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Development of an Enteric Bacterial Enrichment Broth and Its Performance for Isolation of Clinically Significant Bacterial Pathogens from Stool

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Abstract

Background: Early detection and accurate identification of foodborne pathogen outbreaks is an important public health function. Increased clinical adoption of multiplex PCR assays or culture independent diagnostic tests (CIDT) correlates to more stool specimens sent to public health laboratories (PHL) for characterization. Isolation and confirmation of enteric bacterial pathogens can prove difficult to consistently recover. The purpose of this study was to evaluate the performance of a broad-use laboratory developed enrichment broth for isolation of *Campylobacter*, *Salmonella*, *Shigella*, and *Yersinia* strains from stool specimens. **Methods:** The study compared differences in positivity rates among media and enrichment combinations at specific time points. Comparison of direct inoculation (DI), enrichment using a lab-developed Enteric Bacterial Enrichment (EBE) broth and gold-standard isolation methods were conducted to test current utility of this established practice with stool specimens heat injured and non-injured. **Results:** A total of 234 spiked stool samples, 175 non-injured and 59 heat injured, were tested with varying bacterial concentrations. For non-injured stools, direct inoculation performed better for *Campylobacter* and *Yersinia* than enrichment. Conversely, *Salmonella* and *Shigella* recovery and limit of detection increased with enrichment. *Campylobacter* had the highest percent recovery while *Shigella* being the lowest from direct plating at 6-hour and 24-hour enrichment periods. Among broths, EBE performed the best for *Yersinia* and similar to Selenite broth for *Salmonella* and *Shigella*. Generally, heat injured stool had a significantly lower percent of recovery than non-heat injured with a higher limit of detection across organisms. **Conclusion:** Our data suggest there is an only utility for targeted enrichment of CIDT positive *Salmonella* stool specimens.

We highlight the difficulties of formulating an enrichment broth capable of supporting a variety of enteric pathogens with standardized incubation. Increasing demands on PHL infrastructure warrant further examination of enhancing organism isolation and cost analyses for CIDT positive specimens.

Keywords

Enteric Bacteria, Enrichment, Stool, Selective Agar

1. Introduction

Gastroenteritis is a major cause of morbidity worldwide, and clinical presentation alone is unable to distinguish organism etiologies. Early detection and accurate identification of gastrointestinal (GI) bacterial outbreaks is an important public health function. Historically stool culture was the main diagnostic method in clinical microbiology and public health laboratories (PHL) for the identification of GI bacterial pathogens. However, in recent years, multiplex molecular assays have been developed and widely implemented for the detection of GI pathogens directly from clinical stool specimens [1] [2]. These multiplex panels are rapid and sensitive assays although multiple organism detections can cloud interpretation [3]. Traditionally, PHL have received bacterial isolates for organism confirmation and further characterization to aid in disease surveillance. As more laboratories adopt multiplex assays or culture independent diagnostic tests (CIDT), positive stool specimens will be sent to PHL instead of isolates. Recovery and confirmation of these bacterial pathogens will fall to the surveillance laboratories accruing further costs [4] [5].

Gastrointestinal bacterial pathogens can prove difficult to consistently recover for a variety of reasons including transport time and temperature, competition with saprophytic gut flora, and the variance in incubation conditions for cultivation [2]. Tennessee Public Health Laboratory reported pathogen recovery was 57% from referred CIDT-positive stool specimens, which varied greatly based on pathogen type with *Salmonella* (72%) recovered the most frequently in contrast to *Campylobacter* (26%) [6]. If a bacterial isolate cannot be recovered from a positive CIDT specimen, monitoring disease burden and enacting public health interventions could be significantly more difficult.

There have been many reports in the literature concerning the use of enrichment broths and selective agars for the isolation of GI bacteria [7] [8] [9] [10] [11]. Isolation of bacterial pathogens from food matrices using a variety of commercial and in-house developed selective media and enrichment methods is heavily documented [12] [13]. Unfortunately, the use of gold-standard media and enrichment broths in clinical microbiology has not significantly changed over the last 50 years [14] [15] [16] [17]. Well-performing chromogenic agars have been developed but are expensive for routine, high volume use, and are

mainly pathogen specific [18]. Historically, broth enrichment has been used to increase detection of bacterial pathogens especially in times of extended sample transport to the laboratory. Although an overabundance of evidence points to the utility of enrichment, the cost-effectiveness and value of enrichment broths has been questioned [19]. The increased financial burden to PHL having to recover isolates from CIDT positive stool specimens eliminates the potential for pathogen-specific gold-standard enrichment to be used routinely. There is a need to re-evaluate bacterial isolation and screen for broad use methods to increase recovery of GI pathogens. A multi-pathogen broth with standardized incubation conditions, novel in clinical enteric bacterial recovery, can be economically beneficial since it reduces labor and testing cost per sample.

The purpose of this pilot study was to evaluate a broad-use laboratory developed enrichment broth, and to provide comparison of selective agars and enrichment combinations at specific time points for GI pathogen isolation.

