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# **UNIVERSAL ENRICHMENT MEDIA: A SUPPORTIVE TECHNOLOGY FOR MULTIPLEX DETECTION OF PATHOGENS IN FOOD MATRICES**

by  
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**U.S. Army Combat Capabilities Development Command Soldier Center  
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| <b>14. ABSTRACT</b><br>Multiplex detection technologies have progressed in increasing sensitivity; however, pathogen enrichment is still required to properly test food for bacterial targets. Here, a universal enrichment media is evaluated along with its ability to successfully enrich <i>S. Typhimurium</i> , <i>E. coli</i> O157:H7, <i>S. flexneri</i> , <i>Y. enterocolitica</i> , <i>E. coli</i> O78:H11, <i>V. cholerae</i> and <i>V. parahaemolyticus</i> for molecular detection in 16 food matrices while in comparison to a standard enrichment media. Results demonstrate that universal enrichment media can perform equally as effective as the standard recommended enrichment media, no significant difference ( $P > 0.05$ ), for the detection of pathogenic bacteria in a variety of food matrices. Next, an alternative methodology is investigated for simultaneous detection based on the combining of separate enrichment aliquots into a single diagnostic run. Results show the successful detection of <i>S. Typhimurium</i> , <i>S. flexneri</i> , <i>V. cholerae</i> , and <i>C. jejuni</i> in water and bok choy using a single diagnostic test after combining aliquots from separate enrichments. This study demonstrates that a universal enrichment media can be as effective as the standard enrichment media for molecular based testing of foods. Further universal enrichment supports the use of multiplex detection for foodborne illness outbreak investigations and food surveillance programs. The mixing of different enrichment aliquots into a single diagnostic run can be used as an effective alternative methodology for simultaneous detection when culture condition requirements cannot be met with a single enrichment media. Advancing enrichment technology to include multiple bacterial targets is complimentary to multiplex detection platforms. |                    |                                |                                   |  |  |
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| BACTERIA   | ENRICHMENT         | ESCHERICHIA COLI               | TEST AND EVALUATION               |  |  |
| DETECTION  | TECHNOLOGY         | BACTERIAL STRAINS              | MULTIPLEX DETECTION               |  |  |
| INFECTIONS   | FOOD SAMPLES       | CULTURES(BIOLOGY)              | PATHOGENIC BACTERIA               |  |  |
| SENSITIVITY  | WATER SAMPLES      | DIAGNOSIS(GENERAL)             | SALMONELLA ENTERICA               |  |  |
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# UNIVERSAL ENRICHMENT MEDIA: A SUPPORTIVE TECHNOLOGY FOR MULTIPLEX DETECTION OF PATHOGENS IN FOOD MATRICES

## 1. INTRODUCTION

The following report covers work performed at DEVCOM SC October 2017 through September 2019 investigating the application of universal enrichment media for food safety surveillance testing in combination with a DNA based diagnostic system allowing for rapid screening capability while reducing laboratory logistics requirements and reducing method complexities.

Multiplex diagnostic devices have been advantageous for rapid diagnostic testing of samples from patients presenting gastroenteritis (Buss et al. 2015; Binnicker 2015; Huang et al. 2016). Advanced multiplex systems not only can provide a quick time-to-result in comparison to routine laboratory methods for many targets simultaneously, but also require limited sample preparation (decreased hands-on time) prior to loading the instrument (Piralla et al. 2017; Khare et al. 2014; Binnicker 2015). The application of using multiplex diagnostic systems is under investigation for contaminated water and may provide similar advantages in foodborne illness outbreak investigations and food surveillance programs over traditional laboratory methods (Davidson et al. 2018). Reoccurring patterns in etiological data of pathogen/commodity pairings is helpful for determining procedures for food surveillance testing (i.e. selective enrichment combined with specific detection) (Painter et al. 2013). However, many commodities such as shellfish, fresh fruits and vegetables (FF&V), dairy, and meat products can be contaminated with numerous pathogens of concern for food safety (i.e. *Salmonella enterica*, *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Listeria* spp. *Vibrio* spp.)(Dewey-Mattia, Kisselburgh, et al. 2018; Dewey-Mattia et al. 2019; Dewey-Mattia, Manikonda, et al. 2018; Painter et al. 2013). Screening of food samples using standard methods is a daunting task if all possible targets are considered (Zhao et al. 2014; Zhao et al. 2016). Multiplex detection systems would enable more effective surveillance of commodities known to be potentially contaminated with multiple pathogens by taking advantage of not only multi-target testing but also the decreased hands-on time and faster time-to-result over traditional methods.

Though the advantages seem obvious, one very important consideration is the detection sensitivity of the diagnostic system. Pathogens are typically present at very low levels and are surrounded by background microflora, thus requiring an enrichment step to improve pathogen numbers and differentiate them from the background (Nam et al. 2004; Kim and Bhunia 2008). Detection platforms including multiplex PCR assays, microarray biosensors, array-based immunoabsorbent assays, and DNA microarray platforms still require an enrichment step prior to detection (Kim and Bhunia 2008; Bhagwat 2003; Hu Q1 2014). As detection platforms evolve to handle multiple pathogens in a single assay format, it is also important that the enrichment procedures ideally evolve into a single (universal) enrichment media to fully take advantage of the multiplex system's capabilities.

