	83.3	39.0	40.0	92.9	56.7	25.0
PPV (precision)	65.0	29.0	67.0	81.3	40.9	81.2
Accuracy	87.3	51.0	72.7	94.5	66.7	82.3
False Positive Rate	16.7	60.7	60.0	7.0	43.0	75.0