2. Materials and Methods

2.1. Bacterial Pathogens and Sample Preparation

Negative clinical stool specimens from Tripler Army Medical Center (TAMC) were pooled and aliquots created for bacterial spiking of specimens. The following bacterial pathogens and number of strains were included in the study: *Campylobacter* (n = 20), *Salmonella* (n = 10), *Shigella* (n = 6), and *Yersinia* (n = 4). Bacteria were isolated from TAMC clinical stools or obtained from American Type Culture Collection (ATCC) (**Table 1**). Bacterial suspensions were prepared from fresh overnight culture growth to optical density of 0.5 McFarland (~10^8 CFU/mL). Approximately 0.5 gram negative stool was diluted into 8.5 mL sterile saline (0.9% NaCl) and spiked with 1 mL 0.5 McFarland Standard bacterial suspension to generate 10-fold serial dilutions (~1×10^7 - 10^0 CFU/mL) in stool solution. Each stool dilution represented a unique specimen in an attempt to mimic receipt of different bacterial concentration burdens clinically. A stool suspension without bacteria was used as negative control and plated to confirm negative result.

2.2. Culture Media and Enrichment Broths

Bacteria were cultured on agar plates: Hektoen (HEK), Xylose-Lysine-Desoxycolate (XLD), Cefsulodin Irgasan Novobiocin (CIN) (BD Diagnostics, Franklin Lakes, NJ, USA), HardyCHROM SS NoPro agar and Modified Charcoal Cefoperazone Deoxycholate (mCCDA) (Hardy Diagnostics, Santa Maria, CA, USA). Commercial broths used were: GN broth, Selenite broth (SB) (BD Diagnostics, Franklin Lakes, NJ, USA), and EE Mossel broth (EE) (Teknova. Hollister, CA, USA). Results were interpreted based on manufacturer recommendations. Enteric Bacterial Enrichment (EBE) broth was prepared with ingredients from Sigma-Aldrich as follows: 30 g soybean-casein digest, 10 g meat extract, 0.6 g L-cysteine, 1.0 g sodium pyruvate, 0.8 g potassium phosphate, 0.8 g sodium carbonate, 0.6 g bile

salts, and 0.5 g sodium bisulfite per liter of distilled water (Sigma-Aldrich, St. Louis, MO, USA). After autoclaving, 100 μg/ml cycloheximide, 1 μg/ml rifampicin, and 30 mg dissolved hemin were added to broth before use. EBE broth was modified from a successful Food Pathogen Enrichment broth developed to support growth of *Campylobacter* without lysed blood and CO_2 [20].

Table 1. Genera and number of strains used to evaluate media and enrichment broths.

Organism	Clinical*	Reference#	Total
***Campylobacter* sp.**	17	3	20
Campylobacter coli	1	2	3
Campylobacter jejuni	16	0	16
Campylobacter lari	0	1	1
***Salmonella* sp.**	7	3	10
Salmonella Anatum	0	1	1
Salmonella group B	1	0	1
Salmonella group C1	1	0	1
Salmonella group C2	1	0	1
Salmonella group E	1	0	1
Salmonella Paratyphi A	1	1	2
Salmonella Typhumurium	0	1	1
Non-typhoidal *Salmonella*, no group	2	0	2
***Shigella* sp.**	2	4	6
Shigella boydii	0	1	1
Shigella flexneri	0	2	2
Shigella sonnei	2	1	3
***Yersinia* sp.**	1	3	4
Yersinia enterocolitica	1	1	2
Yersinia kristensenii	0	1	1
Yersinia ruckeri	0	1	1

*Clinical strains were isolated from stool specimens; #All reference strains were as follows: *C. coli* ATCC 33559; *C. lari* ATCC 35221; *S.* Anatum E9270; *S.* Paratyphi A ATCC 9150; *S.* Typhimurium ATCC 14028; *S. boydii* ATCC 9207; *S. flexneri* ATCC 9199; *S. flexneri*sero 2b ATCC 12022; *S. sonnei* ATCC 25931; *Y. enterocolitica* ATCC 9610; *Y. kristensenii* ATCC 33639; *Y. ruckeri* ATCC 29473.

2.3. Direct Inoculation and Subculture

Serially diluted stools were inoculated directly onto each media type and into enrichment broth(s) with approximately 100 µL or 2 drops. A thin-layer of mineral oil (3 - 5 drops) was added to each broth prior to incubation. Broths were incubated aerobically at 37˚C for 24 hours. Enrichment broths were subcultured at 6 hours and 24 hours to the appropriate media types for each organism. HEK, XLD, and NoPro SS plates were incubated aerobically at 37˚C for 18 - 24 hours, while mCCDA selective plates were incubated micro aerobically up to 72 hours at 42˚C. CIN for *Yersinia* isolation was incubated at room temperature up to 48 hours. After incubation, media were examined for characteristic pathogen growth.

To simulate dramatic temperature shifts during transport, spiked stool, after initial inoculation, was subjected to a heat shock for 10 minutes at 50˚C. Heat injured stools were directly plated, inoculated to enrichment broth, incubated and subcultured as described above.