Current investigations of multi-pathogen enrichment media for food pathogen testing demonstrates the potential to develop a food surveillance methodology that could employ a single universal enrichment broth. *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* were also simultaneously enriched and detected successfully in a multiplex PCR assay using a multi-pathogen selective enrichment broth, SEL [*Salmonella*, *Escherichia*, *Listeria*], from inoculated meat samples (Kim and Bhunia 2008). A single enrichment media, SSSLE broth, was

demonstrated for detection of *S. Enteritidis*, *S. aureus*, *S. flexneri*, *L. monocytogenes*, and *E. coli* O157:H7 using a multiplex PCR assay in inoculated meat samples (Chen et al. 2015). Artificially contaminated raw beef and ready-to-eat chicken enriched in a selective enrichment broth (SSL) demonstrated a high recovery of *S. Enteritidis*, *S. aureus*, and *L. monocytogenes* and the media proved suitable for simultaneous detection of the pathogens on a multi-pathogen assay (Yu et al. 2010). Multi-pathogen selective enrichment (SVV broth) was formulated for the simultaneous growth of *S. enterica*, *V. parahaemolyticus* and *V. cholerae* then detected on a multi-pathogen real-time PCR assay (Xing-Long Xiao 2010). Additionally, a multi-pathogen selective enrichment (SSS broth) was described for the growth of *S. enterica*, *S. aureus* and *S. dysenteriae* for simultaneous detection on nucleic-acid-based methods (Xiao et al. 2014). Most investigations focus on the use of selective components in the enrichment broth formulations to prevent overgrowth and competition of background flora; however, these selective components can also interfere with the growth of other important pathogens present within a sample. It is for these reasons that a different detection approach is needed, one where a non-selective enrichment media is employed during food surveillance testing which focuses on capitalizing system detection sensitivity.

Previously, simultaneous pre-enrichment of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* from dairy environmental samples using a universal pre-enrichment broth was shown to be as effective as selective primary broths (Nam et al. 2004). Here, the project team demonstrates the use of universal pre-enrichment (UPE) broth for the detection of seven foodborne pathogens in different food matrices over two trials, coupled with a multiplex detection system, BioFire® FilmArray® (FA). Additionally, in the second trial the team compares an aerobic enrichment media (3M *Campylobacter* Enrichment Broth) for the selective enrichment of *Campylobacter* spp. to that of enrichment using Bolton broth under microaerophilic conditions as a means to simplify food surveillance methods for *Campylobacter* with a multiplex detection system. The FA was designed for multiplex pathogen detection in medical diagnostics to include fecal samples from patients presenting gastrointestinal illness, upper respiratory tract infections, bacterial identification in meningitis cases, and culture positive blood samples (Kreiniz et al. 2019; Jennifer Dien Bard 2018). The FA system has also been investigated for use in environmental surveillance testing of sewage water and contaminated river water (Baicus 2017; Davidson et al. 2018). The Gastrointestinal (GI) Assay panel (BioFire®) tests for 22 pathogens associated with gastroenteritis that are also common to contaminated food and water samples. In order to most effectively utilize rapid multiplex detection technology, it is important that associated preparation steps such as enrichment are complimentary. Utilizing a universal enrichment media intended for enrichment of multiple pathogens prior to multiplex detection compliments the multiplex detection platform as a total package technology which evolves to handle multiple pathogens in a rapid, single assay format. The team utilized the GI panel to assay the presence/absence of pathogens in food matrix enrichments for comparison of a UPE broth to various recommended pre-enrichment “standard” broths. There may be instances when universal enrichment of all pathogens in various food matrices is not feasible, which prompted the further demonstration of a methodology for simultaneous detection of four targets in water and Bok Choy using the mixing of different enrichment aliquots into a single GI panel diagnostic run.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial strains and growth conditions

Bacterial isolates were activated from freeze dried preparation obtained from American Type Culture Collection (ATCC, Manassas, VA). *Salmonella enterica* Typhimurium ATCC 14028, *Escherichia coli* O157:H7 ATCC 43894, *Shigella flexneri* ATCC 9380, *Yersinia enterocolitica* ATCC 9610, *Escherichia coli* O78:H11 ATCC 35401, *Vibrio cholerae* ATCC 14035, *Vibrio parahaemolyticus* ATCC 17802, and *Campylobacter jejuni* ATCC BAA 1234 were selected as target organisms for the enrichment comparison trials. *S. enterica* Typhimurium, *E. coli* O157:H7, *E. coli* O78:H11, and *V. cholerae* were incubated aerobically at 37 °C on an orbital shaker (150 rpm) for 24 h in 10 mL of sterile tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, MD). The *S. enterica* Typhimurium, *E. coli* O157:H7, and *E. coli* O78:H11 culture stocks were maintained on tryptic soy agar (TSA) (Difco, Becton Dickinson) at 4 °C throughout the study. *V. cholerae* was stored at -80 °C in 20% v/v glycerol (Fisher Scientific, Thermo Fisher, Waltham, MA) preparations throughout the study. *Y. enterocolitica* was grown aerobically in 10 mL of sterile brain heart infusion broth (BHIB) (Bacto, Becton Dickinson, Sparks, MD) at 30°C statically for 24 h. *S. flexneri* was grown in 10 mL of BHIB at 37 °C aerobically on an orbital shaker (150 rpm) for 24 h. *Y. enterocolitica* and *S. flexneri* cultures were then maintained on brain heart infusion agar (BHIA) (Bacto, Becton Dickinson) at 4 °C throughout the study. *V. parahaemolyticus* was grown in nutrient broth (Difco, Becton Dickinson) supplemented with 3% w/v sodium chloride (NB3%NaCl) (Sigma- Aldrich, St. Louis, MO) at 37 °C for 24 h aerobically on an orbital shaker (150 rpm) and then maintained on nutrient agar (Difco, Becton Dickinson) supplemented 3% with w/v sodium chloride (NA3%NaCl) and stored at 4 °C throughout the study. *C. jejuni* culture was activated in a vented culture flask containing 100 mL of sterile Brucella broth (BB) (Fluka, Sigma-Aldrich, St. Louis, MO) at 42 °C in microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>; Thermo Electron Co., Forma Series II water jacketed CO<sub>2</sub> incubator, Marietta, OH) for 72 h, statically. After incubation, 1 mL was transferred to a vented culture flask containing 100 mL of fresh sterile BB then incubated for 48 h at the same conditions. After incubation, frozen stock preparations were made in Wang's Freezing/Storage Medium (28 g/L Brucella broth powder, 20% v/v glycerol, and 5% laked horse blood) and stored at -80 °C for the duration of the study(Agriculture 2017).

