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# Development of a rapid method to quantify *Salmonella* Typhimurium using a combination of MPN with qPCR and a shortened time incubation

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ABSTRACT

A novel method was developed for the specific quantification of *S.* Typhimurium using a most-probable-number (MPN) combined with qPCR and a shortened incubation time (MPN-qPCR-SIT). For *S.* Typhimurium enumeration, dilutions of samples were transferred into three wells on a microtiter plate and the plate was incubated for 4 h. The *S.* Typhimurium presence in the wells was identified using a qPCR and populations were determined based on an MPN calculation. The $R^2$ between the MPN-qPCR-SIT and conventional MPN exhibited a high level of correlation (0.9335–0.9752), suggesting that the MPN-qPCR-SIT offers a reliable alternative method for *S.* Typhimurium quantification. Although plating and qPCR were limited in their ability to detect low levels of *S.* Typhimurium (e.g. 0.18 log MPN/ml), these levels could be successfully detected with the MPN-qPCR-SIT. Chicken breast samples inoculated with *S.* Typhimurium were incubated at 0, 4, and 24 h and incubated samples were subjected to microbiome analysis. Levels of *Salmonella* and Enterobacteriaceae increased significantly with incubation time. The obvious benefits of the MPN-qPCR-SIT are: 1) a further confirmation step is not required, 2) the detection limit is as low as conventional MPN, but 3) is more rapid, requiring approximately 7 h to simultaneously complete quantification.



## 1. Introduction

Since *Salmonella* species are one of the most prevalent pathogens causing foodborne disease in humans, it is of considerable concern to the food industry to reduce contamination and control transmission to consumers. Effective microbiological testing to not only detect but quantitate *Salmonella* could play a significant role in reducing *Salmonella* occurrence (Crump et al., 2002; De Medici et al., 2003; Koyuncu et al., 2010). Conventional methods based on plating on selective and differential agar media and identifying colonies with either biochemical tests or molecular methodologies have been traditionally used to enumerate target bacteria in food and environmental specimens. Unfortunately, these methods have shown some limitations in specificity and sensitivity for accurate detection. Also, conventional culturing methods require considerable time (5–7 days) and labor before *Salmonella* can be confirmed which can be especially problematic for the food industries (Costello et al., 2002; Kim et al., 2013). For these reasons, development of rapid, reliable, and cost-effective high-throughput enumeration methods would be highly desirable to overcome the disadvantages of conventional methods.

The most probable number method (MPN) approach has been used to obtain quantitative data on bacterial contamination in samples and involves inoculating decimal dilutions into tubes of a broth medium, observing results, calculating the estimated bacteria number using a standard MPN table based on the respective statistical analyses. This method has been used by a wide range of industries (Sutton, 2010) and is also recommended as an official analytical method by governmental agencies such as the U.S. Department of Agriculture (USDA) for low numbers of *Salmonella* in watersheds (Jenkins et al., 2008) and the Food and Drug Administration (FDA) for low numbers of *Staphylococcus aureus* in foods (FDA, 2014). The advantages of an MPN method include simplicity and the ability to detect target bacteria with very low detection limits (below 10 to 100 MPN/g); however, like conventional plating method, it also requires a long incubation time period (greater than

*Abbreviations:* CFU, colony forming unit; MPN, most-probable-number; SIT, shorted incubation time; qPCR, quantitative polymerase chain reaction.

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4 days) to complete quantification since further confirmation steps using biochemical or molecular test must be performed (Martin et al., 2004).

Advanced molecular techniques have been developed over the last few decades to overcome some of the disadvantages associated with culture based methods. Quantitative PCR (qPCR) has been widely used to quantify foodborne pathogens in food or clinical samples. The real-time aspect of qPCR allows rapid quantification of target genes (generally less than 2 h) by quantifying the generated products throughout the ongoing amplification process and exhibiting considerable high accuracy and sensitivity (Corless et al., 2001). However, the detection limit of qPCR is relatively poor compared to MPN methods particularly in food products. For example, the minimum detection levels for *Salmonella* spp. in broth and sterilized milk were reported to be greater than $10^2$ and $10^3$ colony forming unit (CFU)/ml, respectively (Nam et al., 2005).

In this study, we combined MPN and qPCR for quantifying microorganisms to merge the advantages of both methods such as lowering the detection limit of MN and increasing the specificity, reliability and rapidity of qPCR. The purpose of this study was to develop a novel method for rapid quantification of *S.* Typhimurium in poultry products that permits an accurate and rapid estimation of the degree of food and environmental contamination by *S.* Typhimurium using MPN combined with qPCR and a shorted incubation time (MPN-qPCR-SIT). For comparison, the populations of *S.* Typhimurium in a cell suspension and a mixed culture (*S.* Typhimurium with other bacteria from chicken rinsates) were estimated by various quantification methods including the method described here, conventional plating method, conventional MPN, and qPCR. The proposed method was validated using chicken breast inoculated with mixed culture. Since incubation time and background microbiota can be a major factor in the accuracy of enumeration methods, microbiome analysis was also conducted on samples from chicken breast inoculated with mixed cultures at various incubation times to determine bacterial compositional shifts occurring over the incubation time period.