2.4. Bacterial Isolate Identification

Colonies on selective media were subcultured for identification based on morphology and appearance on each media. Up to 3 colonies were selected for downstream identification using standard biochemical methods for *Campylobacter* (Gram stain, oxidase, catalase, and hippurate tests), and VITEK MS or VITEK 2 (bioMérieux, Durham, NC, USA). Data was based on whether a particular bacterial pathogen was present or not.

2.5. Statistical Analysis

Rates of detection were summarized for each pathogen and analysis method (direct plating vs. enrichment broth at 6 and 24 hours). Rates were calculated separately for original stools and heat injured stools. P-values were generated based on the following methods. Multivariable fixed effect logistic regression models, conditioning on isolate number and dilution factor, were used to evaluate detection rate differences among media, broths, and analysis method (direct plating v. enrichment). Models were run separately by heat injured status for each pathogen. McNemar's tests, which are commonly used to analyze paired dichotomous data, were performed to assess differences in positivity between 6 and 24 hour plating for each broth/media combination. Analyses were based on the paired replicates that were plated for 6 and 24 hours for each isolate. All analyses were conducted using SAS statistical software version 9.4 (SAS Institute, Cary, NC, USA).

2.6. Ethics Approval

This protocol was reviewed and approved by Tripler Army Medical Center Scientific Review Committee (Protocol No. TAMC 19N02) and Ethical Committee. The study met the criteria for Research Not Involving Human Subjects to obtain negative stool specimens from routine diagnostic procedures.

3. Results

3.1. Non-Heat Injured Stools

A total of 175 spiked stool samples from 20 *Campylobacter*, 10 *Salmonella*, 6 *Shigella*, and 4 *Yersinia* isolates were analyzed (Table 1). Dilutions from 10^5 to 10^1 CFU/mL were inoculated and subcultured with the exception of *Shigella* spiked to 10^2 CFU/mL. Due to higher prevalence of clinical isolates seen at TAMC more stools were spiked with *Campylobacter* (n = 98) than any other organism followed by *Salmonella* (n = 43), *Shigella* (n = 18) and *Yersinia* (n = 16). Bacterial recovery was compared for direct inoculation and after enrichment of stools, in terms of dilutions, media and broth types. We found that pathogen recovery differed among bacterial types, between direct plating versus enrichment, and at specific enrichment subculture times. Overall rates of positive growth for each media and broth combination at specific enrichment inoculation time points are indicated in Table 2.

Campylobacter isolation was more successful with direct inoculation than enrichment at both subculture time points (92%, 73%, and 66% recovery for DI, 6-hr, and 24-hr, respectively, $p < 0.001$, Figure 1(a)). Direct inoculation recovered at least 90% of strains down to 10^2 CFU/mL far better than isolation at 6-hour (70%) or 24-hour (50%) enrichment (Figure 2(a)). Enrichment required concentrations one to two orders of magnitude higher to achieve the same level of recovery as direct inoculation.

Salmonella strain isolation improved from direct inoculation with 6-hour enrichment and significantly after 24-hour enrichment (32.6%, 38.6%, and 64.7%, respectively; $p = 0.100$ for 6-hr vs. DI and $p < 0.001$ for 24-hr vs. DI). The limit of detection was best for 24-hour enrichment, which showed 73% recovery at 10^3 CFU/mL and 93% recovery at 10^4 CFU/mL. Isolation at 24 hr was 30% - 35% greater than direct inoculation and 6-hour enrichment recovery at the same dilutions (Figure 2(a)). Among media for *Salmonella*, XLD performed better than HEK and SS NoPro ($p < 0.001$ for both comparisons) for direct inoculation and enrichment (Figure 1(a)). Overall, the most successful enrichment broth for *Salmonella* isolation was SB (82%) outperforming EBE (71%), GN (59%), and EE (47%) ($p < 0.001$ for all pairwise combinations with SB except $p = 0.038$ for SB vs. EBE at 24 hr; Figure 3(a)).

