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Assessing *Salmonella* quantification methods: MPN and MPN-LAMP perform equally in artificial contamination, while dPCR lacks sensitivity for naturally contaminated samples

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Abstract

Identifying and quantifying *Salmonella* in chicken carcasses is crucial for food safety and public health. Recent efforts focus on improving detection methods by combining traditional microbiology with molecular biology for better accuracy and speed. This study compared two protocols for *Salmonella* quantification: the conventional most probable number (MPN) and a combined approach integrating MPN with loop-mediated isothermal amplification (LAMP). The second phase evaluated MPN-LAMP and digital polymerase chain reaction for detecting and quantifying *Salmonella* in naturally contaminated chicken samples. The results indicated no statistically significant difference between the conventional MPN and MPN-LAMP methods in the evaluated steps. Both methods had similar performance, suggesting that MPN-LAMP can be used as a faster alternative. Upon application of this methodology to naturally contaminated chicken carcasses, 4 out of 16 samples (25%) exhibited contamination levels exceeding the detection thresholds when quantified using the MPN-LAMP method, with values ranging from 3.6 MPN/g to 15 MPN/g. However, the digital polymerase chain reaction technique could not detect or quantify samples in naturally contaminated chicken carcasses, highlighting challenges in pathogen detection in food. The results emphasize the need to compare methods in routine samples for microbiological surveillance and improve quantification techniques, ensuring food safety and implementing innovative *Salmonella* detection protocols.

Keywords: food safety; *Salmonella* detection; quantification protocols; microbiological control, MPN-LAMP, dPCR.

Practical Application: This study investigated the integration of the most probable number method with loop-mediated isothermal amplification to quantify *Salmonella* in chicken carcasses. The results showed similar performance between most probable number and MPN-LAMP, with loop-mediated isothermal amplification proving to be a faster and more efficient alternative. Additionally, digital polymerase chain reaction (dPCR), an innovative technique, was applied to naturally contaminated samples but failed to detect *Salmonella*, highlighting its limitations in practical applications. The findings emphasize the importance of standardization and exploring different methods for detecting and quantifying *Salmonella*.

1 INTRODUCTION

Salmonella is linked to numerous foodborne illness outbreaks, causing over 78 million cases globally and approximately 28,000 deaths annually (Kirk et al., 2015; Murray et al., 2021). Given the significant public health risks associated with its presence, detecting and quantifying *Salmonella* in chicken carcasses is crucial for ensuring food safety (Machado et al., 2020). To maintain the quality of poultry products, the adoption of robust diagnostic methods is essential (Waghamare et al., 2019).

Conventional methods remain indispensable for the quantitative assessment of *Salmonella*, despite their drawbacks of extended processing time, high costs, and labor-intensive procedures. Their long history of establishment and standardization lends credibility to the results, providing a reliable foundation for microbiological analyses (Corrêa et al., 2018; Forsythe, 2013). Often regarded as the "gold standard," these methods are widely used for detecting and quantifying *Salmonella* in food samples (Neyaz et al., 2024).

A widely accepted conventional microbiological technique for quantifying *Salmonella* in microbiological samples is the most probable number (MPN) method (Machado et al., 2020; Shanker et al., 2014). This approach involves inoculating serial dilutions of the sample into culture tubes, followed by an incubation period. Positive results are interpreted using statistical tables to estimate

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the MPN of bacteria in the original sample. The MPN method has long been a fundamental tool in microbiological surveillance and quality control, particularly in the food industry (Bari & Yeasmin, 2022, Oblinger & Koburger, 1975).

In recent years, efforts have been made to improve the accuracy and efficiency of procedures for detecting, identifying, and quantifying *Salmonella* through innovative approaches (Patel et al., 2024). One such strategy involves integrating traditional techniques with molecular biology. Here, we propose the combination of loop-mediated isothermal amplification (LAMP) with the traditional MPN method (Ahmad et al., 2017; Fu et al., 2021). LAMP is a highly efficient, visually identifiable isothermal nucleic acid amplification technique that, when paired with MPN, provides a sensitive and rapid means of detecting *Salmonella* DNA (Fu et al., 2021; Notomi et al., 2000). This integrated approach enhances the reliability and comprehensiveness of results by combining the robustness of conventional methods with the sensitivity and specificity of molecular techniques (Ahmad et al., 2017; Fu et al., 2021; Ndraha et al., 2023).

