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Digital PCR assay for the specific detection and estimation of *Salmonella* contamination levels in poultry rinse

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ABSTRACT

Strains of *Salmonella* are a frequent cause of foodborne illness and are known to contaminate poultry products. Most *Salmonella* testing methods can qualitatively detect *Salmonella* and cannot quantify or estimate the *Salmonella* load in samples. Therefore, the aim of this study was to standardize and validate a partitioned-based digital PCR (dPCR) assay for the detection and estimation of *Salmonella* contamination levels in poultry rinses. Pure culture *Salmonella* strains were cultured, enumerated, cold-stressed for 48 h, and used to inoculate whole carcass chicken rinse (WCCR) at 1–4 log CFU/30 mL and enriched at 37 °C for 5 h. Undiluted DNA samples with primer and probes targeting the *Salmonella*-specific *invA* gene were used for the dPCR assay. The dPCR assay was highly specific, with a limit of detection of 0.001 ng/μL and a limit of quantification of 0.01 ng/μL. The dPCR assay further showed no PCR reaction inhibition up to 5 μg of crude DNA extract. The assays accurately detected all cold-stressed *Salmonella* in inoculated WCCR samples following a 5-h enrichment. Most importantly, when converted to log, the dPCR copies/μL values accurately estimated the inoculated *Salmonella* levels. The dPCR assay standardized in this study is a robust method for the detection and estimation of *Salmonella* concentration in contaminated food samples. This approach can allow same-day decision-making for poultry processors attempting to maintain limits and controls on *Salmonella* contamination.

1. Introduction

Salmonella enterica is a Gram-negative facultative intracellular anaerobe with over 2500 serovars divided into six subspecies. Virulent strains of *Salmonella* are the second most frequent cause of foodborne illness and are estimated to cause 1.35 million foodborne illnesses in the U.S., with 26,500 hospitalizations resulting in 420 deaths annually (CDC, 2023). *Salmonella* infections have been among the utmost concerns for the United States Department of Agriculture (USDA) as the incidence rates have remained consistent for the past two decades (USDA, 2022b). Over these past two decades, the USDA has implemented policies across industries to achieve the national target, in conjunction with the United States Department of Health and Human Services-Office of Disease Prevention and Human Promotion, of a 25% reduction in *Salmonella* infection rates from 15.3 to 11.5 cases per 100,000 population by the end of 2030 (Healthy People, 2021).

Strains of *Salmonella* are known to contaminate fresh produce, meat, and poultry products (Gould et al., 2013). Among these food categories, *Salmonella* infection in humans is frequently associated with the consumption of contaminated poultry products. According to the Interagency Food Safety Analytics Collaboration (IFSAC) 2022 report, over 23% of foodborne *Salmonella* infections were linked to poultry consumption, of which 17% were chicken and 6% were turkey (USDA, 2022b). Strains of *Salmonella* are attached to the food animals' exterior surface (i.e., hide, feather), and they are further known to colonize their internal organs, which contaminates the meat and poultry products during processing.

Over the last decade, the USDA Food Safety and Inspection Service

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(USDA-FSIS) has made a comprehensive effort (i.e., USDA-FSIS *Salmonella* performance standards) to reduce *Salmonella* infections associated with poultry products (USDA, 2022a). Between 2017 and 2021, FSIS achieved a reduction of *Salmonella*-positive chicken samples by more than 50%; however, this reduction of *Salmonella* contamination has not shown a reduction in the incidence rates of *Salmonella* infections in humans (USDA, 2022b). The current USDA-FSIS *Salmonella* performance standards permit several samples within a sample set to test positive for the presence of *Salmonella*; however, this performance standard does not address the actual *Salmonella* concentration levels (CFU/g) in the samples that test positive.

In the United States, 1532 foodborne outbreaks were reported between 1998 and 2019. Of these 1532 outbreaks, 943 (61%) were confirmed or suspected to be caused by *Salmonella*. Further, outbreak data from 2015 to 2019 showed that outbreaks potentially associated with *Salmonella* had increased to 73% (IFSAC, 2021). Among these foodborne *Salmonella* outbreaks, when estimation of *Salmonella* contamination levels has been possible, 83% of those outbreaks show that these products were contaminated with a load of 100 CFU or greater (Harhay et al., 2021; Teunis et al., 2010). The increase in *Salmonella*-associated outbreaks and higher risk associated with food contaminated with high levels of *Salmonella* emphasize the need to develop robust methods for the estimation of *Salmonella* load in food samples.