### 2.2 Food samples

Food matrices for enrichment studies were selected from outbreak data, published recalls, and literature attributed to the target pathogens. Similar products were used if exact matrices were not available or otherwise specified. Food samples were purchased from a local grocer (Stop and Shop, Natick, MA) and consisted of the following: Cherry Tomatoes (Sunset; One Sweet), Chicken Salad (Willow Tree; w/cranberry and walnut), Ground Beef (Stop and Shop; 80/20%), Romaine Lettuce (Stop and Shop; whole head, unwashed), Organic Green Onions (Stop and Shop; Nature's Promise), Five Layer Bean Dip (Luisa's; pinto beans, salsa, guacamole, sour cream, cheddar cheese), RTE Spring Mix Salad (Stop and Shop), Ground pork (Stop and Shop), Greek Pasta Salad (Stop and Shop), Sauerkraut (Silver Floss; Krrrrisp Kraut), Strawberries (Driscoll's), Bok Choy (Stop and Shop), Shrimp (Stop and Shop; Farm raised), Chinese Cabbage (Stop and Shop), and Shrimp (Gulf Pride; Wild caught). Food matrix samples (10 g) were placed aseptically into sterile Whirl Pak filter homogenizer bags (Nasco, Modesto, CA) for a total of 22

replicates per food in Trial 1 and 30 replicates per food in Trial 2. Food sample replicates were stored at 4 °C overnight prior to inoculation and enrichment procedures.

### 2.3 Food sample inoculation and enrichment

*S. Typhimurium*, *E. coli* O157:H7, *S. flexneri*, *Y. enterocolitica*, *E. coli* O78:H11, and *V. parahaemolyticus* cultures were prepared for food inoculation by transferring a single colony from a streak plate to 10 mL of the appropriate sterile broth and then incubated at the same conditions used previously for 24 h. Following incubation, 100 µL of culture was transferred to a fresh 10 mL tube of sterile broth and re-incubated for 24 h prior to preparation of the inoculum. *V. cholerae* was activated from the frozen stock by transferring 10 µL using a sterile loop and inoculating 10 mL of NB3%NaCl then incubated for 24 h using the same conditions as was done previously. Following incubation, 100 µL was transferred to a fresh sterile broth tube and re-incubated for 24 h prior to the preparation of the inoculum. *C. jejuni* cultures were activated from the frozen stock cultures by transferring 500 µL of the thawed culture stock to 100 mL of sterile BB in a vented culture flask and incubated for 72 h using the same conditions as was done previously, then 1 mL was transferred to a fresh 100 mL of BB and re-incubated for 48 h prior to the preparation of the inoculum.

*S. Typhimurium*, *E. coli* O157:H7, *S. flexneri*, *Y. enterocolitica*, *E. coli* O78H11, and *C. jejuni* inoculums were prepared from the fresh cultures by serial dilution (1:10) in sterile 9 mL butterfields buffer (BBF) (3M, St. Paul, MN). *V. cholerae* and *V. parahaemolyticus* were serially diluted (1:10) in 9 mL of sterile NB3%NaCl to prepare the inoculums. Each food was inoculated with 50 to 100 µL of the appropriate dilution to achieve an inoculation level of ~10 CFU per food sample. The average bacterial load of the inoculum was determined via spread plating, the spike volume on five replicate petri plates and incubating at the appropriate conditions for each organism. *E. coli* O157:H7 and *E. coli* O78:H11 were enumerated on TSA. *S. Typhimurium*, *S. flexneri*, *Y. enterocolitica* were enumerated on BHIA. *V. cholerae* and *V. parahaemolyticus* were enumerated on NA3%NaCl. *C. jejuni* was enumerated on Brucella agar (Fluka, Sigma-Aldrich, St. Louis, MO) supplemented with 5% laked horse blood (BA5%HB) (Innovative Research, Novi, MI).

Two comparison enrichment trials were conducted for this study with Trial 1 containing 11 replicates of each food/pathogen/media combination and Trial 2 containing 15 replicates of each food/pathogen/media combination. In addition to the seven food-borne pathogens included in Trial 1, *C. jejuni* was added to the second trial. Each pathogen/food matrix combination was enriched in a standard media (recommended) and test media (UPE) (Table 1). Standard media included in the comparison studies were Buffered Peptone Water (BPW, Difco), Tryptic Soy Broth with 0.6% Yeast Extract (TSBYE), BHIB, Alkaline Peptone Water pH 8.5 (APW) (10 g/l Bacto peptone [Difco], 10 g/L NaCl [Sigma] adjusted to pH 8.5) and Bolton Broth (BLB) supplement with 5% laked horse blood and Bolton broth selective supplement (Millipore, Billerica, MA). The test media were Universal Pre-Enrichment Broth (UPE) (Sigma Aldrich), UPE + 3% sodium chloride (UPE+), and 3M *Campylobacter* Enrichment Broth (3MB) (3M, St. Paul, MN). Bacteria and food combinations were enriched with either standard or test media (Table 1). Samples were incubated aerobically at 37 °C in closed homogenizer bags without shaking for 24 h with the exception of *C. jejuni*. *C. jejuni* containing samples enriched with BLB were incubated at 42 °C in microaerophilic conditions in open homogenizer bags without shaking for 26 h. *C. jejuni* containing samples enriched in 3MB were incubated at 42 °C aerobically in a tightly closed homogenizer bag for 26 h following the manufacture's

recommended procedures for sample handling and incubation. After enrichment, sample bags were mixed by hand with gentle squeezing, and duplicate 1 mL aliquots were placed into sterile 2 mL cryovials and then frozen at -80 °C until analysis on the FA multiplex PCR system (BioFire, BioMerieux, Salt Lake City, UT).

