Mammalian Cell-Based Immunoassay for Detection of Viable Bacterial Pathogens

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Rapid detection of live pathogens is of paramount importance to ensure food safety. At present, nucleic acid-based polymerase chain reaction and antibody-based lateral flow assays are the primary methods of choice for rapid detection, but these are prone to interference from inhibitors, and resident microbes. Moreover, the positive results may neither assure virulence potential nor viability of the analyte. In contrast, the mammalian cell-based assay detects pathogen interaction with the host cells and is responsive to only live pathogens, but the short shelf-life of the mammalian cells is the major impediment for its widespread application. An innovative approach to prolong the shelf-life of mammalian cells by using formalin was undertaken. Formalin (4% formaldehyde)-fixed human ileocecal adenocarcinoma cell line, HCT-8 on 24-well tissue culture plates was used for the capture of viable pathogens while an antibody was used for specific detection. The specificity of the Mammalian Cell-based ImmunoAssay (MaCIA) was validated with Salmonella enterica serovar Enteritidis and Typhimurium as model pathogens and further confirmed against a panel of 15 S. Enteritidis strains, 8 S. Typhimurium, 11 other Salmonella serovars, and 14 non-Salmonella spp. The total detection time (sample-to-result) of MaCIA with artificially inoculated ground chicken, eggs, milk, and cake mix at 1–10 CFU/25 g was 16–21 h using a traditional enrichment set up but the detection time was shortened to 10–12 h using direct on-cell (MaCIA) enrichment. Formalin-fixed stable cell monolayers in MaCIA provide longer shelf-life (at least 14 weeks) for possible point-of-need deployment and multi-sample testing on a single plate.

Keywords: immunoassay, poultry, mammalian cells, Salmonella, detection, MaCIA, cell-based sensor, stress

INTRODUCTION

Pathogen interaction with the host cells is the crucial first step for initiating infection (Finlay and Falkow, 1997; Kline et al., 2009), and harnessing such interaction may yield a robust detection platform not only to assess pathogenic potential but also its viability. Mammalian cell-based biosensors (CBBs) exploit host-pathogen interactions including pathogen adhesion, activation of host cell signaling events, cell-cycle arrest, apoptosis, and/or cytotoxicity...
Adhesion to the epithelial cells is the crucial first step for enteric pathogens (Kline et al., 2009; Dos Reis and Horn, 2010). For example, *L. monocytogenes* binds to Hsp60 and E-cadherin on the epithelial cell surface through *Listeria* adhesion protein (LAP) and Internalin A (InlA), respectively to initiate adhesion, invasion, translocation, and systemic spread during the intestinal phase of infection (Drolia et al., 2018; Drolia and Bhunia, 2019). Enterohaemorrhagic *E. coli* employs intimin, fimbrial proteins, flagella, and autotransporter proteins to attach to the host cells at different stages of its life cycle during infection (McWilliams and Torres, 2014). Likewise, *Salmonella enterica* utilizes multiple fimbrial adhesins, such as type 1 fimbriae (T1F) and long polar fimbriae (Lpf), and several autotransporter adhesins, such as ShdA and MsL, to promote adhesion to D-mannose receptors on M cells in Peyers Patches and assist colonization in the intestine (Bäumler et al., 1996; Wagner and Hensel, 2011; Bhunia, 2018; Kolenda et al., 2019). Therefore, detecting only adhered pathogens using antibodies is a rational approach. We chose human ileocecal adenocarcinoma cell line, HCT-8, as the target cells for building MaCIA platform on 24-well tissue culture plates. HCT-8 is one of the commonly used model cell lines to study the adhesion of enteric pathogens (McKee and O’Brien, 1995; Dibao-Dina et al., 2015; Hu and Wai, 2017). Unlike other cell lines used, HCT-8 cells can form a fully confluent monolayer in only 5 days.

The objective of this study was to develop a shelf-stable MaCIA platform for the rapid detection of viable bacterial pathogens and to validate its performance using *Salmonella enterica* serovar Enteritidis as a model foodborne pathogen.

*Salmonella enterica* is a major foodborne pathogen of global public health concern. Meat, poultry, eggs, nuts, fruits, and vegetables are common vehicles for *Salmonella* transmission. Each year, *Salmonella* infections contribute to 1.3 billion cases of gastroenteritis and 3 million deaths worldwide (Kirk et al., 2015) and 1.35 million cases, 26,500 hospitalizations, and 420 deaths in the United States (CDC, 2020). Among *Salmonella* serovars, *Salmonella enterica* serovar Enteritidis is one of the most prevalent serovars in the United States. The Centers for Disease Control and Prevention (CDC) has reported eight major outbreaks between 2006 and 2018 resulting in about 4,000 cases (CDC, 2018). In a survey of salmonellosis outbreaks (total 2,447) in the United States between 1998 and 2015, *S. Enteritidis* (29.1%) was reported to be the most prevalent serovar followed by *S. Typhimurium* (12.6%), *S. Newport* (7.6%), and others (Snyder et al., 2019). The frequent occurrence of food-associated *S. Enteritidis* outbreaks with the high number of infections was the motivation for developing a mammalian cell-based functional bioassay for the detection of *S. Enteritidis*.

In this study, we took an innovative approach and developed a shelf-stable Mammalian Cell-based ImmunoAssay (MaCIA) platform for the detection of live pathogenic bacteria. Shelf-life of MaCIA was prolonged by fixing the mammalian cells in formalin (4% formaldehyde) which is a common practice in histology and tissue imaging to preserve the cells by preventing protein degradation (Eltoum et al., 2001). Furthermore, instead of measuring cytotoxicity, we took advantage of the adhesion ability of enteric pathogens to the intestinal cells followed by antibody-based assay for specific detection of the adhered target pathogens. Adhesion to the epithelial cells is the crucial first step for enteric pathogens (Kline et al., 2009; Dos Reis and Horn, 2010). For example, *L. monocytogenes* binds to Hsp60 and E-cadherin on the epithelial cell surface through *Listeria* adhesion protein (LAP) and Internalin A (InlA), respectively to initiate adhesion, invasion, translocation, and systemic spread during the intestinal phase of infection (Drolia et al., 2018; Drolia and Bhunia, 2019). Enterohaemorrhagic *E. coli* employs intimin, fimbrial proteins, flagella, and autotransporter proteins to attach to the host cells at different stages of its life cycle during infection (McWilliams and Torres, 2014). Likewise, *Salmonella enterica* utilizes multiple fimbrial adhesins, such as type 1 fimbriae (T1F) and long polar fimbriae (Lpf), and several autotransporter adhesins, such as ShdA and MsL, to promote adhesion to D-mannose receptors on M cells in Peyers Patches and assist colonization in the intestine (Bäumler et al., 1996; Wagner and Hensel, 2011; Bhunia, 2018; Kolenda et al., 2019). Therefore, detecting only adhered pathogens using antibodies is a rational approach. We chose human ileocecal adenocarcinoma cell line, HCT-8, as the target cells for building MaCIA platform on 24-well tissue culture plates. HCT-8 is one of the commonly used model cell lines to study the adhesion of enteric pathogens (McKee and O’Brien, 1995; Dibao-Dina et al., 2015; Hu and Wai, 2017). Unlike other cell lines used, HCT-8 cells can form a fully confluent monolayer in only 5 days.