Currently, regulatory agencies and other food testing laboratories rely on the selective enrichment of samples, a combination of real-time quantitative polymerase chain reactions (qPCR), and culture-based methods for specific detection and confirmation of *Salmonella* in food samples (Lee et al., 2015). One of the limitations of methods commonly used by the testing laboratories (i.e., real-time PCR-based *Salmonella* detection assay) is the inability to quantify or estimate the *Salmonella* load in the original sample. The Most Probable Number (MPN)-based methods are recommended for the enumeration of *Salmonella* in food samples (Hussong et al., 1984; Santos et al., 2005). However, the MPN-based *Salmonella* detection methods require an extended enrichment period, making the method undesirable for the food industry. Recently, the GENE-UP® QUANT *Salmonella* (bioMerieux Inc. Durham, North Carolina, USA) and BAX SalQuant (Hygiena, Camarillo, California, USA) received AOAC certification for quantification of *Salmonella* in poultry samples. These two commercially available assays rely on plotting the Cq value from test samples on the standard curve for estimating *Salmonella* concentration. However, the best estimates often require the end users to use or generate their own standard curves for each specific food matrix. Due to these, it is evident that even though commercially available methods for estimating *Salmonella* load are available, there is still a need for a better, more straightforward, and more robust *Salmonella* estimation assay.

For over 20 years, qPCR has been the primary technology for the detection of nucleic acid samples and foodborne pathogens. Though the use of qPCR has significantly been used for the detection of foodborne pathogens, the limitations include susceptibility to PCR inhibitors, the need to generate a standard curve for quantification, and susceptibility to variation in Cq-values with change in DNA sample types or lab technician, which affects the reproducibility of qPCR-based relative quantification assays (Pinheiro and Emslie, 2018). Advancements in PCR technology have been made in absolute nucleic acid quantification methods and instrumentation. Currently, droplet digital (ddPCR) and partition-based digital PCR (dPCR) methods are commercially available. The principle behind a dPCR reaction is that the reaction mixture is equally partitioned into thousands of partitions within the dPCR nanoplate. Each partition contains primers, probes, and a master mix to complete the reaction. However, it may or may not contain a target DNA analyte. The PCR reaction is individually performed in each micro-partition. The significant advantage of the dPCR-based absolute quantification approach compared to the qPCR-based relative quantification approach is greater precise quantification of targets without the need to create an external standard curve, the ability to tolerate PCR-inhibitors, the capability to detect targets in highly concentrated DNA samples without any dilution, and less susceptibility to competition from other targets (Bian et al., 2015; Maggi et al., 2020; Moniri et al., 2020; Sedlak et al., 2014; G. Singh et al., 2017; Tiwari et al., 2022; Wang et al., 2018; Wolffs et al., 2006). Moreover, the partition-based dPCR workflow and instrumentation cost is similar to the traditional qPCR-based workflow used by regulatory agencies and private food testing laboratories. Therefore, the aim of this study was to standardize and validate a partition-based dPCR assay for same-day detection and estimation of *Salmonella* levels in poultry rinses.

2. Materials and methods

2.1. Pure culture bacterial strains

Pure culture strains for this study were obtained from a food microbiology laboratory culture collection (Florida State University, Tallahassee, Florida, USA), the Roman L. Hruska U.S. Meat Animal Research Center (Clay Center, Nebraska, USA), and the U.S. National Poultry Research Center (Athens, Georgia, USA). The dPCR assay was validated using 131 pure culture strains among 87 *Salmonella* serovars with 46 *Escherichia coli* strains for specificity testing (Supplementary Tables 1 and 2). The assay was further validated using 16 *Salmonella* strains, which consisted of three serovars [Typhimurium (n = 6), Enteritidis (n = 5), Infantis (n = 5)] inoculated in whole carcass chicken rinse (WCCR)[2] (Table 1). All strains were grown in 10 mL Tryptic Soy Broth (TSB) (Hardy Diagnostics, Santa Maria, CA, USA). Crude DNA from 100 μL all overnight pure culture strains was isolated using the Extracta DNA Prep for PCR protocol (Quantabio, Beverly, MA, USA).