TABLE 1. Bacterial strains and food matrix combinations used for enrichment experiments with a standard media or a test media (two experimental trials).

| <b>Organism</b>                                   | <b>ATCC</b> | <b>Food Matrix</b>       | <b>Standard Media<sup>1</sup></b> | <b>Test Media<sup>2</sup></b> |
|---|-------------|--------------------------|-----------------------------------|-------------------------------|
| <i>Salmonella enterica</i>                        | 14028       | RTE Chicken Salad        | BPW                               | UPE                           |
|   |             | Cherry Tomatoes          | BPW                               | UPE                           |
| <i>Escherichia coli</i><br><i>O157:H7</i>         | 43894       | Ground Beef              | BPW                               | UPE                           |
|   |             | Romaine Lettuce          | BPW                               | UPE                           |
| <i>Shigella flexneri</i>                          | 9380        | Five Layered Bean Dip    | TSBYE                             | UPE                           |
|   |             | Green Onions             | TSBYE                             | UPE                           |
| <i>Yersinia enterocolitica</i>                    | 9610        | RTE Mixed Green<br>Salad | TSBYE                             | UPE                           |
|   |             | Ground Pork              | TSBYE                             | UPE                           |
| <i>Enterotoxigenic</i><br><i>Escherichia coli</i> | 35401       | Sauerkraut               | BHIB                              | UPE                           |
|   |             | Greek Pasta Salad        | BHIB                              | UPE                           |
| <i>Vibrio cholerae</i>                            | 14035       | Aquiculture Shrimp       | APW                               | UPE+                          |
|   |             | Bok Choy                 | APW                               | UPE+                          |
| <i>Vibrio parahaemolyticus</i>                    | 17802       | Raw Shrimp               | APW                               | UPE+                          |
|   |             | Chinese Cabbage          | APW                               | UPE+                          |
| <i>Campylobacter jejuni</i> *                     | BAA-1234    | Ground Beef              | BLB                               | 3MB                           |
|   |             | Strawberries             | BLB                               | 3MB                           |

<sup>1</sup>BPW (Buffered Peptone Water), TSBYE (Tryptic Soy Broth with 0.6% Yeast Extract), BHIB (Brain Heart Infusion Broth), APW (Alkaline Peptone Water pH 8.5), BLB (Bolton Broth w/ 5% laked horse blood and selective supplement)

<sup>2</sup>UPE (Universal Pre-Enrichment Broth), UPE+ (UPE + 3% sodium chloride), 3MB (3M Campylobacter Enrichment Broth)

\*Only included in Trial 2

## 2.4 Multiple target inoculation and enrichment

Sterile Whirl-Pak filter stomacher bags containing 25 g of bok choy (15 replicates) or 25 mL of General Test Water (GTW) (15 replicates) (pH 7.0; 0.2 g/L sea salts [Sigma Aldrich], 0.124 g/L sodium bicarbonate [Sigma Aldrich], 9.3 mg/L tannic acid [Sigma Aldrich]) were inoculated concurrently with *S. Typhimurium*, *S. flexneri*, *V. cholerae*, and *C. jejuni* with total bacteria loads of ~10 CFU of each organism per replicate sample (Agency 1987). The group of 15 replicates for each inoculated sample matrix was divided evenly into three groups for addition of 225 mL UPE, APW or 3MB (Table 2). The samples were homogenized for 30 s at 160 rpm for samples containing UPE and APW or gently hand massaged for samples containing 3MB

following manufacturer's instructions. Samples containing UPE and APW were incubated at 37 °C statically in aerobic conditions for 24 h.

TABLE 2. Treatment combinations of bok choy (n=15) and general test water (n=15) replicate samples co-inoculated with *S. enterica*, *S. flexneri*, *V. cholerae* and *C. jejuni* and enriched using three separate broths (n=5 each) following appropriate conditions.

| Sample Matrix      | Targets                        | Enrichment Broths              | Sample ID |
|--------------------|--------------------------------|--------------------------------|-----------|
| Bok Choy           | <i>S. enterica</i> ATCC 14028  | Universal Pre-Enrichment Broth | Bok A-E   |
|                    | <i>S. flexneri</i> ATCC 9380   | Alkaline Peptone Water, pH 8.5 | Bok F-J   |
|                    | <i>V. cholerae</i> ATCC 14035  | 3M Campy Broth                 | Bok K-O   |
|                    | <i>C. jejuni</i> ATCC BAA-1234 |                                |           |
| General Test Water | <i>S. enterica</i> ATCC 14028  | Universal Pre-Enrichment Broth | GTW A-E   |
|                    | <i>S. flexneri</i> ATCC 9380   | Alkaline Peptone Water, pH 8.5 | GTW F-J   |
|                    | <i>V. cholerae</i> ATCC 14035  | 3M Campy Broth                 | GTW K-O   |
|                    | <i>C. jejuni</i> ATCC BAA-1234 |                                |           |

Samples containing 3MB were incubated at 42 °C for 24 h after each bag was closed tightly (by removing the headspace from the sample and rolling the sample bag top down three turns then using the wire closure). After enrichment, 1 mL aliquots (in duplicate) were removed from each enrichment bag and stored at -80 °C before analysis on the FA system. Samples were analyzed separately and in combination according to the sample combination matrix (Table 3). The combined samples were created by adding 100 µL of three individual enrichment aliquots into sterile 1.5 mL micro centrifuge tubes and vortexed. All individual samples (Table 3: Bok A-O and GTW A-O) as well as a total of 200 µL of each combined sample (Table 3: BokCh1-5 and GTW1-5) were analyzed on the FA.

TABLE 3. Sample ID of individual enrichments and the combined enrichments analyzed using the BioFire® FilmArray® for the presence/absence testing of *S. enterica*, *S. flexneri*, *V. cholerae* and *C. jejuni* in bok choy and general test water

| Sample ID and Combination Matrix |                         |               |               |               |               |               |
|----------------------------------|-------------------------|---------------|---------------|---------------|---------------|---------------|
| Bok Choy                         | Bok A                   | Bok B         | Bok C         | Bok D         | Bok E         |               |
|                                  | Bok F                   | Bok G         | Bok H         | Bok I         | Bok J         |               |
|                                  | Bok K                   | Bok L         | Bok M         | Bok N         | Bok O         |               |
|                                  | <i>Combined Sample*</i> | <i>BokCh1</i> | <i>BokCh2</i> | <i>BokCh3</i> | <i>BokCh4</i> | <i>BokCh5</i> |
| General Test Water               | GTW A                   | GTW B         | GTW C         | GTW D         | GTW E         |               |
|                                  | GTW F                   | GTW G         | GTW H         | GTW I         | GTW J         |               |
|                                  | GTW K                   | GTW L         | GTW M         | GTW N         | GTW O         |               |
|                                  | <i>Combined Sample*</i> | <i>GTW1</i>   | <i>GTW2</i>   | <i>GTW3</i>   | <i>GTW4</i>   | <i>GTW5</i>   |

\*Row of samples generated from the combination of the three individual enrichment samples in the column above.