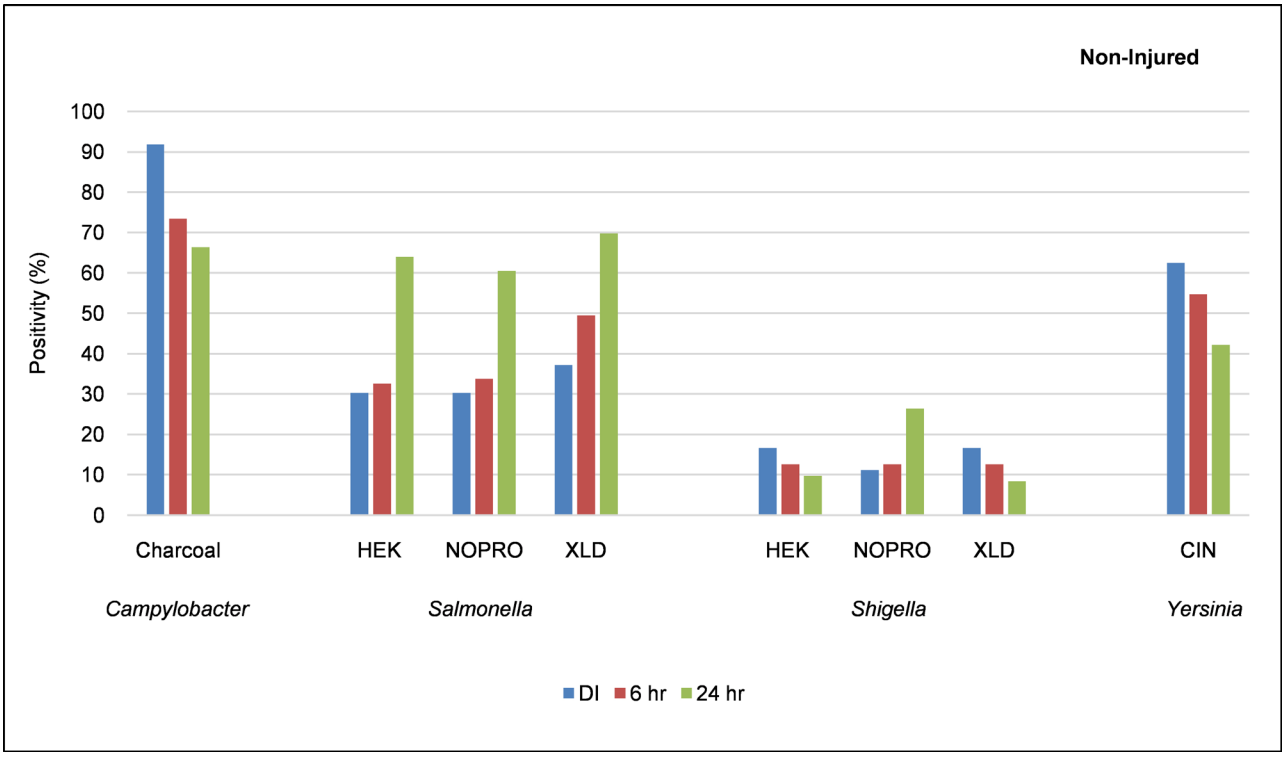
Shigella strains had the lowest percent recovery with no difference in isolation between direct inoculation and enrichment (14.8%, 12.5%, and 14.8% for DI, 6-hr, and 24-hr, respectively). *Shigella* isolation from dilutions between 10^2 - 10^4 CFU/mL was largely unsuccessful, with recoveries less than 20%. Isolation was better at 10^5 CFU/mL (with 55% recovery for direct inoculation, Figure 2(a)). *Shigella* isolate yield was not effective for any agar or enrichment broth although, SS NoPro recovery was considerably better than XLD and HEK after 24-hour enrichment (26% for SS No Pro vs. 8% for XLD, $p = 0.001$, and 10% for HEK, $p = 0.002$; Figure 1(a)).

Table 2. Recovery of *Salmonella*, *Shigella*, and *Yersinia* from non-heat injured and heat injured stools for different media and broth combinations following 6-hour and 24-hour enrichment.