Another innovative technique, digital polymerase chain reaction (dPCR), has emerged as a novel method for quantifying *Salmonella*. This advanced approach leverages microfluidic technology to partition the reaction mixture into numerous individual reactions, each functioning independently (Kuypers & Jerome, 2017; Salipante & Jerome, 2020). By applying the Poisson distribution, dPCR enables the precise determination of the absolute concentration of target genetic material, expressed in copies per microliter (cp/μL), establishing itself as a powerful tool for genetic quantification (Fang et al., 2023; Villamil et al., 2020).

This study aimed to compare the conventional MPN method with the MPN combined with the LAMP technique, called MPN-LAMP, as approaches to quantify *Salmonella* in artificially contaminated poultry samples. Furthermore, it aimed to evaluate the MPN-LAMP and dPCR techniques in the detection and quantification of *Salmonella* in naturally contaminated poultry samples, providing a comprehensive analysis of the effectiveness of these methodologies in detecting the pathogen in practical scenarios.

1.1 Relevance of the work

The significance of this study lies in the comparison of *Salmonella* quantification methods in chicken carcasses, a crucial factor in ensuring food safety and safeguarding public health. The research assessed the integration of traditional microbiological techniques with modern molecular approaches, proposing the most probable number with loop-mediated isothermal amplification (MPN-LAMP) method as a faster alternative to conventional most probable number (MPN), without compromising its effectiveness. The findings also emphasize the use of digital polymerase chain reaction (dPCR) in naturally contaminated samples, underscoring the importance of continuously refining detection methods to enhance microbiological surveillance within the poultry production chain.

2 MATERIALS AND METHODS

2.1 Most probable number study design

A strain of *Salmonella* Typhimurium was resuspended in brain heart infusion (BHI) broth and adjusted to a concentration of 10^8 colony-forming unit (CFU)/mL using the McFarland nephelometric scale. Serial dilutions were then performed to obtain estimated concentrations of 10^5, 10^3, 10^2, 10^1, and 1 CFU/mL, which were used to artificially contaminate broiler meat.

To compare the two techniques, the conventional MPN method was conducted, and at each step, 5 mL aliquots were collected for DNA extraction and subsequent LAMP analysis, defining the MPN-LAMP technique. For this purpose, aliquots from the enrichment broths were collected at four time points: before and after incubation in buffered peptone water (BPW), as well as after incubation in tetrathionate (TT) and Rappaport-Vassiliadis (RV) broths, as outlined in Figure 1.

To conduct the MPN analysis, 19 samples of 25 g of chicken meat, confirmed to be *Salmonella*-negative, were weighed and placed in Stomacher™ bags. Each sample was then individually inoculated with 2.5 mL of *Salmonella* Typhimurium at varying concentrations (10^5, 10^3, 10^2, 10^1, and 1 CFU/mL). After gentle homogenization, the samples were incubated for 30 min.

Following incubation, 225 mL of BPW (Merck™) was added to each sample, followed by 2 min of homogenization. Three 50 mL aliquots were then transferred to sterile conical tubes labeled 1A, 1B, and 1C. From tube 1A, 5 mL was transferred to a new tube (2A) containing 45 mL of BPW and homogenized. Finally, 5 mL from tube 2A was transferred to tube 3A, which also contained 45 mL of BPW. The same procedure was repeated for tubes labeled B and C. The tubes were incubated at 36°C for 24 h.

After incubation, 0.1 mL from each replicate was transferred to 9.9 mL of RV broth (Thermo Scientific™ Oxoid™), while 1 mL was transferred to 9 mL of TT broth (BD™). The RV and TT broths were incubated at 42°C and 36°C, respectively, for 24 h. Following incubation, samples from both broths were plated on deoxycholate-lysine-xylose (XLD) agar (Thermo Scientific™ Oxoid™) and incubated at 36°C for another 24 h. After this period, the plates were examined for the presence of characteristic *Salmonella* colonies. A positive result in the conventional MPN method required the presence of *Salmonella* in the corresponding dilution.