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TABLE 1 | Specificity of mammalian cell-based immunoassay (MaCIA) platform tested against Salmonella and non-Salmonella spp.

| Bacteria                        | CFU/Well       | MaCIA Result* | mAb-2F11 | mAb-F68C |
|---------------------------------|---------------|---------------|----------|----------|
|                                 |               | **Ab450nm ± SD** | **Result** | **Ab450nm ± SD** | **Result** |
| **Salmonella enterica serovars** |               |               |          |          |
| Enteritidis PT21                | 2.0–13 × 10⁷   | 0.95 ± 0.08   | +        | 0.10 ± 0.01 | -          |
| Enteritidis 13ENT1344           | 2.9 × 10⁷      | 1.13 ± 0.16   | +        | NT        | NT         |
| Enteritidis 13ENT1374           | 2.8–3.3 × 10⁷  | 0.91 ± 0.15   | +        | 0.09 ± 0.00 | -          |
| Enteritidis 13ENT1376           | 2.0 × 10⁷      | 1.06 ± 0.03   | +        | NT        | NT         |
| Enteritidis 13ENT1375           | 3.1 × 10⁷      | 1.07 ± 0.15   | +        | NT        | NT         |
| Enteritidis 13ENT1032           | 2.2 × 10⁷      | 1.08 ± 0.25   | +        | NT        | NT         |
| Enteritidis PT1                 | 2.8 × 10⁷      | 1.19 ± 0.04   | +        | NT        | NT         |
| Enteritidis PT4                 | 2.0 × 10⁷      | 1.17 ± 0.06   | +        | NT        | NT         |
| Enteritidis PT6                 | 1.8 × 10⁷      | 1.41 ± 0.04   | +        | NT        | NT         |
| Enteritidis PT7                 | 7.5 × 10⁷      | 0.70 ± 0.06   | +        | NT        | NT         |
| Enteritidis PT8                 | 1.4 × 10⁷      | 1.42 ± 0.06   | +        | NT        | NT         |
| Enteritidis PT13a               | 1.5 × 10⁷      | 0.74 ± 0.02   | +        | NT        | NT         |
| Enteritidis PT13                | 1.1 × 10⁷      | 0.90 ± 0.06   | +        | NT        | NT         |
| Enteritidis PT14b               | 1.3 × 10⁷      | 1.05 ± 0.04   | +        | NT        | NT         |
| Enteritidis PT28                | 1.1 × 10⁷      | 0.53 ± 0.05   | +        | NT        | NT         |
| Typhimurium 13ENT906            | 6.7–8.8 × 10⁷  | 0.33 ± 0.03   | -        | 1.14 ± 0.06 | +          |
| Typhimurium NOS12               | 4.0 × 10⁷      | 0.33 ± 0.03   | -        | 0.98 ± 0.04 | +          |
| Typhimurium NOS3                | 3.3 × 10⁸      | NT            | NT       | 0.80 ± 0.04 | +          |
| Typhimurium NOS10               | 1.3 × 10⁸      | NT            | NT       | 0.90 ± 0.12 | +          |
| Typhimurium NOS2                | 6.7 × 10⁸      | NT            | NT       | 0.73 ± 0.08 | +          |
| Typhimurium NOS4                | 6.7 × 10⁸      | NT            | NT       | 0.89 ± 0.04 | +          |
| Typhimurium NOS1                | 3.3 × 10⁷      | NT            | NT       | 0.84 ± 0.06 | +          |
| Typhimurium ST1                 | 3.8 × 10⁹      | 0.13 ± 0.02   | -        | NT        | NT         |
| Newport 13ENT1060               | 2.3–23 × 10⁷   | 0.32 ± 0.04   | -        | 0.13 ± 0.01 | -          |
| Braenderup 12ENT1138            | 6.3 × 10⁷      | 0.33 ± 0.03   | -        | NT        | NT         |
| Agona 12ENT1356                 | 2.7–13 × 10⁸   | 0.32 ± 0.02   | -        | 0.09 ± 0.01 | -          |
| Hadar 13ENT979                  | 4.3 × 10⁷      | 0.27 ± 0.02   | -        | NT        | NT         |
| Paratyphi 11J85                 | 2.4 × 10⁷      | 0.27 ± 0.05   | -        | NT        | NT         |
| Heidelberg 18ENT1418            | 4.0 × 10⁷      | 0.29 ± 0.04   | -        | NT        | NT         |
| Saintpaul 13ENT1045             | 5.0 × 10⁷      | 0.30 ± 0.04   | -        | NT        | NT         |
| Javanica 13ENT86F               | 0.4–2.7 × 10⁸  | 0.38 ± 0.10   | -        | 0.14 ± 0.07 | -          |
| Infantis 13ENT866               | 2.0 × 10⁷      | 0.32 ± 0.02   | -        | NT        | NT         |
| Bareilly 12ENT1164              | 0.1–4.0 × 10⁸  | 0.32 ± 0.09   | -        | NT        | NT         |
| Pullorum DUP-PVUII 1006         | 1.9 × 10⁷      | 0.34 ± 0.04   | -        | NT        | NT         |
| **Miscellaneous**               |               |               |          |          |
| Listeria monocytogenes F4244    | 0.5–1.6 × 10⁸  | 0.27 ± 0.03   | -        | 0.13 ± 0.01 | -          |
| L. innocua F4248                | 5.0 × 10⁷      | 0.27 ± 0.03   | -        | NT        | NT         |
| Escherichia coli O157:H7 EDL933 | 0.4–1.3 × 10⁹  | 0.28 ± 0.03   | -        | 0.08 ± 0.04 | -          |
| Hafnia alvei 18066              | 3.3–6.3 × 10⁷  | 0.28 ± 0.03   | -        | 0.15 ± 0.02 | -          |
| Citrobacter freundii ATCC8090   | 0.3–1.0 × 10⁸  | 0.29 ± 0.02   | -        | 0.13 ± 0.01 | -          |
| Citrobacter freundii ATCC43864  | 0.4–3.3 × 10⁷  | 0.11 ± 0.00   | -        | 0.11 ± 0.01 | -          |
| Citrobacter freundii ATCC3624   | 0.3–1.3 × 10⁹  | 0.13 ± 0.01   | -        | 0.12 ± 0.02 | -          |
| Serratia marcescens ATCC8100    | 0.6–5.3 × 10⁷  | 0.33 ± 0.02   | -        | 0.12 ± 0.01 | -          |
| Serratia marcescens ATCC3862    | 0.1–1.0 × 10⁸  | 0.11 ± 0.01   | -        | 0.13 ± 0.01 | -          |
| Serratia marcescens B-2544      | 0.6–3.3 × 10⁷  | 0.13 ± 0.01   | -        | 0.11 ± 0.02 | -          |
| Pseudomonas aeruginosa PR999     | 2.25 × 10⁷     | 0.24 ± 0.02   | -        | NT        | NT         |
| Proteus mirabilis B-3402        | 0.7–6.7 × 10⁸  | 0.11 ± 0.01   | -        | 0.11±0.01  | -          |
| Proteus vulgaris DUP-10086      | 0.4–4.0 × 10⁸  | 0.11 ± 0.01   | -        | 0.10 ± 0.02 | -          |
| Klebsiella pneumoniae B-41958   | 6.7 × 10⁸      | 0.10 ± 0.01   | -        | 0.13 ± 0.01 | -          |

*Values are from four independent replicates; Results (±) are decided by comparing to the negative control in each experiment. Values that are significantly different (P < 0.001) from the negative control in each experiment are regarded as +; NT, not tested.
assay steps and total detection time. Overall, the data showed that MaCIA could detect viable S. Enteritidis (1–10 CFU/25 g) in ground chicken, shelled eggs, whole milk, and cake mix using a traditional enrichment set up, but the detection time was shortened to 10–12 h using direct on-cell (MaCIA) enrichment. We also demonstrated the versatility of MaCIA by using a commercial anti-Salmonella reporter antibody for the detection of S. Typhimurium. Formalin-fixed cells in the MaCIA platform permits a longer shelf life (at least 14-week at 4°C), minimum on-site maintenance care, and a stable cell monolayer for point-of-need deployment.

RESULTS

Development of MaCIA (Mammalian Cell-Based ImmunoAssay) Platform

The MaCIA platform was built on a 24-well tissue culture plate, and it consisted of two steps: fixation of mammalian cells and immunoassay for specific detection of adherent target pathogens. We used the formalin-fixed HCT-8 cell line for Salmonella adhesion/capture (30 min) and anti-S. Enteritidis monoclonal antibody, mAb-2F11 (Masi and Zawistowski, 1995), or anti-Salmonella mAb-F68C (Thermo Fisher Scientific; 1.5 h), horseradish peroxidase (HRP)-conjugated second antibody and a substrate for color development (1.5 h). The mAb-2F11 is highly specific for S. Enteritidis (Masi and Zawistowski, 1995; Jaradat et al., 2004), and the Western blot analysis confirmed its specificity without showing any reaction with bands from whole-cell preparations of L. monocytogenes, E. coli O157:H7 or Pseudomonas aeruginosa (Figure 1A).

To fix mammalian cells on the MaCIA platform, HCT-8 cell monolayers in 24 well-plates were treated with a 4% formaldehyde solution for 10 min, followed by three sequential wash using phosphate-buffered saline (PBS, 0.1 M, 4% formaldehyde solution for 10 min, followed by three wash steps). Formalin-fixed (Formalin-fixed HCT-8) HCT-8 cell. The performance of MaCIA was also compared with a live cell-based MaCIA platform to detect S. Enteritidis PT21 that was incubated for 30 min at 37°C. Remarkably, both assay configurations showed strong positive signals toward viable S. Enteritidis, which was significantly (P < 0.0001) higher than the equivalent amounts of dead S. Enteritidis cells (verified by plating) and the negative control (PBS) (Figure 1B).