TABLE 4. Presence/absence testing of inoculated food sample replicates (n=11) enriched with a standard<sup>1</sup> or a test<sup>2</sup> media using the Biofire® FilmArray® (Trial 1)

| Organism                              | Avg. CFU/sample | No. of Pos. / Total Replicates, Enrichment Media |             |                       |             |
|---------------------------------------|-----------------|--|-------------|-----------------------|-------------|
| <i>S. enterica</i> ATCC 14028         | 10.5            | Chicken Salad*                                   |             | Cherry Tomato*        |             |
|                                       |                 | 11/11, BPW                                       | 11/11, UPE  | 11/11, BPW            | 11/11, UPE  |
| <i>E. coli</i> O157:H7 ATCC 43894     | 4               | Ground Beef*                                     |             | Romaine Lettuce*      |             |
|                                       |                 | 11/11, BPW                                       | 11/11, UPE  | 11/11, BPW            | 10/11, UPE  |
| <i>S. flexneri</i> ATCC 9380          | 3.6             | Green Onions*                                    |             | Five Layer Bean Dip*  |             |
|                                       |                 | 11/11, TSBYE                                     | 11/11, UPE  | 11/11, TSBYE          | 10/11, UPE  |
| <i>Y. enterocolitica</i> ATCC 9610    | 74              | RTE Salad*                                       |             | Ground Pork*          |             |
|                                       |                 | 11/11, TSBYE                                     | 11/11, UPE  | 11/11, TSBYE          | 11/11, UPE  |
| <i>E. coli</i> O78:H11 ATCC 35401     | 4.8             | Sauerkraut*                                      |             | Pasta Salad*          |             |
|                                       |                 | 9/11, BHIB                                       | 11/11, UPE  | 11/11, BHIB           | 11/11, UPE  |
| <i>V. cholera</i> ATCC 14035          | 1               | Bok Choy*  |             | Shrimp (farm raised)* |             |
|                                       |                 | 10/11, APW                                       | 11/11, UPE+ | 11/11, APW            | 11/11, UPE+ |
| <i>V. parahaemolyticus</i> ATCC 17802 | 20              | Chinese Cabbage*                                 |             | Shrimp (wild caught)* |             |
|                                       |                 | 11/11, APW                                       | 9/11, UPE+  | 11/11, APW            | 11/11, UPE+ |

<sup>1</sup>BPW (Buffered Peptone Water), TSBYE (Tryptic Soy Broth with 0.6% Yeast Extract), BHIB (Brain Heart Infusion Broth), APW (Alkaline Peptone Water pH 8.5)

<sup>2</sup>UPE (Universal Pre-Enrichment Broth), UPE+ (UPE + 3% sodium chloride)

\*No significant difference (P > 0.05) at the 95% confidence interval between standard and test media

TABLE 5. Presence/absence testing of inoculated food sample replicates ( $n=15$ ) enriched with a standard<sup>1</sup> or a test<sup>2</sup> media using the Biofire® FilmArray® (Trial 2)

| Organism                              | Avg. CFU/sample | No. of Pos. / Total Replicates, Enrichment Media |             |                                    |             |
|---------------------------------------|-----------------|--|-------------|------------------------------------|-------------|
| <i>S. enterica</i> ATCC 14028         | 10              | Chicken Salad*                                   |             | Cherry Tomato*                     |             |
|                                       |                 | 15/15, BPW                                       | 15/15, UPE  | 15/15, BPW                         | 15/15, UPE  |
| <i>E. coli</i> O157:H7 ATCC 43894     | 7.2             | Ground Beef*                                     |             | Romaine Lettuce*                   |             |
|                                       |                 | 15/15, BPW                                       | 15/15, UPE  | 15/15, BPW                         | 15/15, UPE  |
| <i>S. flexneri</i> ATCC 9380          | 2.8             | Green Onions*                                    |             | Five Layer Bean Dip*               |             |
|                                       |                 | 14/15, TSBYE                                     | 15/15, UPE  | 13/15, TSBYE                       | 13/15, UPE  |
| <i>Y. enterocolitica</i> ATCC 9610    | 69              | RTE Salad*                                       |             | Ground Pork*                       |             |
|                                       |                 | 15/15, TSBYE                                     | 15/15, UPE  | 14/15, TSBYE                       | 15/15, UPE  |
| <i>E. coli</i> O78:H11 ATCC 35401     | 5               | Sauerkraut*                                      |             | Pasta Salad                        |             |
|                                       |                 | 15/15, BHIB                                      | 13/15, UPE  | 14/15, BHIB                        | 15/15, UPE  |
| <i>V. cholera</i> ATCC 14035          | 15.2            | Bok Choy*  |             | Shrimp (farm raised)*              |             |
|                                       |                 | 15/15, APW                                       | 15/15, UPE+ | 15/15, APW                         | 15/15, UPE+ |
| <i>V. parahaemolyticus</i> ATCC 17802 | 8.2             | Chinese Cabbage* <sup>t</sup>                    |             | Shrimp (wild caught)* <sup>t</sup> |             |
|                                       |                 | 15/15, APW                                       | 11/15, UPE+ | 15/15, APW                         | 11/15, UPE+ |
| <i>C. jejuni</i> ATCC BAA-1234        | 7               | Strawberries*                                    |             | Ground Beef*                       |             |
|                                       |                 | 15/15, BLB                                       | 14/15, 3MB  | 15/15, BLB                         | 15/15, 3MB  |

<sup>1</sup>BPW (Buffered Peptone Water), TSBYE (Tryptic Soy Broth with 0.6% Yeast Extract), BHIB (Brain Heart Infusion Broth), APW (Alkaline Peptone Water pH 8.5), BLB (Bolton Broth w/ 5% laked horse blood and selective supplement)