For the MPN-LAMP protocol, aliquots from each dilution were collected, and their genomic material was extracted for LAMP analysis. The number of positive replicates in each dilution was compared using the MPN determination table, as described in a previous study (Blodgett, 2010). The MPN values obtained from both techniques were then compared, considering confidence interval values. A representative diagram of the methodology is illustrated in Figure 1, outlining the steps used in the study.

2.2 DNA extraction

The samples were extracted using an internal protocol based on magnetic beads, adapted from a previous study (Possebon

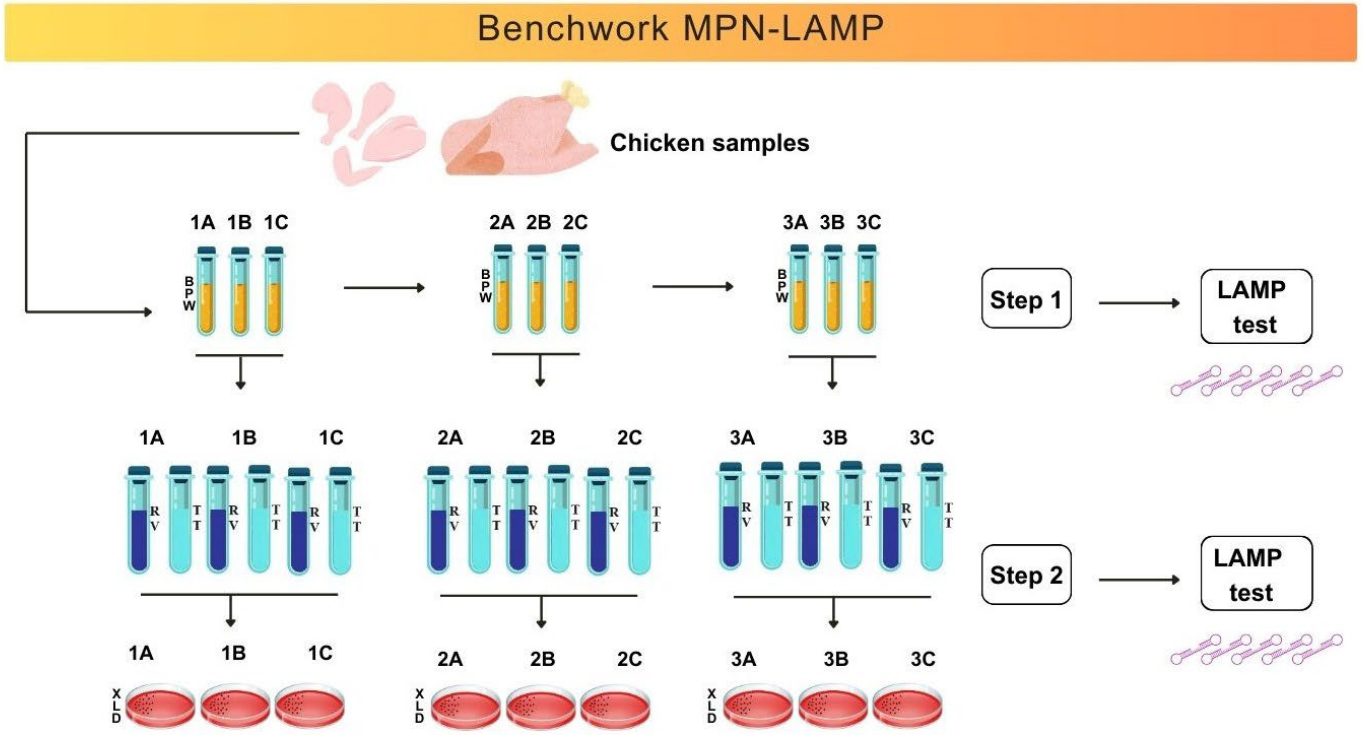

Figure 1. Scheme of MPN-LAMP steps.
Samples (5 mL) were collected before and after the incubation of the BPW broth (Step 1) and after the incubation of the TT and RV broths (Step 2) for LAMP analysis.

et al., 2022), utilizing the KingFisher™ Flex Purification System (Thermo Fisher Scientific, USA). After homogenization, 200 µL of the sample was used for extraction. The lysis and DNA-binding steps were carried out using 350 µL of lysis buffer (5.5 M guanidine isothiocyanate [GITC], 50 mM Tris-Cl [pH 8], 20 mM ethylenediaminetetraacetic acid [EDTA] [pH 8], 2% N-lauroylsarcosine, and 0.1% defoamer), along with 350 µL of isopropanol and 40 µL of magnetic beads. The extraction process included two wash steps: the first with a mixture of 250 µL of GITC and 250 µL of isopropanol, and the second with 500 µL of 80% ethanol. Finally, the samples were eluted in 100 µL of nuclease-free water.