The performance of MaCIA was also compared with traditional sandwich ELISA where mAb-2F11 was used as capture and anti-Salmonella pAb-3238 (Abdelhaseib et al., 2016) was used as the reporter. MaCIA gave positive results when tested with viable S. Enteritidis cells (1 × 10⁶ CFU/mL), which is significantly higher (P < 0.0001) than that of the equivalent numbers of dead cells or the PBS control. On the other hand, both viable and dead S. Enteritidis cells showed positive signals with sandwich ELISA, though the signals for viable cells were slightly higher than those of the dead cells (Figure 1C). Furthermore, the total detection time (after addition of bacteria to the wells of assay plates) required for sandwich ELISA was 5.5 h, while 4 h for MaCIA (Figure 1C).

Specificity of the MaCIA Platform

Next, the specificity of the MaCIA was determined by testing a panel of 15 S. Enteritidis strains, eight S. Typhimurium strains, 11 other Salmonella serovars, and 14 non-Salmonella spp. at ~1 × 10⁶ to 1 × 10⁷ CFU/mL each. The data showed that MaCIA was highly specific toward all tested viable strains of S. Enteritidis or S. Typhimurium serovars depending on the reporter antibody used and the signals were significantly (P < 0.001) higher than the signals obtained for other Salmonella serovars or non-Salmonella species (Figures 2A,B and Table 1). Thus, any sample showing a significantly higher signal (P < 0.001) than the negative control was considered positive. Furthermore, samples containing live S. Enteritidis cells gave significantly (P < 0.0001) higher absorbance values (signals) than that of the values obtained for dead cells or the PBS control (Figure 2B). The specificity of MaCIA toward viable cells was not affected when tested against a mixture containing equal amounts of viable and dead pathogens.
FIGURE 2 | Continued
S. Enteritidis cells (Figure 2C), and non-S. Enteritidis bacteria (Figure 2D). Immuno-stained confocal images, the Z-stacking (three-dimensional image), and Giemsa stain images confirmed increased adhesion of viable S. Enteritidis cells to HCT-8 cells than that of the dead S. Enteritidis cells (Figures 2E–G). Confocal imaging further revealed the absence of non-specific binding of mAb-2F11 to the HCT-8 cell monolayer (Figures 2E,F). Furthermore, MaCIA successfully detected S. Enteritidis cells when exposed to various stressors for 3 h (Hahm and Bhunia, 2006) including cold (4°C), heat (45°C), acidic pH (4.5) and NaCl (6.5%) for 3 h before analysis. +, Positive control (bacteria without any stress exposure); −, No bacteria; dead, heat-killed S. Enteritidis cells. Error bars represent SEM. **** \( P < 0.0001; ** P < 0.001; * P < 0.01; ns, no significance.\)

### Detection Sensitivity of MaCIA

To determine assay sensitivity, S. Enteritidis cells were serially diluted using either PBS or ground chicken suspended in buffered peptone water (BPW) and added to the wells containing formalin-fixed HCT-8 cell monolayers (30-min post-fixation). After a 30-min incubation at 37°C with test samples, the monolayers were washed, probed with mAb-2F11, and the color was developed. An initial bacterial concentration of 1 \( \times 10^6 \) to 1 \( \times 10^8 \) CFU/mL showed significantly \( P < 0.001 \) higher signal than the wells containing 1 \( \times 10^5 \) CFU/mL or dead cells (1 \( \times 10^6 \) cells) suspended in PBS (Figure 4A) or ground chicken slurry (Supplementary Figure 1) and the absorbance values showed strong correlation \( R^2 = 0.9344 \) with S. Enteritidis cell numbers (1 \( \times 10^6 \) CFU/mL to 1 \( \times 10^8 \) CFU/mL) (Figure 4B). Furthermore, MaCIA also showed a similar concentration-dependent rise in signals when bacteria were suspended in ground chicken, liquid egg, milk, and cake mix slurry (Figure 4C). However, the detection sensitivity varied depending on the food matrix tested. In milk, the detection limit was determined to be 1 \( \times 10^5 \) CFU/mL while in ground chicken, 1 \( \times 10^6 \) CFU/mL, in cake mix, 1 \( \times 10^7 \) CFU/mL, and in egg, 1 \( \times 10^8 \) CFU/mL (Figure 4C). These results indicate that assay sensitivity for MaCIA for the detection of S. Enteritidis varies from 1 \( \times 10^5 \) CFU/mL to 1 \( \times 10^8 \) CFU/mL depending on the food matrix tested.

### Further Optimization of MaCIA

#### One-Step Antibody Probing Method

To shorten the detection time, we explored if a one-step antibody probing approach is feasible. Ground chicken was inoculated with S. Enteritidis at 6 \( \times 10^2 \) CFU/25 g in a stomacher bag. After 10-h enrichment at 37°C, the...
enriched chicken samples (1 mL) were added to the fixed HCT-8 cell monolayer for 30 min, followed by PBS wash (3 times). The cell monolayers were probed with an antibody cocktail that contained both primary (mAb-2F11) and secondary (anti-mouse HRP-conjugated IgG) antibodies, followed by the colorimetric substrate. Data showed that the signal obtained from the one-step antibody probing was comparable to the results when the sequential antibody probing method was used (Figure 5A). This experiment indicates that one-step antibody probing is equally effective as the sequential antibody probing method, thus shortening the assay time by 2.5 h.

**On-Cell Food Sample Enrichment**

Direct on-cell (MaCIA platform) enrichment of test samples was pursued to simplify the assay procedure and to reduce the sample handling steps. S. Enteritidis inoculated food suspensions (with an initial inoculation of 10 CFU/mL) were directly added to the wells (1 mL/well) containing formalin-fixed HCT-8 cell monolayers and incubated at 37°C. The assay was performed after 6, 7, 8, and 9-h on-cell sample enrichment followed by sequential antibody probing (3 h). After 7-h on-cell enrichment, both ground chicken and egg samples gave positive results while the whole milk and cake mix needed 9-h enrichment to give positive results when compared with

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**FIGURE 5** | Mammalian cell-based immunoassay assay optimization. (A) One-step antibody probing vs sequential antibody probing against a bacterial cell concentration of 8.75 × 10⁶ CFU/mL of S. Enteritidis. (B) Analysis of time (h) required for positive MaCIA result during on-cell enrichment of S. Enteritidis PT21 (~10 CFU/mL) inoculated into different food products. (C) Light microscopic images of formalin-fixed HCT-8 cell monolayers after on-cell enrichment for 7–9 h. Magnification (400×). (D) MaCIA analysis of skin swab samples after on-cell enrichment (7 h). Samples with higher concentrations were also significantly (P < 0.001) different than the negative control. Error bars represent SEM. ****P < 0.0001; **P < 0.01; ns, no significance. Cut-off for positive: P < 0.001.
uninoculated food samples (Figure 5B). A similar result was obtained when the food samples were tested in a blinded fashion (Supplementary Figure 2). Total assay time (sample-to-result) for on-cell enrichment was estimated to be 10–12 h. Remarkably, the HCT-8 cell monolayers remained intact without any visible damage during on-cell enrichment (Figure 5C). Due to the limitation in the amount of sample volume (1 mL/well), that can be tested, the “on-cell enrichment” option is suitable only when the starting S. Enteritidis concentration is above 10 CFU/mL (2.5 × 10^3 CFU/25 g); hence it may not be suitable for routine testing of bulk-food samples that may contain < 100 CFU/g.

We then examined if the on-cell enrichment set up is suitable for testing surface swab samples. Skin swabs from inoculated chicken thigh parts (1.35 × 10^9 to 1.35 × 10^5 CFU/50 cm^2 at 4°C for 24 h) were resuspended in 1.1 mL of BPW, and 1 ml of each suspension was added to the wells of MaCIA. After 7-h on-cell enrichment followed by sequential immunoprobing (3 h), MaCIA generated significantly (P < 0.0001) higher signals than that of the values obtained from the negative control (swabbed suspension of the uninoculated sample) (Figure 5D). These data indicate that MaCIA is suitable for testing surface swab samples, and results can be obtained in less than 12 h.