<sup>2</sup>UPE (Universal Pre-Enrichment Broth), UPE+ (UPE + 3% sodium chloride), 3MB (3M Campylobacter Enrichment Broth)

\*No significant difference ( $P > 0.05$ ) at the 95% confidence interval between the standard and test media

<sup>t</sup>Significantly different at the 90% confidence interval ( $P < 0.1$ ) between the standard and test media

## **2.5 Detection procedure**

The FilmArray molecular diagnostic system was used for presence/absence testing of pathogen targets in each respective food matrix enrichment. Samples were analyzed using the Gastrointestinal (GI) Panel (BioFire®, BioMerieux) following sample loading procedures outlined in the GI Panel instruction booklet (BioFire Diagnostics 2017). The GI panel tests for 22 pathogens associated with gastroenteritis and performs sample preparation, amplification, detection, and analysis of patient derived stool specimens directly on board the test panel after loading. No alteration to sample preparation or loading procedures was required to analyze the food sample enrichment solutions. Sample enrichment frozen aliquots were thawed at room temp in a biological safety cabinet, vortexed for 3 s to mix, and then 200 µL was added to the sample loading tube containing GI panel loading buffer. The loading tube containing the sample and buffer was inverted three times to mix prior to loading into a hydrated GI panel test. Individual sample analysis was completed in ~ 1 h after loading the sample into the GI panel.

## **2.6 Statistical Analysis**

Enrichment comparison data within each trial (1 and 2) were analyzed in 2 x 2 contingency tables for significant difference ( $P < 0.05$ ) of the Positive/Negative ratio between the recommended media and the test media for each food/bacteria pairing using Fisher's exact test of independence in GraphPad Prism 8 statistical software (GraphPad Software, San Diego, CA).

### 3. RESULTS

Two trials were completed for the comparison of UPE to multiple standard pre-enrichment broth and pathogen combinations in a variety of food matrices (Table 4 and 5). The inoculation level achieved for each pathogen in the food sample replicates was well below the reported limit of detection (LOD) of the GI panel for both experimental trials (Table 4 and 5) (BioFire Diagnostics 2017).

The majority of food samples in Trial 1 had no difference in ratio of positive/negative results of each pathogen food enrichment media pairing (Table 4). Most samples had 100% positive results for the 11 replicates for both the standard enrichment broth and the UPE or UPE+. Two instances of disagreement between UPE and the standard media were seen with *E. coli* O78:H11 in sauerkraut and *V. parahaemolyticus* in Chinese cabbage. Sauerkraut inoculated with *E. coli* O78:H11 enriched in BHIB had 9 positive samples (11 total) while those samples enriched in UPE had all 11 samples found positive. Chinese cabbage samples inoculated with *V. parahaemolyticus* enriched in UPE+ had 9 positive samples in comparison to enrichment in APW where all 11 samples were found positive. No significant difference ( $P > 0.05$ ) was found between the positive/negative ratio of the *E. coli* O78:H11 inoculated sauerkraut samples enriched in BHIB and UPE. Similarly no significant difference ( $P > 0.05$ ) was found between the positive/negative ratio for *V. parahaemolyticus* inoculated Chinese cabbage samples enriched in APW and UPE+.

The number of replicates were increased in the second trial to 15 for each pathogen/food/enrichment media pairing. As was seen in Trial 1, most food sample replicates had 100% positive results in Trial 2. As an exception in Trial 2, the five layer bean dip replicate samples enriched in TSBYE or UPE were 86% positive for *S. flexneri*. There were seven instances of disagreement found between the standard enrichment broth and UPE, UPE+ or 3MB in Trial 2 (Table 5). Specifically, replicate samples of *S. flexneri* in green onion, *Y. enterocolitica* in ground pork, *E. coli* O78:H11 in sauerkraut and pasta salad, *V. parahaemolyticus* in Chinese cabbage and wild caught shrimp, and *C. jejuni* in strawberries had a disagreement in the positive/negative ratio of samples. No significant difference was found between the positive/negative ratio for any of the pathogen, food and enrichment media pairings at the 95% confidence interval ( $p < 0.05$ ); however, the positive/negative ratios of Chinese cabbage and wild caught shrimp samples containing *V. parahaemolyticus* and enriched in UPE+ or APW were significantly different at the 90% confidence interval ( $P < 0.1$ ). Chinese cabbage samples and wild caught shrimp enriched in UPE+ had 11 of 15 samples found positive while all 15 samples enriched in APW were found positive.

A multiple target inoculation and enrichment was conducted using *S. enterica*, *S. flexneri*, *V. cholerae*, and *C. jejuni* with bok choy and GTW as the test matrices. The inoculation level of each pathogen achieved was 13.4, 7.4, 15.8 and 2.8 total CFU/sample respectively. After addition of 225 mL of enrichment broth and homogenization of the inoculated 25 g sample, the concentration of *S. enterica*, *S. flexneri*, *V. cholerae*, and *C. jejuni* was estimated to be 0.05, 0.02, 0.06, and 0.01 CFU/mL homogenate respectively. Total volume of the homogenate was 250 mL. The inoculation level of each pathogen in the enrichment homogenate was well below the LOD of each pathogen on the GI panel (BioFire Diagnostics 2017). *S. enterica*, *S. flexneri*, and *V. cholerae* were detected in both bok choy (Table 6) and GTW (Table 7) after enrichment with either UPE or APW for all five replicates. *C. jejuni* was not detected in samples enriched with UPE or APW and was only detected in bok choy and GTW samples enriched with 3MB for all

five replicates (Table 6 and 7). After combining equal volume aliquots of UPE, APW and 3MB into one GI panel test kit, all four targets were detected in both bok choy and GTW samples for all five replicates (Table 6 and 7).