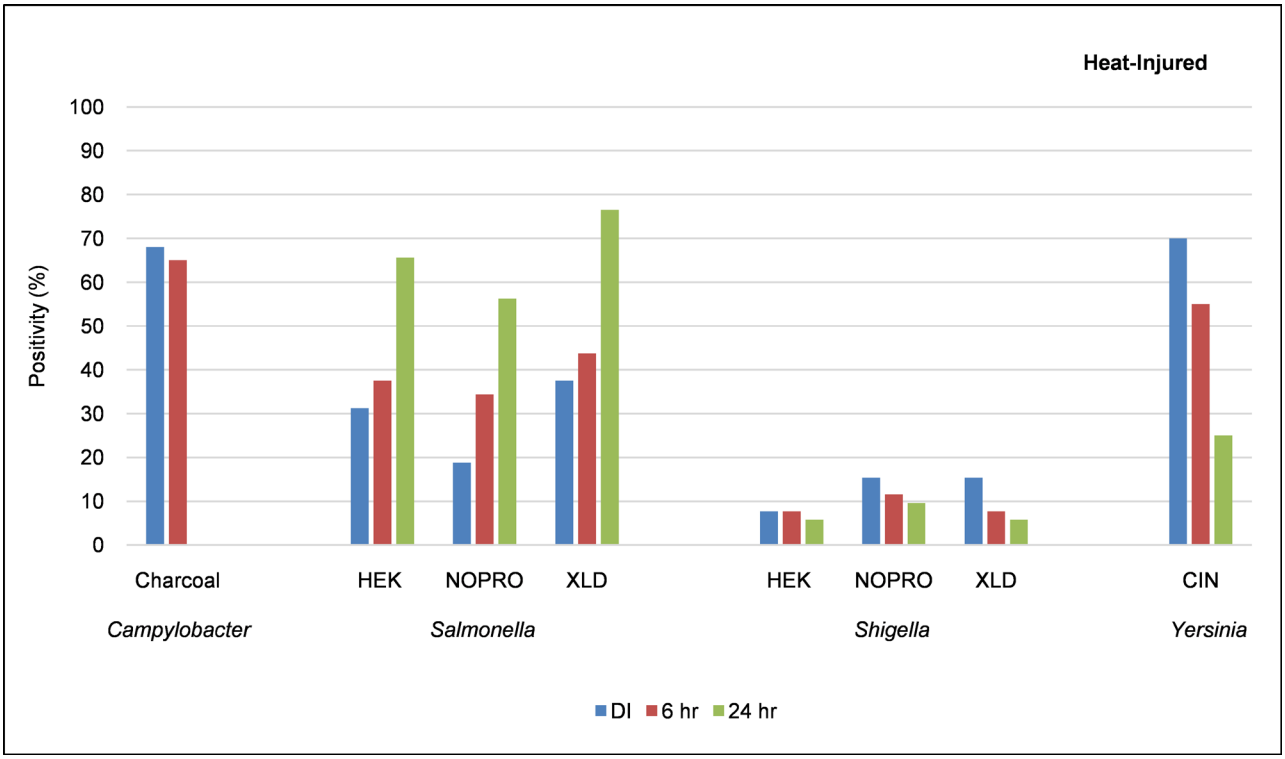
Salmonella	Non-heat injured stools								p-value
	6 hr				24 hr				
	Total	Negative	Positive	%Positive	Total	Negative	Positive	%Positive	
Broth/Media									
EE/HEK	43	32	11	26	43	25	18	42	0.016
EE/NOPRO	43	35	8	19	43	22	21	49	<0.001
EE/XLD	43	28	15	35	43	22	21	49	0.070
GN/HEK	43	37	6	14	43	17	26	60	<0.001
GN/NOPRO	43	30	13	30	43	19	24	56	0.003
GN/XLD	43	28	15	35	43	17	26	60	0.013
EBE/HEK	43	32	11	26	43	11	32	74	<0.001
EBE/NOPRO	43	33	10	23	43	19	24	56	<0.001
EBE/XLD	43	23	20	47	43	7	36	84	<0.001
SB/HEK	43	15	28	65	43	9	34	79	0.070
SB/NOPRO	43	16	27	63	43	8	35	81	0.022
SB/XLD	43	8	35	81	43	6	37	86	0.688
Shigella	Non-heat injured stools								p-value
	6 hr				24 hr				
	Total	Negative	Positive	%Positive	Total	Negative	Positive	%Positive	
Broth/Media									
EE/HEK	18	16	2	11	18	18	0	0	0.480
EE/NOPRO	18	15	3	17	18	16	2	11	1.000
EE/XLD	18	15	3	17	18	18	0	0	0.248
GN/HEK	18	17	1	6	18	15	3	17	0.500
GN/NOPRO	18	16	2	11	18	13	5	28	0.250
GN/XLD	18	18	0	0	18	18	0	0	--
EBE/HEK	18	15	3	17	18	18	0	0	0.248
EBE/NOPRO	18	15	3	17	18	11	7	39	0.125
EBE/XLD	18	15	3	17	18	15	3	17	1.000
SB/HEK	18	15	3	17	18	14	4	22	1.000
SB/NOPRO	18	17	1	6	18	13	5	28	0.125
SB/XLD	18	15	3	17	18	15	3	17	1.000
Yersinia	Non-heat injured stools								p-value
	6 hr				24 hr				
	Total	Negative	Positive	%Positive	Total	Negative	Positive	%Positive	
Broth/Media									
EE/CIN	16	7	9	56	16	12	4	25	0.063
GN/CIN	16	9	7	44	16	13	3	19	0.125
EBE/CIN	16	3	13	81	16	4	12	75	1.000
SB/CIN	16	10	6	38	16	8	8	50	0.500

Continued

Salmonella	Heat injured stools								p-value
	6 hr				24 hr				
	Total	Negative	Positive	%Positive	Total	Negative	Positive	%Positive	
Broth/Media									
EE/HEK	16	12	4	25	16	11	5	31	1.000
EE/NOPRO	16	12	4	25	16	13	3	19	1.000
EE/XLD	16	12	4	25	16	8	8	50	0.125
GN/HEK	16	12	4	25	16	6	10	63	0.031
GN/NOPRO	16	11	5	31	16	7	9	56	0.219
GN/XLD	16	11	5	31	16	1	15	94	0.002
EBE/HEK	16	13	3	19	16	4	12	75	0.004
EBE/NOPRO	16	15	1	6	16	8	8	50	0.016
EBE/XLD	16	12	4	25	16	6	10	63	0.031
SB/HEK	16	3	13	81	16	1	15	94	0.625
SB/NOPRO	16	4	12	75	16	0	16	100	0.134
SB/XLD	16	1	15	94	16	0	16	100	1.000
Shigella	Heat injured stools								p-value
	6 hr				24 hr				
	Total	Negative	Positive	%Positive	Total	Negative	Positive	%Positive	
Broth/Media									
EE/HEK	13	11	2	15	13	13	0	0	0.480
EE/NOPRO	13	13	0	0	13	12	1	8	1.000
EE/XLD	13	12	1	8	13	13	0	0	1.000
GN/HEK	13	13	0	0	13	11	2	15	0.480
GN/NOPRO	13	11	2	15	13	13	0	0	0.480
GN/XLD	13	13	0	0	13	12	1	8	1.000
EBE/HEK	13	12	1	8	13	13	0	0	1.000
EBE/NOPRO	13	10	3	23	13	10	3	23	1.000
EBE/XLD	13	12	1	8	13	13	0	0	1.000
SB/HEK	13	12	1	8	13	12	1	8	1.000
SB/NOPRO	13	12	1	8	13	12	1	8	1.000
SB/XLD	13	11	2	15	13	11	2	15	--
Yersinia	Heat injured stools								p-value
	6 hr				24 hr				
	Total	Negative	Positive	%Positive	Total	Negative	Positive	%Positive	
Broth/Media									
EE/CIN	10	4	6	60	10	8	2	20	0.125
GN/CIN	10	8	2	20	10	8	2	20	1.000
EBE/CIN	10	1	9	90	10	7	3	30	0.031
SB/CIN	10	5	5	50	10	7	3	30	0.500