**Comparison of MaCIA With the USDA/FDA Detection Methods**

To compare the performance of MaCIA with USDA/FDA detection methods, S. Enteritidis inoculated food samples (ground chicken, egg, milk and cake mix held at 4°C for 24 h) were also tested in parallel using the US Department of Agriculture (USDA-FSIS, 2013) or Food and Drug Administration (FDA, 2001) reference method.

**Growth Kinetics of S. Enteritidis in Different Foods**

Freshly grown (37°C, 18 h) S. Enteritidis PT21 culture was inoculated (<10 CFU/ml) into 25 g of each ground chicken, egg, whole milk, or cake mix in 225 mL BPW in a stomacher bag (Seward Inc., Bohemia, NY, United States) and held at 4°C for 24 h. Inoculated food samples were then incubated at 37°C and bacterial counts were determined every 2-h intervals until 18 h. The growth data of S. Enteritidis in all tested food samples were fitted with the Gompertz model to generate a growth curve (Figure 6A). The R^2 values of Gompertz fitted growth curves of S. Enteritidis PT21 in ground chicken, egg, whole milk, and cake mix were 0.99, 0.99, 0.96, and 0.99, respectively. Based on the Gompertz modeled growth curve equations, the lag phase duration (LPD) and exponential growth rate (EGR) were estimated to be 2.204–2.427 h and 0.767–0.934 log (CFU/mL)/h, respectively (Supplementary Table 1). Utilizing LPD, EGR, and the MaCIA detection limit data, we were able to estimate the required enrichment time for each food product, assuming the starting S. Enteritidis concentration is 1 CFU/25 g (Supplementary Table 1). The required enrichment time for ground chicken, egg, milk, and cake mix was estimated to be 14, 19, 16, and 16 h, respectively (Supplementary Table 1).

**Sample-to-Result Time**

To confirm the sample-to-result time, we inoculated the selected food samples with S. Enteritidis at 0, 9, or 45 CFU/25 g (Figure 6B) and 0, 2 or 45 CFU/mL (Figure 6C). After a specified enrichment period, we analyzed the samples using MaCIA. All S. Enteritidis-inoculated samples produced significantly higher signals (P < 0.0001) than the uninoculated food samples (Figures 6B,C) even in the presence of background microflora (Supplementary Figure 3). The sample-to-result time was estimated to be 16–21 h. Analysis of food samples by the USDA-FSIS or FDA-BAM method followed by polymerase chain reaction (PCR) assay using three sets of primers targeting invA,
IE-1, and IE-2 genes (Figure 6D) confirmed the presence of S. Enteritidis in these food samples. Note, the USDA method needed 72 h, while the FDA method needed 72–168 h to confirm the presence of Salmonella in the inoculated food samples.

Formalin-Fixation Prolongs the Shelf-Life of MaCIA

The bottleneck for widespread use of cell-based sensors is its limited shelf-life. As we have demonstrated earlier (Figure 1B), the performance of MaCIA prepared with live HCT-8 cells is equally sensitive to the formalin-fixed HCT-8 cells (30 min after fixation). In this experiment, we investigated if the prolonged storage (4, 8, and 14 weeks at 4°C or 4 weeks at room temperature) of formalin-fixed HCT-8 cell would uphold MaCIA’s performance. Data showed that formalin-fixed HCT-8 cells stored for 4–12 weeks at 4°C generated comparable results to that of live HCT-8 cells when tested with viable S. Enteritidis PT21 at a concentration of $1 \times 10^7$ CFU/ml and signals were significantly higher ($P < 0.0001$) than the signals generated by an equivalent amount of dead S. Enteritidis cells or the PBS control (no bacteria) (Figure 7A). The light microscopic photomicrographs further confirmed that the cell monolayer and the cellular morphology in formalin-fixed HCT-8 cells were unaffected after 14 weeks of storage at 4°C or even after bacterial exposure and the subsequent three PBS wash (Figure 7B). These results indicate that formalin fixation was able to prolong the shelf-life of HCT-8 cells up to 14 weeks without affecting their performance, thus showing a promising application of the MaCIA for point-of-need deployment.

DISCUSSION

The conventional culture-based detection methods (sample-to-result) take 4–7 days to obtain the results (FDA, 2001; USDA-FSIS, 2013; Bell et al., 2016), and the so-called rapid methods take at least 24–48 h (Bhunia, 2014; Lee et al., 2015; Ricke et al., 2018; Rajapaksha et al., 2019). This is a major inconvenience for the food industries since some foods have a limited shelf-life. Furthermore, holding of products until the microbiological safety assessment can also increase the cost of storage. Therefore, products are released into the supply chain even before obtaining test results. Such practice is very costly, resulting in more than hundreds of recalls each year and the loss of millions of pounds of food (Buzby et al., 2014; Elkhishin et al., 2017). Therefore, rapid, accurate, and user-friendly viable pathogen detection tools are in high demand to lower recalls, reduce food waste and financial loss, and prevent foodborne outbreaks.

Mammalian cell-based assays are highly attractive functional screening tools to assess the presence of viable pathogens or active toxins in near-real-time (Bhunia, 2011, 2014; To et al., 2020). CBB monitors host-hazard interaction (Banerjee and Bhunia, 2009); therefore, non-pathogenic, non-hazardous, dead, or non-toxic agents do not yield false results. However, the major drawback is its short self-life, i.e., the mammalian cells may not survive on the sensor platform for a prolonged period without the proper growth conditions. Mammalian cells have stringent requirements for specialized growth media and growth conditions for survival, such as temperature and CO$_2$-controlled humidified environment. Limited self-life of cells is a monumental deterrent for CBB’s widespread application affecting its deployment for point-of-need use. To overcome the limitation, we employed formalin (4% formaldehyde) to preserve the functionality of the mammalian cells. We used the human ileocecal cell line, HCT-8, as our model cell line, which maintained its functionality after formalin-fixation, at least for 14 weeks at 4°C. The fixed HCT-8 cells showed selective interaction with viable or even stress-exposed Salmonella, while dead cells had negligible or no interaction at all (Figures 1–3). Further specificity of the assay was accomplished by immunoprobing the adhered bacterial cells using a specific antibody. The MaCIA was found to be highly specific for the detection of S. Enteritidis or S. Typhimurium without showing any cross-reaction with other Salmonella serovars or non-Salmonella species tested. The assay was further validated for its ability to detect S. Enteritidis in inoculated ground chicken, egg, whole milk, and cake mix in the presence of background natural microflora. A brief sample enrichment step also allows the resuscitation of stressed or injured cells before detection (Wu, 2008).

In the MaCIA platform, HCT-8 cells were used as a capture element instead of an antibody, which is traditionally used in a sandwich ELISA. In this study, HCT-8 cells out-performed the antibody (Figure 1C), and 30 min incubation was sufficient for optimal capture of viable bacteria by HCT-8 cells (Jaradat and Bhunia, 2003; Barrila et al., 2017) while 2 h was needed for sandwich ELISA. Improved bacterial capture by HCT-8 is attributed to the formation of a three-dimensional structure by mammalian cell monolayer (Figure 2E), creating a larger surface area for bacteria to bind. Furthermore, HCT-8 cell possesses surface receptor molecules for specific interaction with Salmonella adhesion factors. S. Enteritidis utilize type 1 fimbria to recognize and bind to high-mannose oligosaccharides, which are carried by various glycoproteins on the host cell surface (Kolenda et al., 2019). Long polar fimbriae also mediate adhesion of Salmonella to Peyer’s patches on the host cell (Bäumler et al., 1996). Besides, MaCIA was able to differentiate viable cells from dead Salmonella cells while sandwich immunoassay was unable. Lack of adhesion of dead Salmonella to HCT-8 may be due to the loss or denaturation of bacterial adhesins (Figure 2G). While in sandwich ELISA, bacterial surface antigens from dead cells were still able to bind the capture-antibody. MaCIA also showed strong signals when tested with stress-exposed S. Enteritidis cells suggesting a brief stress exposure (3 h) does not affect bacterial ability to interact with the HCT-8 cells (Figure 3) while such exposure caused a 20–48% reduction in ELISA signal for Salmonella in a previous study (Hahm and Bhunia, 2006).