TABLE 6. Presence/absence testing of bok choy samples co-inoculated with *S. enterica* ATCC 14028, *S. flexneri* ATCC 9380, *V. cholerae* ATCC 14035, and *C. jejuni* ATCC BAA-1234 and enriched with UPE, APW or 3MB using BioFire® FilmArray®.

| Enrichment       | Sample ID | Target     |          |                 |               |
|------------------|-----------|------------|----------|-----------------|---------------|
|                  |           | Salmonella | Shigella | Vibrio cholerae | Campylobacter |
| UPE              | Bok A     | +          | +        | +               | -             |
|                  | Bok B     | +          | +        | +               | -             |
|                  | Bok C     | +          | +        | +               | -             |
|                  | Bok D     | +          | +        | +               | -             |
|                  | Bok E     | +          | +        | +               | -             |
| APW              | Bok F     | +          | +        | +               | -             |
|                  | Bok G     | +          | +        | +               | -             |
|                  | Bok H     | +          | +        | +               | -             |
|                  | Bok I     | +          | +        | +               | -             |
|                  | Bok J     | +          | +        | +               | -             |
| 3MB              | Bok K     | -          | -        | -               | +             |
|                  | Bok L     | -          | -        | -               | +             |
|                  | Bok M     | -          | -        | -               | +             |
|                  | Bok N     | -          | -        | -               | +             |
|                  | Bok O     | -          | -        | -               | +             |
| <i>Combined*</i> | BokCh 1   | +          | +        | +               | +             |
|                  | BokCh 2   | +          | +        | +               | +             |
|                  | BokCh 3   | +          | +        | +               | +             |
|                  | BokCh 4   | +          | +        | +               | +             |
|                  | BokCh 5   | +          | +        | +               | +             |

\*Samples analyzed after combination of aliquots from the enrichments of UPE (Universal Pre-Enrichment Broth), APW (Alkaline Peptone Water), and 3MB (3M Campylobacter Enrichment Broth)

TABLE 7. Presence/absence testing of general test water samples co-inoculated with *S. enterica* ATCC 14028, *S. flexneri* ATCC 9380, *V. cholerae* ATCC 14035, and *C. jejuni* ATCC BAA-1234 and enriched with UPE, APW or 3MB using BioFire® FilmArray®.

| Enrichment       | Sample ID | Target     |          |                 |               |
|------------------|-----------|------------|----------|-----------------|---------------|
|                  |           | Salmonella | Shigella | Vibrio cholerae | Campylobacter |
| UPE              | GTW A     | +          | +        | +               | -             |
|                  | GTW B     | +          | +        | +               | -             |
|                  | GTW C     | +          | +        | +               | -             |
|                  | GTW D     | +          | +        | +               | -             |
|                  | GTW E     | +          | +        | +               | -             |
| APW              | GTW F     | +          | +        | +               | -             |
|                  | GTW G     | +          | +        | +               | -             |
|                  | GTW H     | +          | +        | +               | -             |
|                  | GTW I     | +          | +        | +               | -             |
|                  | GTW J     | +          | +        | +               | -             |
| 3MB              | GTW K     | -          | -        | -               | +             |
|                  | GTW L     | -          | -        | -               | +             |
|                  | GTW M     | -          | -        | -               | +             |
|                  | GTW N     | -          | -        | -               | +             |
|                  | GTW O     | -          | -        | -               | +             |
| <i>Combined*</i> | GTW 1     | +          | +        | +               | +             |
|                  | GTW 2     | +          | +        | +               | +             |
|                  | GTW 3     | +          | +        | +               | +             |
|                  | GTW 4     | +          | +        | +               | +             |
|                  | GTW 5     | +          | +        | +               | +             |

\*Samples analyzed after combination of aliquots from the enrichments of UPE (Universal Pre-Enrichment Broth), APW (Alkaline Peptone Water), and 3MB (3M Campylobacter Enrichment Broth)

#### 4. DISCUSSION

Food commodities and food processing environments can be contaminated with many different pathogenic bacteria. Screening for their presence/absence can be very challenging following traditional methods. Application of multiplex detection systems would provide an advantage for screening of multiple targets simultaneously, thus helping to ensure product safety. However, to utilize multiplex technology, enrichment of the sample for pathogenic organisms is currently required based on established LODs. Obtaining a universal enrichment broth to screen for multiple pathogens simultaneously compliments multiplex detection platforms and takes full advantage of the system's rapid multiplex capability. UPE broth has been successfully demonstrated for enrichment of *Salmonella*, *Listeria*, and shiga toxin-producing *E. coli* (STEC) from dairy farm environmental samples, milk products, beef, poultry, and radish sprouts in comparison to the standard recommended media (Nam et al. 2004; Hammack et al. 2003; Masashi Kanki 2009). This laboratory investigation demonstrates using UPE and a modified version (UPE+) to enrich food borne pathogens in a variety of inoculated food matrices for its application with multiplex detection. UPE formulation was modified with the addition of 3% sodium chloride (UPE+) to support *V. parahaemolyticus*, which has a salt requirement for growth, but otherwise UPE was not altered for any other comparison study. Pathogenic organisms for testing were selected from the FA multiplex detection assay, (GI panel) and the inoculation levels were targeted below the LOD for the assay. UPE performed equally well for enrichment in comparison to the standard media of each food/pathogen combination (Table 4 and 5). The majority of samples had no significant difference found between the positive/negative ratios at the 95% confidence interval using Fisher's exact test in both comparison trials. Commonly, one to two sample replicates did not have agreement between the standard media and test media for the pairwise comparisons in both trials. Notably, performance of UPE+ decreased in Trial 2 for the detection of *V. parahaemolyticus*. The positive/negative ratios of Chinese cabbage and wild caught shrimp samples containing *V. parahaemolyticus* and enriched in UPE+ or APW were significantly different at the 90% confidence interval ( $P < 0.1$ ) in Trial 2, but were not significant in Trial 1. The inoculation level was slightly higher in Trial 1 than Trial 2 (20 vs 8.2 CFU/sample, respectively). This factor could have positively influenced the growth and subsequent detection rates seen in Trial 1 versus that of Trial 2. In the preliminary growth screening, the team cultured *V. parahaemolyticus* in UPE supplemented with 3% NaCl (UPE+) without modification to an alkaline pH. The UPE+ formulation worked well in Trial 1, but the noticeable decrease in Trial 2 indicates that more comparison testing is required to ascertain what effect, if any, inoculation load has on UPE+ performance for the detection of *V. parahaemolyticus*. Although growth has been reported in media at pH 5 to 11.0, the optimum pH range for growth of *V. parahaemolyticus* is 7.6 to 8.6 (Beauchat 1975). Evaluation of UPE+ at an alkaline pH is also warranted to improve growth/enrichment performance of the media for *Vibrio spp.*