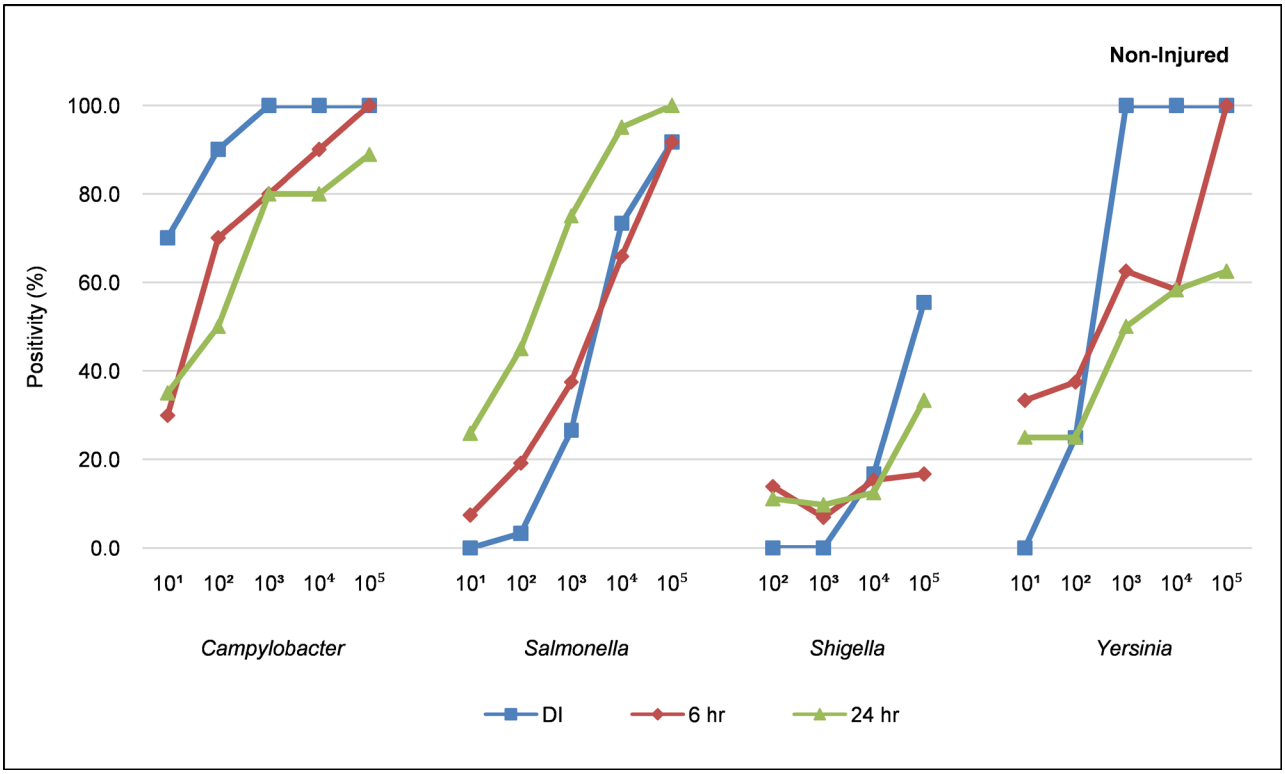


(a)