The sensitivity of MaCIA was found to be about $1 \times 10^6$ CFU/mL to $1 \times 10^7$ CFU/mL, which is in agreement with a typical ELISA where antibodies serve as the capture molecule (Mansfield and Forsythe, 2000; Eriksson and Aspán, 2007) or ELISA with bacteriophage as a recognition molecule (Galikowska et al., 2011). However, MaCIA has the potential to outperform ELISA in some aspects, due to its ability to differentiate viable from dead bacteria. Viable pathogens that can adhere and invade
into intestinal cells are of food safety concerns. MaCIA is a better choice over ELISA for the food industry when viable pathogens in food are the target. False-positive results generated by either ELISA or PCR due to the presence of non-viable pathogens could lead to unnecessary recalls, food waste, and economic loss. On the other hand, assays with higher sensitivity may be useful for detecting samples with low bacterial concentration, but enrichment is considered a necessary step to ensure accuracy (Bhunia, 2014). Assuming a 25 g sample contains 1 CFU of bacteria unless one performs a test on the entire 25 g sample, there is a high possibility that one would not be able to accurately detect the bacteria even with a sensor that has the sensitivity to detect 1 CFU. So, the sensitivity of an assay not only depends on the limit of detection but also on the sample size. Therefore, we proposed to perform MaCIA in concert with the traditional enrichment step, to offer a more reliable and accurate testing result.

The assay sensitivity was also affected by the food matrices tested. Ground chicken, raw eggs, whole milk, and cake mix
were chosen since these products were implicated in *Salmonella* outbreaks, and they also represent foods with high protein, fat, or carbohydrate contents. In milk, the detection limit for *S. Enteritidis* was $1 \times 10^5$ CFU/mL while in ground chicken, $1 \times 10^6$ CFU/mL, in cake mix, $1 \times 10^7$ CFU/mL, and in egg, $1 \times 10^8$ CFU/mL (Figure 4C). Among the foods tested, eggs exhibited the highest interference while milk had the least. Egg contains about 13 g protein and 11 g fat per 100 g while whole milk contains only 3.15 g of protein and 3.25 g of fat per 100 g (Kuang et al., 2018).

Mammalian cell-based immunoassay is highly specific for *S. Enteritidis* and did not show any non-specific reaction with other *Salmonella* serovars, non-*Salmonella* organisms, or natural microflora present in uninoculated food samples. The specificity of MaCIA is attributed to the specificity of the reporter antibody, mAb-2F11 used, that binds the O-antigen (LPS) on the surface of *S. Enteritidis* (Masi and Zawistowski, 1995; Jaradat et al., 2004). The advantage of the MaCIA platform is that the specificity depends on the primary reporter antibody used. We have demonstrated that using a commercial anti-*Salmonella* mAb-F68C (Thermo-Fisher) as a reporter antibody, *Salmonella* enterica serovar Typhimurium can be detected on the MaCIA platform (Table 1). These results indicate that the MaCIA platform is versatile and can be adapted for a different target pathogen using an appropriate antibody.

The accuracy of MaCIA for *S. Enteritidis* was also confirmed by comparing the results with the reference methods, such as the FDA-BAM, USDA-FSIS, and PCR (Figure 6D). The three primer sets that were used in PCR (Supplementary Table 3) target IE-1, IE-2 in *S. Enteritidis*, and InvA in *S. Enteritidis* and *S. Typhimurium* (Fratamico and Strobaugh, 1998; Wang and Yeh, 2002; Paiaó et al., 2013), which again confirm the accuracy of MaCIA for its ability to detect *S. Enteritidis* from spiked food samples.

The major advancement of the MaCIA is its extended shelf-life, at least for 14 weeks, that was achieved through formalin-fixation of HCT-8 cells. Formalin is routinely used to preserve tissues and cells and it protects protein from denaturation (Eltoum et al., 2001). Therefore, receptor molecules on formalin-fixed HCT-8 cells, remained active and enabled viable *Salmonella* binding without diminishing MaCIA's performance. Previously, many attempts have been made to extend the shelf-life (functionality) of cells in CBB; however, none were satisfactory. Bhunia et al. (1995) used ultra-low temperature (freezing at $-80$°C and $-196$°C) to extend the shelf-life of cells (up to 8 weeks) before performing the cytotoxicity assay for *L. monocytogenes*. However, the major drawback was the generation of high background signal originating from freeze-injured or dead mammalian cells. Banerjee et al. (2007) used modified growth conditions that included 5% fetal calf serum without any exogenous CO$_2$ and was able to extend the viability of the lymphocyte cell line for 6–7 days at room temperature. Curtis et al. (2009) used an automated media delivery system integrated with a thermoelectric controller to keep endothelial cells healthy up to 16 weeks. More recently, Jiang et al. (2018) used a screen-printed hydrogel-encapsulated rat basophilic leukemia mast cell-based electrochemical sensor for the detection of quorum sensing molecules for fish spoilage and the sensor-generated stable signal for 10 days. However, these attempts required incorporating mammalian cells in a specially designed external device to ensure the success of detection.

**CONCLUSION**

In conclusion, the present study demonstrates that MaCIA is a highly specific functional cell-based assay coupled with an immunoassay for the rapid and specific detection of the viable target pathogen. *S. Enteritidis* was used as a model pathogen which was successfully detected from food samples (ground chicken, shelled egg, whole milk, and cake mix) in 16–21 h using a conventional sample enrichment set up. The assay time (sample-to-result) was shortened to 10–12 h when an on-cell (on the MaCIA platform) sample enrichment was used. Thus, MaCIA could serve as a universal platform for other pathogens provided an appropriate cell line and a pathogen-specific antibody is used. The extended shelf-life of mammalian cells made MaCIA an attractive screening tool for point-of-need deployment. Furthermore, the MaCIA platform (24-well tissue culture plate) is suitable for testing at least 10 samples (plus positive and negative controls) in duplicate on a single plate thus reducing overall cost per sample testing.

**MATERIALS AND METHODS**

**Mammalian Cell Culture**

HCT-8 cell line (ATCC, Manassas, VA, United States) was maintained in Dulbecco's modified Eagles medium (DMEM; Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Bio-Techne Sales Corp, Minneapolis, MN, United States) at 37°C with 5% CO$_2$ in cell culture flasks (T25). For all experiments, HCT-8 cells were seeded in 24-well tissue culture plates (Fisher Scientific) at a density of $5 \times 10^4$ cell/mL/well. Media were replaced on day 4 and a final cell density of $2 \times 10^5$ cell/mL (monolayer) was achieved on day 5. Cell monolayers were washed twice with PBS (0.1 M, pH 7.0) and used immediately (Live HCT-8 cell assay) or exposed to 4% formaldehyde (Polysciences Inc., Warrington, PA) of 500 µL/well and incubated at room temperature for 10 min (Formalin-fixed HCT-8). Formaldehyde solution was removed and the cell monolayers were washed three times with PBS. Formalin-fixed cells were stored in 1 mL PBS/well for 14 weeks at 4°C or until use.

**Bacterial Culture and Growth Media**

Bacterial strains (Table 1) were stored as 10% glycerol stocks at $-80$°C. To revive frozen cultures, each strain was streaked onto tryptic soy agar (TSA; Thermo Fisher Scientific, Rochester, NY, United States) plate and incubated at 37°C for 18 h to obtain pure colonies. A single colony of each strain was inoculated and propagated in tryptic soy broth containing 0.5% yeast extract (TSBYE; Thermo Fisher Scientific) at 37°C for 18 h with shaking at 120 rpm.
Development and Specificity of MaCIA

HCT-8 cell monolayers were prepared and maintained as described above in 24-well plates. Overnight grown bacterial cultures (Table 1) were diluted in PBS to achieve a cell concentration of $1 \times 10^7$ CFU/mL. To obtain dead cells, cell suspensions were treated with heat (80°C for 10 min) or formaldehyde (4% for 10 min) and plated on TSA to ensure bacterial inactivation. One milliliter of bacterial cell suspensions was added into each well containing HCT-8 cells and incubated for 30 min at 37°C (Jaratad and Bhunia, 2003; Barrila et al., 2017). Cell monolayers (live or formalin-fixed) were washed 2–3 times with PBS gently and sequentially probed with either mAb-2F11 (3.06 μg/mL) (Jaratad et al., 2004) or mAb-F68C (0.2 μg/mL; Catalog # MAI-7443; Thermo Fisher Scientific) as primary antibodies, and anti-mouse HRP-conjugated secondary antibodies for 1.5 h each at room temperature. Both antibodies were suspended in PBS containing 3% BSA and incubated with PBS to remove unattached bacterial cells (as above). After immunoprobing with mAb-2F11, the monolayers were washed and probed with Alexa Fluor 488 conjugated anti-mouse antibody at room temperature for 1.5 h. The slides were mounted using an antifade reagent (Cell-Signaling). Images were acquired using the Nikon A1R confocal microscope with a Plan Ap VC oil immersion objective (Drolia et al., 2018) and were processed with the Nikon Elements software at the Purdue Bindley Bioscience Imaging Facility.