2.2. Oligonucleotides

Salmonella-specific primer and hydrolysis probe targeting the *invA* gene validated in our previous study were used for the dPCR assay (Table 2) (Singh and Mustapha, 2013).

2.3. Whole carcass chicken rinse preparation

Whole chickens were purchased from local supermarkets (Tallahassee, Florida). The chicken was placed into a sterilized autoclave bag

Table 1
Sixteen *Salmonella* Strains used for inoculating chicken rinses.

Serovar	Strains
Enteritidis	PT30
	96037
	CRJJGF00005
	CRJJGF00007
	CRJJ00008
Infantis	25
	36
	48
	82
	94
Typhimurium	1808
	1810
	1880
	1896
	1898
	14028

[2] whole carcass chicken rinse (WCCR).

Table 2
Oligonucleotides used in the study.

Name	Primer Sequence	Target Gene	Product Size	Reference
150-invF	5′-CCAGTTTATCGTTATTACCAAAGG-3′	*invA*	198 bp	Singh and Mustapha (2013)
151-invR	5′-ATCGCACCGTCAAAGGARC-3′			
79-invP	/56-FAM/CT CTG GAT G/ZEN/G TAT GCC CGG TAA ACA/3IABkFQ/			

along with 400 mL neutralizing Buffered Peptone Water (nBPW) (Hardy Diagnostics, Santa Maria, CA, USA) and was hand shaken for 2 min to prepare chicken rinses (USDA, FSIS, MLG 4.14, 2023). The whole carcass chicken rinse (WCCR) was then individually transferred to autoclaved media bottles. All WCCRs were tested for the presence of *Salmonella* using the standard method from MLG 4.14 (USDA, 2023). Any WCCR samples that produced a black colony on Xylose Lysine Deoxycholate (XLD) (Hardy Diagnostics, Santa Maria, CA, USA) plates were isolated, purified by streaking, and the identity of the isolate was verified using VITEK 2 GN ID card (bioMerieux Inc. Hazelwood, MO, USA). Antibiotic susceptibility of the isolates was characterized using VITEK 2 AST-GN69 cards (bioMerieux Inc. Hazelwood, MO, USA) following the manufacturer's instructions. Only rinses that tested negative for the presence of *Salmonella* were used for the validation study. Thirty-milliliter aliquots of WCCR were transferred to sterile 50 mL centrifuge tubes, stored at −20 °C, and used for assay validation described below.

2.4. Inoculation of Salmonella in WCCR

Sixteen *Salmonella* strains were sub-cultured twice at 37 °C in 10 mL of tryptic soy broth (TSB) (Hardy Diagnostics, Santa Maria, CA, USA). After the second subculture, the cultures were serially diluted in maximum recovery diluent (MRD). The serial dilutions were plated on plate count agar (PCA) (Hardy Diagnostics, Santa Maria, CA, USA). The PCA plates were aerobically incubated at 37 °C for approximately 24 h. The MRD was stored in a refrigerator at 4 °C for 40–48 h to facilitate environmental cold-stressing of the strains, which can impact their lag phase (Harhay et al., 2021). Before inoculation, the 50 mL conical tubes of chicken rinse were thawed overnight in the refrigerator at 4 °C. Counts from PCA plates were used to calculate the appropriate dilution and volume needed for inoculating 30 mL of WCCR at 1, 2, 3, and 4 log CFU/30 mL. The inoculum volume used to inoculate WCCR at log 1 was spread-plated on PCA to enumerate the *Salmonella* inoculation load. In each experiment set, two non-inoculated WCCR samples and one water no template control were used as negative controls. All samples were inoculated in duplicates, and the study was independently performed twice.