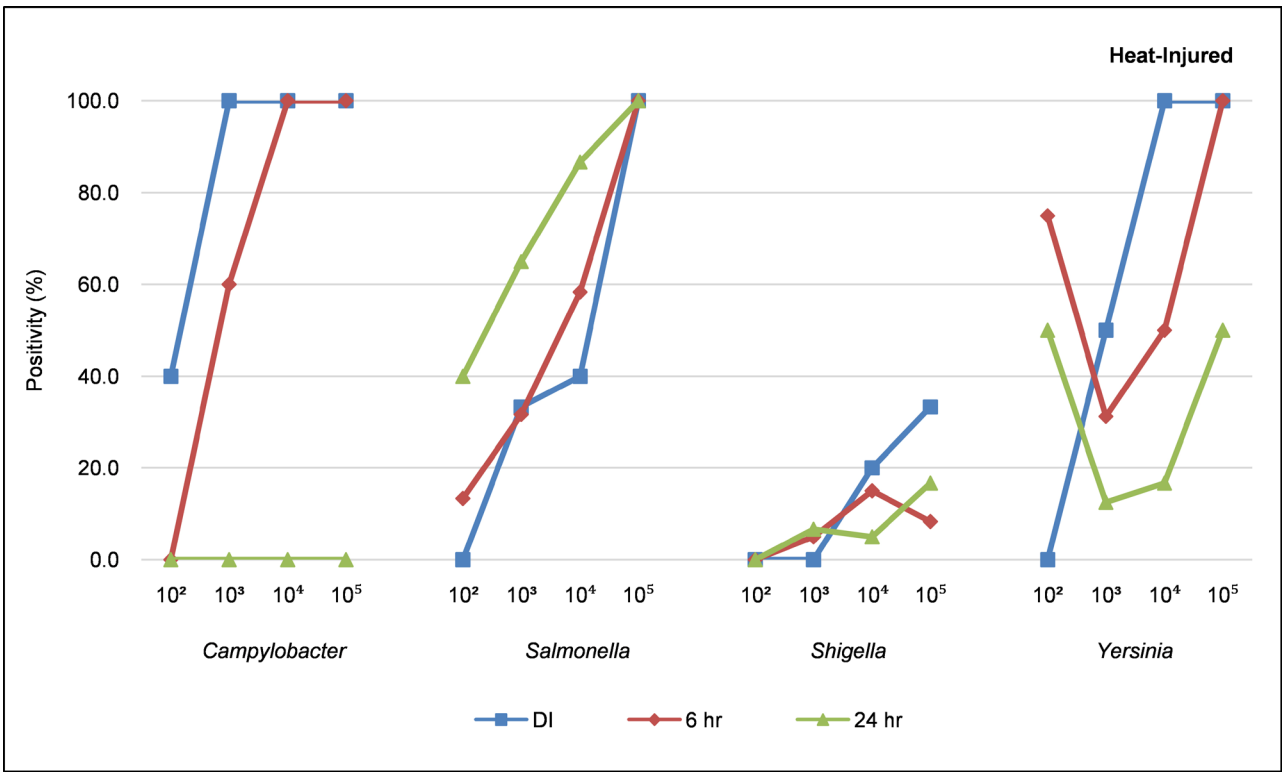


(b)

Figure 1. Comparison between percent recovery of isolates by media type for each enteric bacterial pathogen based on inoculation time point for all dilutions, non-injured (a) and heat injured (b). Abbreviations: Direct inoculation (DI); Hektoen agar (HEK); HardyChrom SS NoPro (NOPRO); Xylose Lysine Deoxycholate (XLD) agar; Cefsulodin, Irgasan, Novobiocin (CIN) agar.