2.3 Loop-mediated isothermal amplification technique

For the LAMP assay, a 25 µL reaction was prepared using 12.5 µL of WarmStart Colorimetric LAMP 2X Master Mix (New England BioLabs, USA), 1.6 µM of FIP and BIP primers, 0.2 µM of F3 and B3 primers, and 0.8 µM of Loop-F and Loop-B primers. Additionally, 1 µL of the sample was added, and the volume was adjusted with nuclease-free water. The reaction was incubated at 65°C for 30 min, followed by enzyme inactivation at 85°C for 5 min. After the reaction, samples displaying a yellowish color were considered positive. The primers used for the reaction are listed in Table 1.

2.4 MPN-LAMP Salmonella quantification in naturally contaminated samples

The MPN-LAMP and dPCR techniques were used to quantify *Salmonella* in 16 samples of naturally contaminated chicken carcasses, all of which tested positive using conventional analysis at the Food Sanitary Inspection Laboratory, School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP). Microbiological analyses followed the ISO 6579-1:2017 methodology for *Salmonella* detection (International Organization for Standardization [ISO], 2017). For each sample, 25 g of chicken meat from different regions of the carcass was collected and homogenized in Stomacher™ bags with 225 mL of BPW. After aliquots were taken for dPCR, the MPN-LAMP technique was applied to all samples.

2.5 Digital polymerase chain reaction

For *Salmonella* quantification in naturally contaminated poultry carcasses, a 50 mL aliquot of the rinse solution (BPW 1%), collected before incubation, was set aside for genetic material extraction and subsequent dPCR analysis. The aliquot was centrifuged at 10,000 × *g* for 15 min, after which the supernatant was discarded, and the pellet was resuspended in 1 mL of nuclease-free water. DNA extraction followed the previously described protocol. To prepare the dPCR reaction, 4 µL of QIAcuity® EG PCR Kit Master Mix, 0.48 µL each of forward and reverse primers targeting the *Salmonella invA* gene (primer sequences listed in Table 1), 5.04 µL of nuclease-free water, and 2 µL of the sample were combined, yielding a final volume of 12 µL. The reaction was performed using QIAcuity® 8.5k nanoplates, which contain 8,500 compartments in a 24-well format. The dPCR amplification was conducted on the QIAcuity One® system, with an initial denaturation at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C, and 15 s at 72°C.

Fluorescence images were captured in the green channel (FAM) with an exposure time of 250 ms and a gain of 3. Final data were analyzed using QIAcuity Software Suite 2.1.8.23.

2.6 Statistical analysis

The MPN data obtained by the different techniques (MPN and LAMP-MPN in different sampling stages) were tabulated, and distribution analysis was performed, with normality tests (Shapiro-Wilk) and graphic analyses (histogram and quantile--quantile graph). The Kruskal-Wallis test was used to compare the MPN medians obtained with the different techniques. Alpha significance level was set to 0.05. For the analysis, the greater than (>) signs were not considered. Also, the 95% confidence interval was considered for comparing the outcomes of the mentioned techniques, being considered statistically similar when the 95% confidence intervals overlapped.

3 RESULTS AND DISCUSSION

The comparison between conventional MPN and MPN--LAMP techniques is summarized in Table 2, which presents the MPN values obtained from both methods. No statistically significant differences were observed between conventional MPN and MPN-LAMP techniques across various culture steps. When considering the 95% confidence interval for MPN-LAMP performed in BPW and conventional MPN, 94.73% (18/19) of the samples showed concordant results.

Table 1. Sequences of primers used in the LAMP and dPCR reactions.