2.5. WCCR enrichment and DNA extraction

A modified 2 × BPW (mBPW) (Hardy Diagnostics, Santa Maria, CA, USA) containing filter-sterilized novobiocin (15 mg/L) and sodium pyruvate (1 g/L) was used for enrichment. Fifteen mL of pre-warmed (42 °C) 2 × mBPW were added to each inoculated WCCR before aerobically incubating the 50 mL conical tube at 37 °C for 5 h. Then, each was centrifuged at 3200×*g* for 5 min at 4 °C using a refrigerated centrifuge (Beckman Coulter, Indianapolis, Indiana, USA). DNA from the obtained cell pellets was isolated using the Extracta DNA Prep for PCR protocol (Quantabio, Beverly, MA, USA). The cell pellet was resuspended with 200 μL of extraction reagent (Quantabio, Beverly, MA, USA). The samples were vortexed until the cell pellet was fully resuspended, then heated at 95 °C for 10 min to facilitate cell lysis. Following the lysis step, samples were allowed to cool for 5 min at room temperature before centrifuging at 20,000×*g* for 2 min. Lastly, 60 μL of supernatant was transferred to a new 1.5 mL centrifuge tube and diluted with 90 μL of stabilization buffer reagent (Quantabio, Beverly, MA, USA). The obtained mother stock DNA concentrations were quantified using a Nanodrop One Spectrophotometer (Thermo Fisher, Wilmington, DE, USA).

2.6. Digital PCR

dPCR assay was performed on the QIAcuity One system (QIAGEN, Hilden, Germany) with a 4x QIAcuity Probe PCR kit (QIAGEN, Hilden, Germany). For the detection of pure culture strains, the 24-well 8.5k nanoplate (QIAGEN, Hilden, Germany) was used. Each16 μL dPCR reaction consisted of 3.2 μL of 10 ng *Salmonella* or non-*Salmonella* strain DNA, 4 μL of the 4x QIAcuity Probe Master mix, 800 nM of both the forward and reverse primers, and 400 nM probe. A 14.5 μL aliquot of each reaction mixture was loaded per well. For the quantitation of *Salmonella* in chicken rinse, the 24-well 26K nanoplate (QIAGEN, Hilden, Germany) was used. Each 50 μL dPCR reaction mix consisted of 9.9 μL of mother stock DNA, 12.43 μL of the 4x QIAcuity Probe Master mix, 800 nM of both the forward and reverse primers, and 400 nM probe. A 45 μL aliquot of each reaction mixture was loaded per well. Amplification was carried out with an initial denaturation at 95 °C for 2 min, followed by 40 cycles of two-step PCR amplification of denaturation at 95 °C for 15s and annealing and extension at 60 °C for 30s. Images were acquired in the green (FAM) channel with an exposure duration of 500 ms (ms) at a gain of 6. The final data was analyzed using the QIAcuity Software Suite 2.0.20.

2.7. Reanalysis of saturated dPCR samples

Our workflow used undiluted mother-stock DNA for the assay. DNA samples showing close to the saturation level of positive partition were re-tested by diluting the DNA sample by a factor of 10 or 100 to generate more precise absolute quantification data. Data generated using these diluted samples were multiplied by the dilution factor, and the values were used to estimate *Salmonella* concentration.

2.8. Effect of variation between chicken carcass samples

During the standardization and validation of the study, multiple chicken carcasses obtained from the same store and brand were processed in parallel, and the obtained WCCRs were portioned in 50 mL centrifuge tubes and stored at −20 °C until needed. To show that the observed variation between the results of replicate one and two was associated with different batches of WCCR, we conducted a study with three strains (*Salmonella* Infantis 25, *Salmonella* Infantis 82, and *Salmonella* Enteritidis CRJJ00008), which were tested in parallel with WCCRs obtained from two different chicken carcasses. The rinses were inoculated in duplicates at log 1 and 2 CFU/30 mL concentrations, enriched and tested by dPCR assay as described above.

2.9. Limits of detection (LOD) and limits of quantification (LOQ)

Pure culture DNA of three *Salmonella* strains (Enteritidis CRJJ00008, Infantis 25, and Typhimurium 14028) were serially diluted to 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng/μL concentrations. dPCR assay was performed in a single replicate using serially diluted DNA samples to determine LOD and LOQ. The LOD was determined by observing data for the lowest DNA concentration, which showed at least one positive partition. The LOQ was determined as the lowest DNA concentration with a coefficient of variation below 25% (Cai et al., 2017; Floren et al., 2015).

2.10. dPCR data analysis

Thresholds for each experiment were manually set slightly above the negative cluster. The dPCR data were analyzed using the QIAcuity Software Suite (version 2.0.20). All the sample concentrations (copies/μL) values obtained for each sample were log transformed and used to estimate the *Salmonella* load in the samples. The log inoculum levels and log values obtained from dPCR for each sample were compared with a one-sample *t*-test (GraphPad Prism Version 9.4.1, Boston, MA, USA).