Immunofluorescence and Giemsa Staining

After exposure of formalin-fixed HCT-8 cell monolayers to viable or dead S. Enteritidis ($1 \times 10^8$ cells/ml) for 30-min, the wells of the chambered slides (Fisher Scientific) were washed with PBS to remove unattached bacterial cells (as above). After immunoprobing with mAb-2F11, the monolayers were washed and probed with Alexa Fluor 488 conjugated anti-mouse antibody for 1.5 h at room temperature in the dark, followed by three PBS wash. Note, antibody concentrations used were the same as above. The monolayers were counterstained with DAPI (500 ng/mL; Cell-Signaling) for nuclear staining and the slides were mounted using an antifade reagent (Cell-Signaling). Images were acquired using the Nikon A1R confocal microscope with a Plan Ap VC oil immersion objective (Drolia et al., 2018) and were processed with the Nikon Elements software at the Purdue Bindley Bioscience Imaging Facility.

For Giemsa staining, the formalin-fixed HCT-8 cell monolayers were exposed to viable or dead S. Enteritidis cells as above, air-dried, and immersed in Giemsa staining solution for 20 min. Giemsa staining solution was prepared using a 20-fold dilution of the KaryoMAX Giemsa staining solution (Thermo-Fisher) in deionized water. The slides were examined under a Leica DAS Microscope at the magnification of 1,000×.

Sensitivity of MaCIA

HCT-8 cell monolayers were prepared and maintained as described above in 24-well tissue culture plates. Overnight grown fresh S. Enteritidis PT21 culture was serially diluted to obtain $1 \times 10^8$ CFU/mL to $1 \times 10^6$ CFU/mL using PBS or homogenized 25 g food samples (Supplementary Table 2) in 225 mL BPW (Becton Dickinson, Sparks, MD, United States). One milliliter of each diluted sample was added onto HCT-8 cell monolayer and was incubated at 37°C for 30 min. The remaining steps were the same as above.

Detection of Stressed Cells Using MaCIA

Freshly prepared S. Enteritidis cells ($2.17 \times 10^8$ CFU/ml) suspended in TSB were exposed to cold (4°C), heat (45°C), acidified TSB (pH, 4.5) and 5.5% NaCl for 3 h, as reported before (Hahn and Bhunia, 2006). Bacterial cells were washed with PBS and added onto the fixed HCT-8 monolayer for 30-min and probed with mAb-2F11 as above.

Salmonella Growth Kinetics Assessment

Overnight-grown S. Enteritidis PT21 cultures were serially diluted in PBS to achieve a concentration of $1 \times 10^6$ CFU/mL. One hundred microliters of the diluted culture were added into 25 g of each ground chicken, whole fat milk, liquid eggs, and...

Sandwich ELISA

High-affinity (4HBX) ELISA plates (Thermo Fisher Scientific) were coated with mAb-2F11 for 2 h at 37°C, followed by 3 times wash with PBS-T (PBS containing 0.01% Tween-20). Freshly prepared BSA-PBS solution (1 mg/mL) was used for blocking at 37°C for 2 h. After washing with PBS-T, overnight grown cultures (Table 1) were added into the plates at a concentration of $1 \times 10^7$ CFU/mL. To obtain dead cells, cell suspensions were exposed to cold (4°C), heat (45°C), acidified TSB (pH, 4.5) and 5.5% NaCl for 3 h, as reported before (Hahn and Bhunia, 2006). Bacterial cells were washed with PBS and added onto the fixed HCT-8 monolayer for 30-min and probed with mAb-2F11 as above.

Western Blot

The whole-cell lysate of L. monocytogenes F4244, P. aeruginosa PRJ99, E. coli EDL933, and S. Enteritidis PT21 overnight cultures (5 mL each) was prepared by sonication (Branson, Danbury, CT, United States). Bacterial samples were sonicated in an ice bucket (three 10 s cycles at 30-s intervals) and centrifuged for 10 min at 14,000 rpm (Eppendorf) at 4°C to separate the soluble fraction (supernatant) from the bacterial debris (pellet). The protein concentration was determined by the BCA method (Thermo Fisher Scientific). Equal amounts of proteins were separated on SDS-PAGE gel (10% polyacrylamide) and electro-transferred to polyvinylidene difluoride (PVDF) membrane (Fisher Scientific) (Singh et al., 2016; Drolia et al., 2018). Primary and secondary antibodies were diluted as above. Membranes were first probed with mAb-2F11 at 4°C overnight, and then with anti-mouse HRP conjugated antibody at room temperature for 1.5 h. LumiGLO reagent (Cell-Signaling Technology) was used to visualize the bands using the Chemi-Doc XRS system (Bio-Rad).
cake mix with 225 mL BPW and were incubated at 4°C for 24 h. The samples were then incubated at 37°C for 20 h with shaking at 120 rpm and enumerated on XLD (xylose lysine deoxycholate) agar plates (Remel, San Diego, CA) at every hour. S. Enteritidis counts in artificially inoculated samples at earlier stages of growth was determined by directly plating 1, 0.5, 0.1 mL of the sample on XLD plates with four repeats (1, 2, and 3 h); and S. Enteritidis counts from the later stages of growth (3 h and after) was obtained after serially diluting the samples in PBS. The growth of S. Enteritidis in food samples enriched using BPW was modeled using the Gompertz equation (Silk et al., 2002; Kim and Bhunia, 2008) through Prism software version 8.0. Lag-phase duration (LPD) and exponential growth rate (EGR) were calculated from the Gompertz model and were used to determine an enrichment time required for each food product to reach an optimum S. Enteritidis concentration required for detection by MaCIA, assuming the initial concentration was 1 CFU/25 g of sample.

Food Sample Testing With MaCIA and Validation With the FDA and USDA Methods

Food samples (ground poultry, milk, egg, or cake mix) were inoculated with variable concentrations of S. Enteritidis PT21. To simulate cold storage, inoculated foods were stored at 4°C for 24 h. Samples (25 g in 225 mL BPW) were then homogenized or pummeled using hands and incubated at 37°C for 14–19 h (Supplementary Table 1) with shaking at 120 rpm. One milliliter of enriched food sample was added into each well of MaCIA for 30 min, followed by immunoprobing as above.

For direct on-cell enrichment, the homogenized food suspensions (1 ml of each food sample) were dispensed into wells containing formalin-fixed HCT-8 cells (MaCIA) and incubated for 7–9 h. After the removal of food samples, wells were washed 3 times with PBS before immunoprobing and color development. Salmonella counts in enriched food samples (inoculated or uninoculated) were enumerated on XLD plates. The presence of background bacteria in uninoculated food samples was assessed on TSA plates after incubation at 37°C for 24 h. For the blind test, the inoculation of the samples was performed by XB, while the MaCIA test was done by LX in a blinded fashion without prior knowledge of samples that were inoculated with Salmonella.

Inoculated food samples were also analyzed by the FDA-BAM (FDA, 2001) or USDA-FSIS (2013) method as before. The ground chicken was processed according to the USDA-FSIS method, while shelled egg, whole milk, and cake mix were prepared based on the FDA-BAM. Twenty-five gram of each prepared sample was then enriched in 225 mL of BPW (ground chicken), trypticase soy broth (shelled egg), and lactose broth (whole milk and cake mix) at 37°C for 24 h followed by sequential enrichment in RV (Rappaport-Vassiliadis) broth and TT (tetraphionate) broth at 42°C for 24 h. Samples were then plated on selective BGS (Brilliant Green Agar with Sulfadiazine) or XLD agar plates to isolate colonies, which were further confirmed by PCR assay.