Primers	Sequence (5' > 3')	Reference
	LAMP	
FIP	GACGACTGGTACTGATCGATAGTTTTTCAACGTTTCCTGCGG	Xu et al., 2019
BIP	CCGGTGAAATTATCGCCACACAAAACCCACCGCCAGG	
F3	GGCGATATTGGTGTTTATGGGG	
B3	AACGATAAACTGGACCACGG	
Loop-F	GACGAAAGAGCGTGGTAATTAAC	
Loop-B	GGGCAATTCGTTATTGGCGATAG	
	dPCR	
INVA5F	GATTTGAAGGCCGGTATTATTG	Barbau-Piednoir et al., 2013
INVA5R	ATAAACTTCATCGCACCGTCA	

Table 2. Comparison of conventional most probable number obtained through different techniques in samples of poultry carcass artificially contaminated with *Salmonella*.

Sample	MPN	LAMP-MPN BPW	LAMP-MPN TT	LAMP-MPN RV	LAMP-MPN TT/RV
1	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)
2	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)
3	> 1100 (420 to –)	1100 (180–4100)	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)
4	> 1100 (420 to –)	> 1100 (420 to –)	> 1100 (420 to –)	290 (90–1000)	> 1100 (420 to –)
5	1100 (180–4100)	38 (8.7–110)	1100 (180–4100)	1100 (180–4100)	> 1100 (420 to –)
6	1100 (180–4100)	210 (40–430)	1100 (180–4100)	1100 (180–4100)	1100 (180–4100)
7	460 (90–2000)	460 (90–2000)	460 (90–2000)	36 (8.7–94)	460 (90–2000)
8	460 (90–2000)	150 (37–420)	460 (90–2000)	460 (90–2000)	460 (90–2000)
9	460 (90–2000)	460 (90–2000)	460 (90–2000)	460 (90–2000)	460 (90–2000)
10	460 (90–2000)	28 (8.7–94)	28 (8.7–94)	28 (8.7–94)	28 (8.7–94)
11	460 (90–2000)	150 (37–420)	460 (90–2000)	460 (90–2000)	460 (90–2000)
12	240 (42–1000)	1100 (180–4100)	1100 (180–4100)	460 (90–2000)	1100 (180–4100)
13	210 (40–430)	21 (4.5–42)	210 (40–430)	35 (8.7–94)	210 (40–430)
14	150 (37–420)	93 (18–420)	150 (37–420)	> 1100 (420 to –)	> 1100 (420 to –)
15	93 (18–420)	35 (8.7–94)	43 (9–180)	43 (9–180)	43 (9–180)
16	43 (9–180)	9.2 (1.4–38)	9.2 (1.4–38)	9.2 (1.4–38)	9.2 (1.4–38)
17	23 (4.6–94)	7.4 (1.3–20)	3.6 (0.17–18)	3.6 (0.17–18)	3.6 (0.17–18)
18	3.6 (0.17–18)	11 (3.6–38)	28 (8.7–94)	20 (4.5–42)	35 (8.7–94)
19	3.6 (0.17–18)	3.6 (0.17–18)	3.6 (0.17–18)	3.6 (0.17–18)	3.6 (0.17–18)
Median	460	150	460	460	460

In parentheses, the confidence intervals of each result obtained.

MPN: Conventional most probable number; LAMP: Loop-mediated isothermal amplification; LAMP-MPN BPW: Most probable number associated with the LAMP technique performed in buffered peptone water broth; LAMP-MPN TT: Most probable number associated with the LAMP technique performed in tetrathionate (TT) broth; LAMP-MPN RV: Most probable number associated with the LAMP technique performed in Rappaport-Vassiliadis (RV) broth; LAMP-MPN TT/RV: Most probable number associated with the LAMP technique considering the results of TT and RV, similar to the conventional technique; $p = .7587$.

Other authors have successfully combined the LAMP and MPN techniques (Ahmad et al., 2017; Kanitkar et al., 2017). In a study focusing on *Escherichia coli* and *Enterococcus faecalis*, MPN-LAMP proved effective in replacing the conventional MPN method. This approach demonstrated simplicity, sensitivity, and rapid response, facilitating efficient sampling (Ahmad et al., 2017). Additionally, another study developed an MPN--LAMP assay using a polymethylmethacrylate microchip to assess fecal contamination. The findings indicated the feasibility and reliability of the MPN-LAMP technique (Fu et al., 2021).