3. Results

3.1. Assay standardization

Our dPCR assay standardized in this study was able to accurately quantify all 16 strains belonging to the three *Salmonella* serovars inoculated. As expected, our *Salmonella*-specific probe showed 100% inclusivity and exclusivity for all 131 pure culture *Salmonella* strains that were of 87 different serovars and 46 *E. coli* strains (Singh and Mustapha, 2013). The LOD of pure culture DNA samples per serovar (*Salmonella* Enteritidis, *Salmonella* Infantis, and *Salmonella* Typhimurium) was 0.1, 0.1, and 1 pg/reaction, respectively, while the LOQ was 10 pg/reaction for all the strains tested.

3.2. Assay validation of lab-inoculated WCCR

The average inoculation level of 30 mL chicken rinse samples inoculated at log 1 for each serovar Enteritidis (n = 5), Infantis (n = 5), and Typhimurium (n = 6) strains were 1.23, 0.98, and 1.19 log CFU, respectively, with 1.15 log CFU being the average of all 16 strains used in the study. The dPCR analysis software generated sample concentrations of 18–6235, 44–20505, and 53–15264 copies/μL for Enteritidis, Infantis, and Typhimurium strains, respectively, inoculated at log 1–4. When these concentration values (copies/μL) were converted into their respective log values, they closely correlated to their initial inoculum counts (Table 3). The log-transformed values for Enteritidis strains were 1.01, 1.85, 2.84 and 3.71. Similarly, samples inoculated with Infantis strains resulted in log 1.24, 2.05, 3.09, and 4.00, while Typhimurium samples were 1.53, 2.41, 3.29, and 3.93. When the data for all strains were analyzed, the log values were 1.26 ± 0.21, 2.10 ± 0.23, 3.06 ± 0.19, 3.88 ± 0.13 at each inoculation level (Table 4). Compared to the initial inoculum counts, the *Salmonella* levels estimated by the dPCR assay were not statistically different ($p > 0.05$). The experimental design included 30 non-inoculated WCCR and 16 water no template controls. Among these 46 samples, a few negative control samples generated less than ten positive partitions; the average concentration (copies/μL) for non-inoculated WCCR was 0.4 and 0.2 copies/μL for water no template controls.

Table 4
The total average of copies concentration and log of copies for the 16 strains of *Salmonella* serovars.

Log	dPCR Log Value ± SD	Inoculation (CFU)	Inoculation Log Value
1	1.26 ± 0.21	14.0	1.15
2	2.10 ± 0.23	139.7	2.15
3	3.06 ± 0.19	1396.7	3.15
4	3.88 ± 0.13	13966.7	4.15

3.3. Effect of variation between chicken carcass samples

Three strains (Infantis 25, Infantis 82, and Enteritidis CRJJ00008) were inoculated at log 1 and 2 concentrations; the inoculum concentrations were 1.6, 1.15, and 1.46 log CFU, respectively. The dPCR log values from the first set of chicken rinses for Infantis 25, Infantis 82, and Enteritidis CRJJ00008 were estimated at 0.79, 1.47, and 1.45, respectively, for samples inoculated at log 1 samples. Samples inoculated at log 2 were estimated to be 1.31, 2.32, and 2.06, respectively. When the same inoculum concentration was used to spike the second set of chicken rinses, the estimated dPCR log values of Infantis 25, Infantis 82, and Enteritidis CRJJ00008 were log 0.35, 1.07 and 0.86 for log 1 samples, and log 1.11, 2.07 and 1.66 for log 2 samples, respectively. *Salmonella* log levels estimated by dPCR using two rinses, when compared with a paired *t*-test (GraphPad Prism Version 9.4.1), showed that parallelly inoculated rinses from different chickens were significantly different ($p < 0.05$). These observed differences (Supp. Table 3), when samples inoculated at the same concentration, can be attributed to differences in the residual level of sanitizers and chicken composition, protein and fat levels in chicken rinse samples used during poultry processing can interfere with the PCR reaction, eventually resulting in differences between the *Salmonella* estimation levels.