## 2. Materials and methods

### 2.1. Bacterial strain

*Salmonella* Typhimurium ATCC 14028 marker strain (nalidixic acid resistance strain) was stored at −20 °C in tryptic soy broth (TSB; Becton Dickinson and Company, Sparks, MD) supplemented with nalidixic acid (NA, 20 μg/ml) also containing 20% glycerol. To prepare a working stock of the bacterial culture, one loop of glycerol stock culture was inoculated into fresh TSB+NA and then enriched at 37 °C for 24 h. One loop of enriched culture was streaked onto tryptic soy agar (TSA; Becton Dickinson and Company) supplemented with NA, incubated 37 °C and maintained at 4 °C, with subculturing performed at monthly intervals.

### 2.2. Preparation of S. Typhimurium pure cell suspension

One colony on TSA supplemented with NA was expanded by growing cultures in 10 ml TSB with NA at 37 °C for overnight. The *S.* Typhimurium pure cell culture was centrifuged at 14,000 rpm for 5 min (Centrifuge 5804 R, Eppendorf, Germany). The supernatants were discarded and the cell pellets were washed twice with sterile phosphate buffered saline (PBS; Amresco, Solon, OH). After centrifugation, the final cell pellet was resuspended in the same buffer. A portion of the cell suspension was diluted into sterile PBS to obtain incrementally different bacterial concentrations (approximately 1 to $1 \times 10^5$ CFU/ml).

### 2.3. Preparation of mixed culture (S. Typhimurium with non-target bacteria from the chicken carcass rinsate)

Whole chicken carcasses were purchased from a local market (Fayetteville, AR, USA). Chicken carcass rinsates were obtained based on the U.S. Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) method (MLG 4.08). Chicken carcasses were transferred to sterile bags and 400 ml of sterile PBS were added to each bag. The sterile bag was manually shaken for 2 min assuring that all surfaces were rinsed. Chicken carcass rinsates were transferred to sterile bottles and then stored at −20 °C. Prior to experiments, the chicken rinsates were tested for previous contamination with *S.* Typhimurium. Confirmation was accomplished by streaking on xylose lysine tergitol 4 agar (XLT4; Becton Dickinson) after enrichment in TSB 37 °C for 24 h and qPCR. Briefly, 10 ml of chicken rinsate were added to 90 ml of fresh TSB and subsequently homogenized. After incubation at 37 °C for 24 h, one loopful of enrichment cultures was streaked on XLT4 and incubated at 37 °C for 24 h. In addition, 1 ml of enriched culture was utilized to DNA extraction and qPCR was performed as described in subsection 2.5.3. Chicken rinsates with no presumptive *Salmonella* colonies on XLT4 and negative result with qPCR were used to further experiments. Equal volumes from both *S.* Typhimurium pure cell culture suspensions (with different cell populations prepared by the above mentioned method, *S.* Typhimurium populations: approximately 1 to $1 \times 10^5$ CFU/ml) and chicken carcass rinsates were transferred into sterile tubes and vortexed; this was used as a mixed culture.

### 2.4. Spiking mixed culture (S. Typhimurium with non-target bacteria from the chicken carcass rinsate) on chicken breast

Chicken breasts were purchased from a local market. Prior to inoculation with the bacteria, absence of *Salmonella* on the chicken breasts was confirmed with enrichment in TSB for overnight followed by streaking onto XLT4 and qPCR. The 25 g of chicken breast was aseptically taken and homogenized with 225 ml of sterile TSB using a stomacher at 230 rpm for 2 min. The next procedure was same with above mentioned methods in section 2.3 and chicken breast samples with negative results on XLT4 and qPCR were used for additional experiments. The absence of *Salmonella* was confirmed for all chicken breast samples used in this experiment. Mixed cell suspensions possessing various concentrations of *S.* Typhimurium were prepared as described in the previous section (2.3) and 1 ml of mixed suspension inoculated to 25 g of chicken breast meat (a total of sixteen chicken breast samples were used and *S.* Typhimurium populations were approximately $1 \times 10^1$ to $1 \times 10^6$ CFU/ml). Samples were homogenized with 225 ml of TSB+NA using a stomacher at 230 rpm for 2 min.

### 2.5. Enumeration methods

The populations of *S.* Typhimurium in pure cell suspension, in mixed cell suspension, and on chicken breast inoculated with mixed cell suspension was determined with various enumeration methods including conventional selective plating, conventional MPN, qPCR, and the MPN-qPCR-SIT developed in this study. The procedure for each quantification method is presented in Fig. 1.

#### 2.5.1. Conventional spread plating method

To quantify *S.* Typhimurium, 1 ml aliquots of prepared samples (pure bacterial cell cultures, mixed bacterial cell cultures, and mixed bacterial cell cultures on chicken breast) were serially diluted in 9 ml of sterile PBS. A sample (100 μl) of undiluted and each corresponding dilution were subsequently spread-plated in duplicate on TSA+NA (for the pure cell culture experiment) or