The MPN-LAMP method emerges as a promising alternative, offering a cost-effective and efficient solution for quantitative analysis. The data further suggest that performing LAMP-MPN directly in BPW broth is feasible and advantageous, given the time savings compared to aliquots obtained after selective enrichment. Based on these findings, we proceeded with the second part of the study, utilizing LAMP-MPN in BPW broth for naturally contaminated samples.

In the second stage of the study, which aimed to apply the methodology to naturally *Salmonella*-infected chicken carcasses, 4 out of 16 samples (25%) had *Salmonella* concentrations above the MPN-LAMP detection limit. However, none of the 16 naturally contaminated samples could be detected by the dPCR method. To ensure assay validity, positive controls were included, and the valid partitions generated by the software during the run were evaluated. Two positive controls were used: *Salmonella* Typhimurium, quantified at 11,523.2 cp/µL, and *Salmonella* Pullorum, quantified at 15,317.7 cp/µL. The number of valid partitions per sample ranged from 7,846 to 8,288, out of a total of 8,500 available partitions. The results are available in Table 3 and Figure 2.

Table 3. *Salmonella* MPN-LAMP enumeration data obtained from 16 chicken carcass samples.

Sample	LAMP-MPN BPW	dPCR
1	Undetectable	Undetectable
2	Undetectable	Undetectable
3	Undetectable	Undetectable
4	Undetectable	Undetectable
5	Undetectable	Undetectable
6	Undetectable	Undetectable
7	Undetectable	Undetectable
8	Undetectable	Undetectable
9	3.6 MNP/g	Undetectable
10	Undetectable	Undetectable
11	Undetectable	Undetectable
12	Undetectable	Undetectable
13	15 MNP/g	Undetectable
14	7.2 MNP/g	Undetectable
15	11 MNP/g	Undetectable
16	Undetectable	Undetectable
Total	4	0

Note: 'Undetectable' indicates values below the detection limit.

The quantifications obtained through MPN-LAMP in naturally contaminated samples were consistent with values reported in the literature. The lowest detected concentration was 3.6 MPN/g, while the highest was 15 MPN/g, with an average of 9.2 MPN/g. This average closely aligns with findings from a previous study, which reported a mean concentration of 10.6 MPN/g (Rortana et al., 2021). Additionally, a survey on *Salmonella* in raw chicken meat documented MPN-LAMP quantifications ranging from 1.2 to 55.6 MPN/g (Rosniawati et al., 2021). Although the obtained values were within the expected range, the MPN--LAMP technique exhibited limited sensitivity in naturally contaminated poultry carcasses.

The high complexity of the food matrix, characterized by the presence of salts, enzymes, proteins, and lipids, can significantly influence molecular processes: from DNA extraction, where these components may hinder cell lysis and degrade genetic material, to the amplification step, where they can affect polymerase activity (Moon et al., 2022; Schrader et al., 2012). This, and the low pathogen concentration in the sample, may be associated with the results obtained in the MPN-LAMP reaction and dPCR.

The application of dPCR for pathogen detection in food samples presents several challenges, including microbiological injuries caused by food processing, the inherent complexity of food matrices, natural inhibitors, low bacterial loads, and uneven pathogen distribution (Lei et al., 2021; Ndraha et al., 2023). Additionally, the small reaction volume, of only 12 µL per assay, must be considered (Kuypers & Jerome, 2017).

In this study, BPW 1% without incubation was used, which may have hindered the detection of extremely low levels of *Salmonella*, potentially falling below the technique's detection limit. While dPCR has demonstrated high specificity and low detection limits (0.001 ng/µL) in artificially contaminated chicken carcasses, the quantification of naturally contaminated samples appears to be more challenging (Velez et al., 2024).

Despite its advantages, dPCR still faces obstacles in practical applications, highlighting the need for further assay development and standardization. Addressing these limitations through in-depth investigations is essential for optimizing dPCR and improving its effectiveness in food pathogen detection.