For PCR assay, DNA was extracted from the isolated colonies by the boiling method (Kim and Bhunia, 2008; Kim et al., 2015). The primer sequences and the putative product sizes for each amplicon are listed in Supplementary Table 3 (Wang and Yeh, 2002; Paião et al., 2013). PCR reaction mixture (25 µL) contained 1 µL of DNA template, 0.2 µM of each primer, 2.5 mM MgCl2, 200 µM of dNTP, 1 x GoTaq flexible buffer of buffer and 1 U of GoTaq Flexi DNA polymerase (Promega) (Singh et al., 2014). The PCR amplification was performed in the ProFlex PCR system with an initial denaturation at 94°C for 3 min, 35 amplification cycles consisting of 1 min of denaturation at 94°C, 1.5 min of annealing at 50°C, and 1.5 min of elongation at 72°C. DNA amplicons were analyzed using agarose gel (1.5%, wt/vol) electrophoresis containing ethidium bromide (0.5 µg of /mL).

Swab Sample Testing

Chicken thigh cuts (procured from a local grocery store) were inoculated with overnight grown S. Enteritidis PT21 at 1.35 × 10^3 to 1.35 × 10^5 CFU per 50 cm² evenly on the skin of chicken thighs. Inoculated samples were stored at 4°C for 24 h. BPW-soaked sterile rayon tipped swab applicators (Puritan, Guilford, ME, United States) were used to swab the chicken skin and were vortexed in 1.1 mL of BPW. One milliliter of the sample was added into each well of MaCIA and incubated at 37°C for 7 h for on-cell enrichment, followed by immunoprobing as above. The rest of the swabbed sample (0.1 mL) was used to enumerate Salmonella on XLD plates.

Statistical Analysis

All data were analyzed using GraphPad Prism software (San Diego, CA, United States). The unpaired t-test was used when comparing two datasets. Tukey’s multiple comparison test was also used when comparing more than two datasets. All data were presented with mean ± standard error of the mean (SEM).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LX, XB, RD, and AB conceived and designed the experiments. LX, XB, ST, YL, and RD performed the experiments. LX, XB, and AB analyzed the data. LX and AB wrote the manuscript. All authors approved the final version.

FUNDING

This material is based upon work supported by the U.S. Department of Agriculture, Agricultural Research Service, under Agreement No. 59-8072-6-001, and the USDA National Institute of Food and Agriculture (Hatch accession no. 1016249). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.
ACKNOWLEDGMENTS

We thank S. Lelièvre, B. M. Applegate, T. Kwok, and S. Chittiboyina for advice and D. Liu, Z. Tang, W. Lv, M. Samaddar for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.575615/full#supplementary-material

Supplementary Figure 1 | Detection sensitivity of MaCIA tested against the different concentrations of S. enterica serovar Enteritidis cells suspended in ground chicken slurry (in buffered peptone water). D. dead cells.

REFERENCES

Abdelhaseib, M. U., Singh, A. K., Bailey, M., Singh, M., El-Kbateib, T., and Bhunia, A. K. (2016). Fiber optic and light scattering sensors: complimentary approaches to rapid detection of Salmonella enterica in food samples. Food Control 61, 135–145. doi: 10.1016/j.foodcont.2015.09.031

Banerjee, P., and Bhunia, A. K. (2009). Mammalian cell-based biosensors for pathogens and toxins. Trends Biotechnol. 27, 179–188. doi: 10.1016/j.tibtech.2008.11.006

Banerjee, P., and Bhunia, A. K. (2010). Cell-based biosensor for rapid screening of pathogens and toxins. Biosens. Bioelectron. 26, 99–106. doi: 10.1016/j.bios.2010.05.020

Banerjee, P., Lenz, D., Robinson, J. P., Rickus, J. L., and Bhunia, A. K. (2008). A novel and simple cell-based detection system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for rapid detection of pathogens and toxins. Lab. Invest. 88, 196–206. doi: 10.1038/landev.3700703

Banerjee, P., Morgan, M. T., Rickus, J. L., Ragheb, K., Corvalan, C., Robinson, J. P., et al. (2007). Hybridized Ped-2E9 cells cultured under modified conditions can sensitively detect Listeria monocytogenes and Bacillus cereus. Appl. Microbiol. Biotechnol. 73, 1423–1434. doi: 10.1007/s00253-006-0622-0

Barrila, J., Yang, J., Crabbe, A., Sarker, S. F., Liu, Y., Ott, C. M., et al. (2017). Three-dimensional organotypic co-culture model of intestinal epithelial cells and macrophages to study Salmonella colonization patterns. NPJ Microgravity 3:10.

Bäumler, A. J., Tsolis, R. M., and Heffron, F. (1996). The Ipf fimbrial operon mediates adhesion of Salmonella typhimurium to murine Peyer's patches. Proc. Nat. Acad. Sci. U.S.A. 93, 279–283. doi: 10.1073/pnas.93.1.279

Bell, R. L., Jarvis, K. G., Ottesen, A. R., Mcfarland, M. A., and Brown, E. W. (2016). Recent and emerging innovations in Salmonella detection: a food and environmental perspective. Microb. Biotechnol. 9, 279–292. doi: 10.1111/1751-7915.12399

Bhunia, A. K. (2011). Rapid pathogen screening tools for food safety. Food Technol. 65, 38–43.

Bhunia, A. K. (2014). One day to one hour: how quickly can foodborne pathogens be detected? Future Microbiol. 9, 935–946. doi: 10.2217/fmb.14.61

Bhunia, A. K. (2018). “Salmonella enterica,” in Foodborne Microbial Pathogens: Mechanisms and Pathogenesis, ed. A. K. Bhunia (New York, NY: Springer), 271–287.

Bhunia, A. K., Westbrook, D. G., Story, R., and Johnson, M. G. (1995). Frozen stored murine hybridoma cells can be used to determine the virulence of Salmonella monocytogenes. J. Clin. Microbiol. 33, 3349–3351. doi: 10.1128/jcm.33.12.3349-3351.1995

Buzby, J. C., Farah-Wellis, H., and Hyman, J. (2014). The Estimated Amount, Value, and Calories of Postharvest Food Losses at the Retail and Consumer Levels in the United States. Washington, DC: USDA-ERS, 121. Economic Information Bulletin Number.

CDC (2018). Salmonella—reports of selected Salmonella outbreak investigations. Atlanta, GA: CDC.}

Supplementary Figure 2 | Experimental set-up of the blind test using an on-cell (MaCIA) enrichment method. (A,B) The checkerboard filled areas correspond with sample a; the diagonal stripes filled areas correspond with sample b; No pattern-filled area corresponds with negative control for each food product. The numbers in the table represent the concentration (CFU/mL) of the inoculant, S. Enteritidis PT21. (C) Blind test using on-cell enrichment. Positive samples were inoculated with 25 CFU/mL cold-stored S. Enteritidis PT21. Neg: negative control. a, b: blind tested samples.

Supplementary Figure 3 | Tryptic soy agar (TSA) plates showing the presence of background bacterial populations from different food samples except for the eggs.

Supplementary Table 1 | Proposed enrichment time for different food products before testing with MaCIA.

Supplementary Table 2 | Total detection time required for each method.

Supplementary Table 3 | PCR primer sequences used.

CDC (2020). Salmonella. Atlanta, GA: CDC.

Curtis, T. M., Widder, M. W., Brennan, L. M., Schwager, S. J., Van Der Schalie, W. H., Fey, J., et al. (2009). A portable cell-based impedance sensor for toxicity testing of drinking water. Lab Chip 9, 2176–2183. doi: 10.1039/b91314h

Dibao-Dina, A., Follet, J., Ibrahim, M., Vlandas, A., and Senez, V. (2015). Electrical impedance sensor for quantitative monitoring of infection processes on HCT-8 cells by the waterborne parasite Cryptosporidium. Biosens. Bioelectron. 66, 69–76. doi: 10.1016/j.bios.2014.11.009

Dos Reis, R. S., and Horn, F. (2010). Enteropathogenic Escherichia coli, Salmonella, Shigella and Yersinia: cellular aspects of host-bacteria interactions in enteric diseases. Gut Pathog. 2:8. doi: 10.1186/1757-4749-2-8

Drolia, R., and Bhunia, A. K. (2019). Crossing the intestinal barrier via Listeria adhesion protein and internalin A. Trends Microbiol. 27, 408–425. doi: 10.1016/j.tim.2018.12.007

Drolia, R., Tenguria, S., Durkes, A. C., Turner, J. R., and Bhunia, A. K. (2018). Listeria adhesion protein induces intestinal epithelial barrier dysfunction for bacterial translocation. Cell Host & Microbe 23, 470–484. doi: 10.1016/j.chom.2018.03.004

Elkhashim, M. T., Gooneratne, R., and Hussain, M. A. (2017). Microbial safety of foods in the supply chain and food safety. Adv. Food Technol. Nutr. Sci. Open J. 3, 22–32.