3.4. Identification of presumptive Salmonella isolates

During the preparation of our WCCRs, sixteen rinses showed the presence of black colonies on XLD plates. One of these 16 isolates failed to sub-culture, so it was excluded. The remaining 15 isolates, when tested with the VITEK 2 system and genus-specific qPCR assay, confirmed that 13 strains were *Salmonella,* and two isolates were identified as *Citrobacter braakii* with an identification probability of 95% or higher. Antibiotic susceptibility of these *Salmonella* strains shows that 12 strains were resistant to cefazolin, tobramycin, and gentamicin, with an additional nine also resistant to ampicillin (Supp. Table 4).

4. Discussion

To align with the U.S. poultry industry priorities, these validation studies were performed using Key Performance Indicator (KPI) *Salmonella* strains, i.e., Enteritidis, Typhimurium, and Infantis. These serovars

Table 3
The average concentration and its respective log converted values for all the strains of each serogroup.

Serogroup	Log	Concentration (copies/μL)	Log $_{10}$ of copies/μL	Inoculation Amount
***S.* Enteritidis**	1	18	1.01	16.8
	2	162	1.85	
	3	1167	2.81	
	4	6235	3.71	
***S.* Infantis**	1	44	1.24	9.6
	2	293	2.05	
	3	3485	3.09	
	4	20505	4.00	
***S.* Typhimurium**	1	53	1.53	15.5
	2	429	2.41	
	3	2887	3.29	
	4	15264	3.93	

are of particular concern due to being collectively responsible for 33% of reported *Salmonella* illnesses frequently associated with foodborne outbreaks and human infections (USDA, 2022a). These serovars, especially *Salmonella* Enteritidis, have high pathogenicity and have been linked to many outbreaks with high case counts compared to other serovars (IFSAC, 2021; Teunis et al., 2010). The Centers for Disease Control and Prevention (CDC) frequently isolates Enteritidis and Typhimurium strains from clinical samples. Strains of Enteritidis and Infantis are of most importance to the poultry industry as these serovars have not seen declines in the past ten years and have adapted to live in poultry, rendering them important sources of infection for eggs and poultry products (Collins et al., 2022; Mughini-Gras et al., 2021; Tack et al., 2019).

The standardization of this robust workflow underwent extensive troubleshooting and optimization. Poultry industry stakeholders' inputs were collected for the development of the testing workflow. They expressed a preference for a low-cost diluent for the preparation of WCCR. Therefore, we initially explored the applicability of phosphate-buffered saline (PBS) and compared it with nBPW, which is part of FSIS MLG 4.14. PBS showed major variations with chickens obtained from the same retail store when inoculated at the same level. Therefore, further studies were conducted following the standard FSIS protocols that recommend the use of nBPW. Even though nBPW is expensive, it has an excellent buffering capacity, which enables it to neutralize any sanitizer residues (i.e., chlorine, peracetic acid) present on the poultry carcasses, facilitating the growth of stressed *Salmonella* strains. Adopting nBPW into our workflow generated results that facilitated reproducible detection and estimation of stressed *Salmonella* strains in WCCR samples without significant variation. Since the implementation of nBPW, there has been a threefold increase in the number of samples that have tested positive for *Salmonella* (Hinton et al., 2019). Vuia-Riser et al. compared the applicability of PBS, BPW, and nBPW as a dilution buffer for preparing chicken rinses, which were sanitized by peracetic acid and cetylpyridinium chloride. According to the study, nBPW exhibited better performance as a diluent and significantly increased the chances of detecting *Salmonella* in chicken rinses compared to other diluents. This was true irrespective of the type or concentration of chemical sanitizer used (Vuia-Riser et al., 2018).

BPW is the most commonly used media for enriching *Salmonella*. This study evaluated the applicability of 2 × BPW and supplements (i.e., iron, novobiocin, sodium pyruvate, and yeast extract) for promoting *Salmonella* growth within the shortest enrichment time. The use of 2 × BPW with iron as a growth enhancer generated inconsistent results, which may be due to other microorganisms also utilizing the iron supplement for their own growth. Similarly, yeast extract as a supplement resulted in lower *Salmonella* counts on XLD plates. Therefore, 2 × BPW supplemented with novobiocin and sodium pyruvate was selected as the final enrichment media.