4 CONCLUSION

The newly proposed MPN-LAMP assay demonstrated performance similar to conventional MPN for *Salmonella* detection in artificially contaminated poultry samples, significantly reducing both time and laboratory work. However, when applied to naturally contaminated samples, it revealed the need for further efforts to enhance the sensitivity of MPN. Conversely, direct quantification of *Salmonella* in poultry samples by dPCR requires additional studies to validate its potential and efficacy in this context. Enhancing these techniques could substantially contribute to strengthening efforts aimed at ensuring the safety of poultry products.

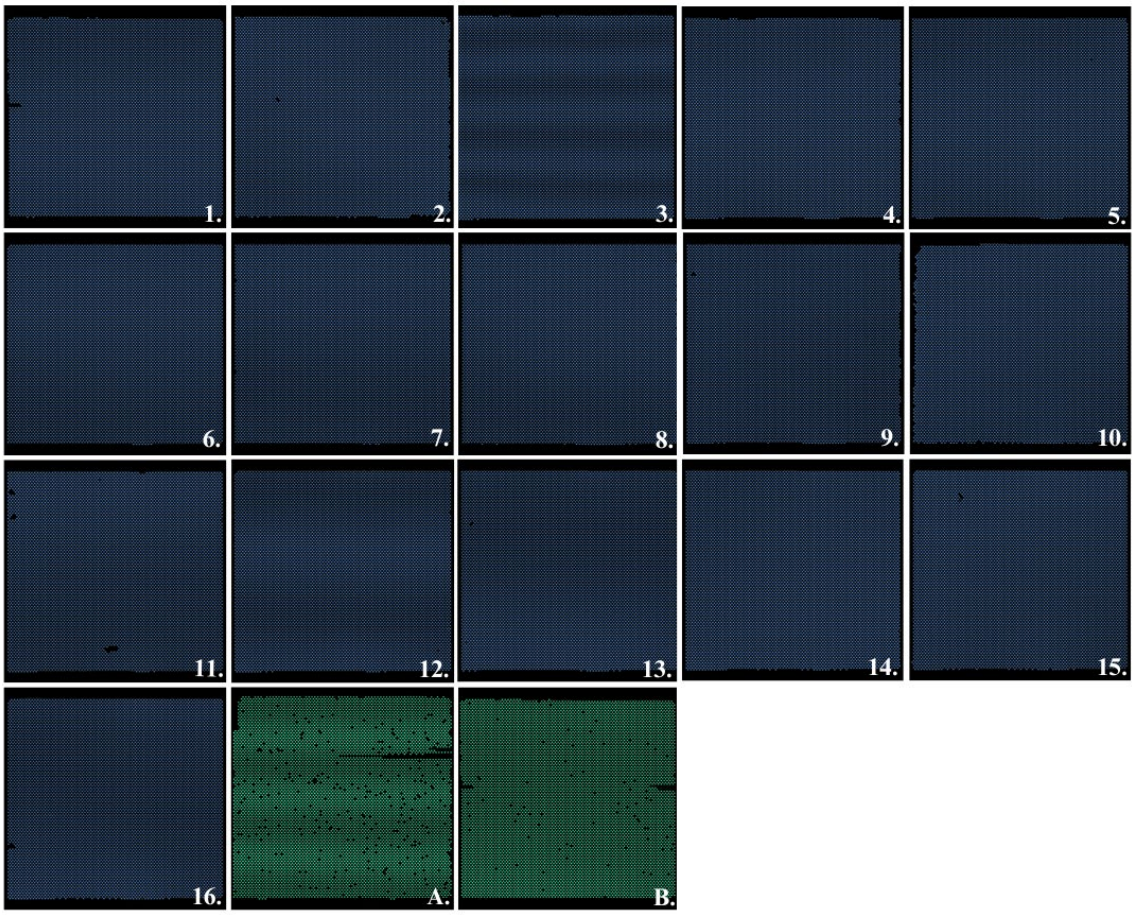

Figure 2. Images generated by the QIAcuity Software Suite® after the dPCR run depict naturally contaminated samples 1–16, where no quantification occurred.

The partitions in blue represent the reference channel. The positive controls of the reaction are represented in A. *Salmonella* Typhimurium and B. *Salmonella* Pullorum.

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