Elton, I., Fredenburgh, J., and Grizzle, W. E. (2001). Advanced Concepts in Fixation: 1. Effects of fixation on immunohistochemistry, reversibility of fixation and recovery of proteins, nucleic acids, and other molecules from fixed and processed tissues. 2. Developmental methods of fixation. J. Histotechnol. 24, 201–210. doi: 10.1179/his.2001.24.3.201

Eriksson, E., and Aspan, A. (2007). Comparison of culture, ELISA and PCR techniques for Salmonella detection in faecal samples for cattle, pig and poultry. BMC Vet. Res. 3:21. doi: 10.1186/1746-6148-3-21

FDA (2001). Bacteriological Analytical Manual Online, 8th Edn. Arlington, VA: AOAC International.

Finlay, B. B., and Falkow, S. (1997). Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. 61, 136–169. doi: 10.1128/MMBR.61.1.136-169.1997

Fratamico, P. M., and Strobaugh, T. P. (1998). Simultaneous detection of Salmonella spp and Escherichia coli O157:H7 by multiplex PCR. J. Indust. Microbiol. Biotechnol. 21, 92–99.

Galkowska, E., Kunowski, D., Tokarska-Pietrzak, E., Dzidziuszko, H., Iosi, J. M., Golec, P., et al. (2011). Specific detection of Salmonella enterica and Escherichia coli strains by using ELISA with bacteriophages as recognition agents. Eur. J. Clin. Microbiol. Infect. Dis. 30, 1067–1073. doi: 10.1007/s10096-011-1193-2

Gray, K. M., Banada, P. P., O’neal, E., and Bhunia, A. K. (2005). Rapid Ped-2E9 cell-based cytotoxicity analysis and genotyping of Bacillus species. J. Clin. Microbiol. 43, 5865–5872. doi: 10.1128/JCM.43.12.5865-5872.2005

Hahn, B. K., and Bhunia, A. K. (2006). Effect of environmental stresses on antibody-based detection of Escherichia coli O157:H7, Salmonella enterica
serotype Enteritidis and Listeria monocytogenes. J. Appl. Microbiol. 100, 1017–1027. doi: 10.1111/j.1365-2672.2006.02814.x

Hu, L., and Wai, T. T. (2017). Comparing invasive effects of five foodborne bacterial pathogens in human embryonic intestine 407 cells and human ileocecum HCT-8 cells. Asian Pacific J. Trop. Biomed. 7, 937–944. doi: 10.1016/ j.apjtb.2017.09.004

Jaradat, Z. W., and Bhunia, A. K. (2003). Adhesion, invasion and translocation characteristics of Listeria monocytogenes serotypes in Caco-2 cell and mouse models. Appl. Environ. Microbiol. 69, 3640–3645. doi: 10.1128/aem.69.6.3640-3645.2003

Jaradat, Z. W., Bzikot, J. H., Zawistowski, J., and Bhunia, A. K. (2004). Optimization of a rapid dot blot immunoassay for detection of Salmonella enterica serovar Enteritidis in poultry products and environmental samples. Food Microbiol. 21, 761–769. doi: 10.1016/j.fm.2004.01.010

Jiang, D., Liu, Y., Jiang, H., Rao, S., Fang, W., Wu, M., et al. (2018). A novel screen-printed mast cell-based electrochemical sensor for detecting spoilage bacterial quorum signaling molecules (N-acyl-homoserine-lactones) in freshwater fish. Biosens. Bioelectron. 102, 396–402. doi: 10.1016/j.bios.2017.11.040

Kasturi, N. D., and Drgon, T. (2017). Real-time PCR Method for detection of Salmonella spp. in environmental samples. Appl. Environ. Microbiol. 83: e00644-17.

Kim, H., and Bhunia, A. K. (2008). SEL, a selective enrichment broth for simultaneous growth of Salmonella enterica, Escherichia coli O157:H7, and Listeria monocytogenes. Appl. Environ. Microbiol. 74, 4833–4846. doi: 10.1128/ aem.02756-07

Kim, K. P., Singh, A. K., Bai, X., Leprun, L., and Bhunia, A. K. (2015). Novel PCR assays complement laser biosensor-based method and facilitate Listeria species detection from food. Sensors 15, 22672–22691. doi: 10.3390/s150922672

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., Ngamwongsatit, P., Banada, P. P., Panbangred, W., and Bhunia, A. K. (2008). WST-Masi, A., and Zawistowski, J. (1995). Detection of live and heat-treated Salmonella enterica serovar Enteritidis and Typhimurium in naturally infected broiler chickens by a multiplex PCR-based assay. Braz. J. Microbiol. 44, 37–42. doi: 10.1590/s1517-83822013000500002

Jayapaksha, P., Elbourne, A., Gangadoo, S., Brown, R., Cozzolino, D., and Chapman, J. (2019). A review of methods for the detection of pathogenic microorganisms. Analyst 144, 396–411. doi: 10.1039/c8an01488d

Ricke, S. C., Kim, S. A., Shi, Z., and Park, S. H. (2018). Molecular-based identification and detection of Salmonella in food production systems: current perspectives. J. Appl. Microbiol. 125, 313–327. doi: 10.1111/jam.13888

Schlaberg, R., Chiu, C. Y., Millner, S., Procop, G. W., Weinstock, G., Professional, C., et al. (2017). Validation of metagenomic next-generation sequencing tests for universal pathogen detection. Arch. Pathol. Lab. Med. 141, 776–786. doi: 10.5858/arpa.2016-0539-va

Silk, T. M., Roth, T. M. T., and Donnelly, C. W. (2002). Comparison of growth kinetics for healthy and heat-injured Listeria monocytogenes in eight enrichment broths. J. Food Prot. 65, 1333–1337. doi: 10.4315/0362-028x-65.8.1333

Singh, A. K., Leprun, L., Drolia, R., Bai, X., Kim, H., Aroonnaual, A., et al. (2016). Virulence gene-associated mutant bacterial colonies generate differentiating two-dimensional laser scatter fingerprints. Appl. Environ. Microbiol. 82, 3256–3268. doi: 10.1128/aem.04129-15

Singh, A. K., Bettasso, A. M., Bae, E., Rajwa, B., Dundar, M. M., Forster, M. D., et al. (2014). Laser optical sensor, a label-free on-plate Salmonella enterica colony detection tool. mBio 5:e01619-13.

Snyder, T. R., Boktor, S. W., and Mikanatha, N. M. (2019). Salmonellosis outbreaks by food vehicle, serotype, season, and geographical location. United States, 1998 to 2015. J. Food Prot. 82, 1191–1199. doi: 10.4315/0362-028x-jfp-18-494

To, C., Banerjee, P., and Bhunia, A. K. (2020). “Cell-based biosensor for rapid screening of pathogens and toxins,” in Handbook of Cell Biosensors, ed. G. Thonoud (Cham: Springer International Publishing), 1–16. doi: 10.1007/978-3-319-47405-2_102-1

To, C. Z., and Bhunia, A. K. (2019). Three dimensional Vero cell-platform for rapid and sensitive screening of Shiga-toxin producing Escherichia coli. Front. Microbiol. 10:949. doi: 10.3389/fmicb.2019.00949

USDA-FSIS (2013). Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products. Method Number MLG 4.06. Available at: http://www.fsis.usda.gov/wps/wcm/connect/700c05fe-06a2-83822013005000002

Wagner, C., and Hensel, M. (2011). “Adhesive mechanisms of Salmonella enterica,” in Bacterial Adhesion, eds D. Linke and A. Goldman (Berlin: Springer), 17–34. doi: 10.1007/978-94-007-0940-9_2

Wang, S. J., and Ye, D. B. (2002). Designing of polymerase chain reaction primers for the detection of Salmonella Enteritidis in foods and faecal samples. Lett. Appl. Microbiol. 34, 422–427. doi: 10.1046/j.1272-7655.2002.0114.x

Ye, Y., Guo, H., and Sun, X. (2019). Recent progress on cell-based biosensors for analysis of food safety and quality control. Biosens. Bioelectron. 126, 389–404. doi: 10.1016/j.bios.2018.10.039

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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