After determining the optimal supplements and strength of the enrichment media, we proceeded to optimize the enrichment time and extraction volume of the enrichment for DNA isolation. Optimization of the assay was initially done on qPCR as the assay conditions were easily transferrable to dPCR (Gutiérrez-Aguirre et al., 2015). The applicability of zero to 6 h of enrichment time was explored during the assay optimization. Data collected for samples enriched for 4 h and below were not consistent. The data obtained from samples enriched for five and 6 h showed no difference in results and reproducibility. Therefore, a 5-h enrichment time was the most suitable for the detection and estimation of *Salmonella* at 1–4 log CFU/30 mL. Upon comparing DNA isolated from 200 μL, 1 mL, 2 mL, and 45 mL enriched samples, it was found that higher extraction volumes produced better amplification with lower Cq values. Since dPCR reactions can tolerate DNA in the microgram range, a volume of 45 mL was selected. This eliminated pipetting and DNA quantification steps and provided a simple and reproducible workflow.

One interesting aspect of the dPCR assay observed in our study was the possibility of saturation of the dPCR reaction wells for samples inoculated at higher concentrations. DNA concentrations of our enriched WCCR ranged from 213 to 1439 ng/μL, with an average of 352 ng/μL. Interestingly, approximately only 30 ng of pure culture *Salmonella* DNA was necessary to saturate the 26K dPCR reaction wells. Our samples inoculated with logs 3 and 4 showed a tendency to saturate the reaction wells, with 85 out of the 128 samples completely saturating the reaction wells. These results were mainly observed for log 4 samples, with 60 out of the 85 samples showing complete saturation. Log 3 samples were frequently saturated by the Typhimurium samples, observed in 16 of 25 samples, indicating a faster growth rate of the Typhimurium strains than other serovar tested in the study. Reanalysis of DNA samples showing saturation was performed using diluted DNA samples. *Salmonella* estimation values obtained from diluted and non-diluted DNA were not significantly different ($p > 0.05$). The combined values for log 3 samples before reanalysis was log 3.6 ± 0.24, while after reanalysis with diluted samples was log 3.65 ± 0.28. The average estimation value for undiluted log 4 samples was log 3.7 ± 0.28; after dilution, it was log 3.93 ± 0.48 (Supplementary Table 5). Compared to relative quantification-based qPCR assays for *Salmonella* quantification, which can quantify up to log 9, our dPCR assay can only quantify up to log 4 of *Salmonella* in WCCR (Basu, 2017). This may be considered a limitation of our dPCR assay. However, it is highly unlikely to find chicken carcass samples contaminated at levels higher than log 4 after the application of sanitizers used by the poultry industry. However, if any sample shows saturation on the 26K dPCR reaction well, it must undergo an effective intervention treatment to eliminate *Salmonella* irrespective of knowing the accurate *Salmonella* contamination level.

Digital PCR has emerged as a robust and cost-effective diagnostic technology that can be leveraged for the development of pathogen detection assays and enhancing food safety. dPCR simplifies the measurement of a target sequence by converting it into a set of positive and negative partitions (Quan et al., 2018). Despite its inception in 1999, it has only gained significant traction over the past 15 years (Basu, 2017; Lievens et al., 2016; Vogelstein and Kinzler, 1999). Of the two currently available dPCR technologies, ddPCR requires the use of multiple instruments, which significantly increases the cost associated with the equipment and the assay time, making it unaffordable for the food industries. In contrast, our study used the partition-based dPCR, which is very similar to the qPCR in terms of workflow, has a higher acceptance for food testing, requires minimal sample preparation time, and uses a simplified partitioning technology. As this technology advances, we expect to see more widespread use of digital PCR in food safety.

5. Conclusion

This study standardized hydrolysis-probe-based dPCR assay to detect and estimate the *Salmonella* load in chicken rinse samples. The extensively validated assay generated reproducible absolute quantification results with minimal sample-to-sample variations, high resistance to PCR inhibitors, and high tolerance to DNA concentration. The standardized workflow is best suited for private and federal food testing laboratories, as it can enable same-day detection and estimation of *Salmonella*.

CRediT authorship contribution statement

Frank J. Velez: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Nethraja Kandula:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Yotam Blech-Hermoni:** Software, Writing – review & editing. **Charlene R. Jackson:** Resources, Writing – review & editing. **Joseph M. Bosilevac:** Resources, Writing – review & editing. **Prashant Singh:** Conceptualization, Data curation, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100807.

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