*Campylobacter* is an example of a difficult to enrich pathogen with increasing importance to food surveillance programs, making it desirable to be included in a multiplex assay. *Campylobacter* was the third most bacterial etiological agent (confirmed) in food borne illness outbreaks in 2017 (Dewey-Mattia et al. 2019). Furthermore, between 2012 and 2014 *Campylobacter* was responsible for the greatest incidence of GI infections (10.30 per 100,000 active service members) in the U.S. Armed Forces worldwide (Sarah E. Hill 2017). The United States Food and Drug Administration recommended methods to enrich food and water samples

to test for the presence of *Campylobacter* to include the use of Bolton broth using selective supplements and lysed horse blood under microaerophilic conditions (i.e. bubbling microaerobic gas mixture, CO<sub>2</sub> generating sachets, microaerobic CO<sub>2</sub>/O<sub>2</sub> incubator) (Anonymous 1998). Besides generating microaerobic conditions, several O<sub>2</sub>-quenching agents have been traditionally added to enrichment broths and agar plates for the isolation of *Campylobacter* spp. (Zhou et al. 2011). With the advancement of DNA-based biosensors and automation for bacterial detection, enrichment broths could be screened for the presence of *Campylobacter* spp. in a shorter time, with greater sensitivity and without the generation of any microaerobic condition (Zhou et al. 2011). In Trial 2, the performance of 3MB was compared to the traditional method of using BLB w/selective supplement, and the addition of laked horse blood under microaerophilic conditions for the enrichment/detection of *Campylobacter* in strawberries and ground beef. No statistical difference ( $P > 0.05$ ) was found between positive/negative ratio of ground beef or strawberry samples spiked with *Campylobacter jejuni* and enriched in BLB vs. 3MB. Eliminating the need to incubate enrichment samples for the presence of *Campylobacter* under a modified atmosphere greatly simplifies the process for surveillance. Simplifying the process for *Campylobacter* enrichment will allow for easier inclusion of this organism into a multiplex testing protocol and no longer needing to invest in systems to generate microaerobiosis will result in reduced cost for *Campylobacter* testing (Zhou et al. 2011).

Multiplex testing for difficult-to-culture pathogens (i.e. unique growth requirements, specialized equipment, and slow growth) that cannot be otherwise enriched in a single universal media can also be accomplished via combining of enrichment aliquots into a single multiplex test. The method is only limited by the LOD of the detection system in regards to how many aliquots can be combined within a single test. A single food sample can be divided up into multiple portions and then each portion can be enriched with different media spanning the desired organisms for surveillance. The team investigated this approach and included *Campylobacter* testing into multiplex surveillance in combination with a universal media. Simultaneous detection of four targets was carried out (*S. enterica*, *S. flexneri*, *V. cholerae*, and *C. jejuni*) in GTW and bok choy using the mixing of different enrichment aliquots into a single GI panel diagnostic run (Table 3). Experimental conditions were selected to simulate the requirement of multiple enrichment broths to be used on one food and water sample contaminated with multiple pathogens, with some requiring differing conditions and media formulations for optimal enrichment. The GTW and bok choy samples inoculated with all four organisms were enriched in UPE, APW, or 3MB (Table 2). The enrichments of each sample were analyzed on the FA GI panel for presence/absence testing as individual samples and in combination (Table 3). Samples enriched in UPE or APW had positive results for *Salmonella*, *Shigella*, and *V. cholerae* and all UPE and APW enrichment samples were negative for *Campylobacter* (Table 6 and 7). *Salmonella*, *Shigella* and *Vibrio* all have salt tolerance and can grow in the presence of sodium chloride at 3%, as is the concentration in APW (Jack R. Matches 1972; Anonymous 1998; Zaika 2002). Additionally, *Vibrio cholerae* has an obligate requirement for sodium ion (Na<sup>+</sup>) and can be satisfied by trace amounts of Na<sup>+</sup> normally found in most media constituents; thus, *V. cholerae* demonstrated growth in UPE (Table 6 and 7). *Campylobacter* was not expected to enrich in either UPE or APW in the presence of competitive organisms at the aerobic conditions (37 °C for 24 h) used to enrich the GTW and bok choy samples. *Campylobacter* was only found positive in samples enriched with 3MB while the remaining three organisms were not detected in 3MB due to the media's selectivity (Table 6 and 7). All four organisms were detected only after the enrichment broth aliquots were combined into

a single test panel (Table 6 and 7). This strategy for using multiple enrichment broths and combined analysis on a single test panel will allow for surveillance efforts to include more targets of interest while at the same time reducing costs.

Food commodities are susceptible to contamination with multiple organisms and foodborne illness causing organisms can occur in a wide variety of food matrices. Food surveillance programs and food borne illness outbreak investigations can greatly benefit from using multiplex diagnostics not only from a larger screening panel, but also a faster time to result. Utilizing the multiplex diagnostics can only be effective at detection if pathogen levels are properly enriched to detectable quantities (Kim and Bhunia 2008; Bhagwat 2003). Here, the project team demonstrated how a universal enrichment broth combined with multiplex diagnostics could be utilized successfully to enhance food surveillance and outbreak investigations. Additionally, an alternative method, based on combining several sample enrichments into a single multiplex diagnostic test, could also be used to integrate a multiplex assay into food surveillance programs for organisms that require specific conditions or selective enrichment. Together, these methodologies and findings provide valued advancements for using universal enrichment as a complimentary technology with multiplex detection in the field of foodborne illness outbreak investigations and food surveillance programs

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