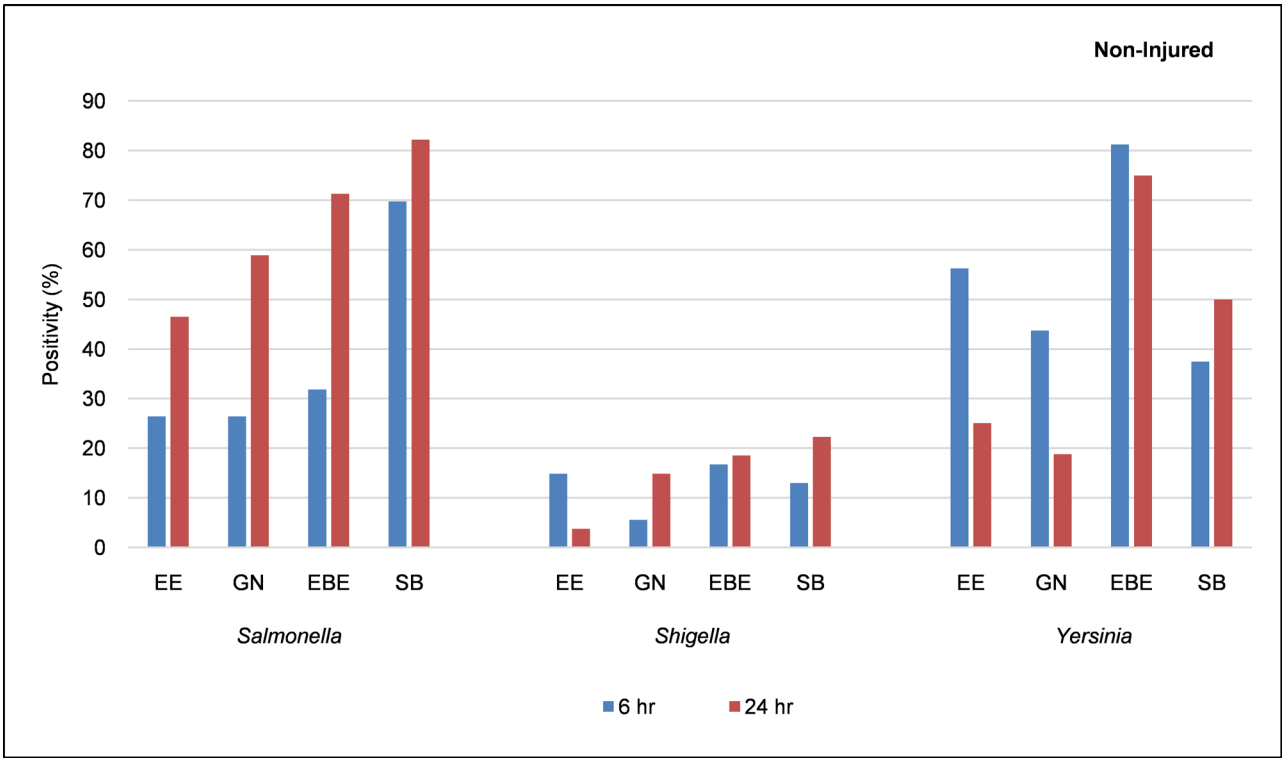


(a)

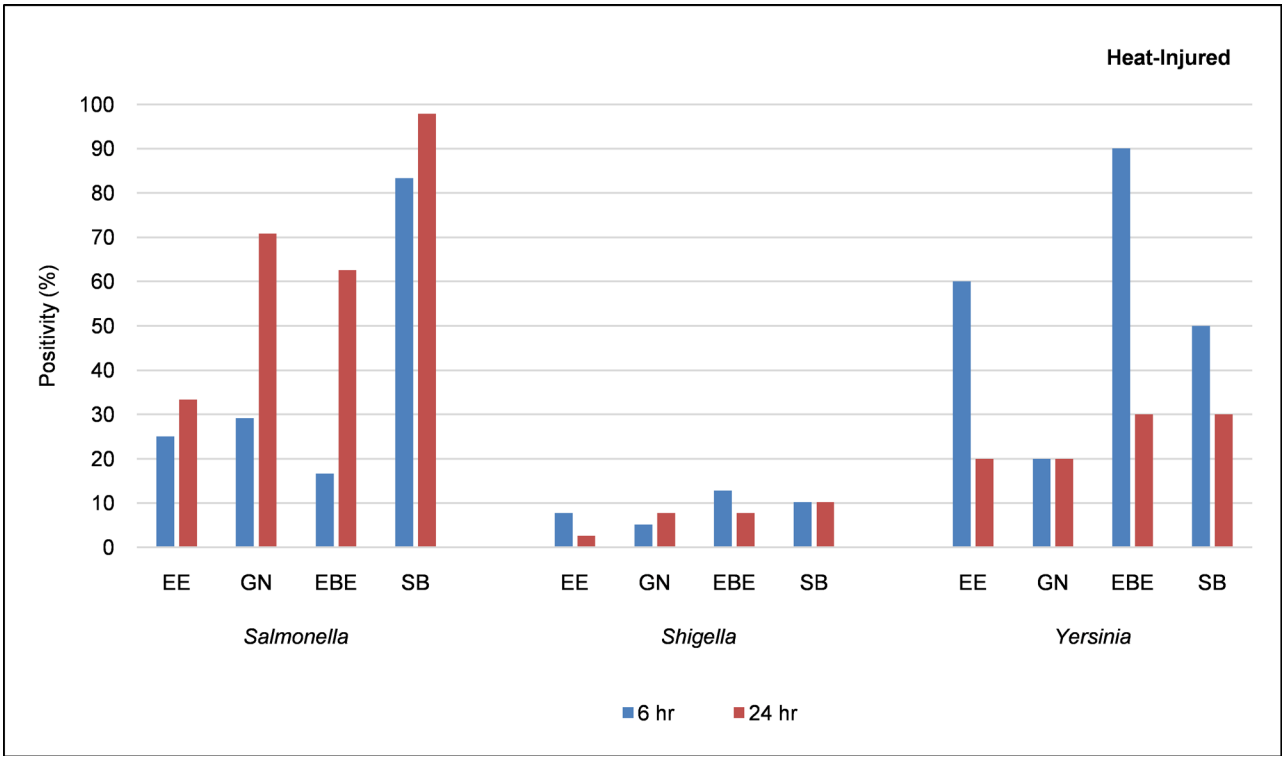


(b)

Figure 2. Isolation percentage based on each enteric bacterial pathogen stool dilution at direct inoculation (DI) and enrichment time points for (a) non-injured and (b) heat injured stools.



(a)



(b)

Figure 3. Comparison of isolates recovered by enrichment broth for *Salmonella, Shigella*, and *Yersinia* based on inoculation time point for all dilutions, non-injured (a) and heat injured (b). Abbreviations: EE Mossel (EE) broth; GN broth (GN); Enteric Bacterial Enrichment (EBE) broth; Selenite broth (SB).

Direct inoculation of *Yersinia* stools was better than enrichment, although not significant (63% for DI vs. 55% for 6-hr and 42% for 24-hr). Recovery with 6-hour enrichment outperformed 24-hour enrichment, $p = 0.039$, **Figure 1(a)**). Bacterial concentrations were consistently recovered with direct inoculation down to 10^3 CFU/mL, but isolation of lower loads was better with 6-hour enrichment, although based on small sample size (**Figure 2(a)**). EBE broth performed much better than other broths in recovering *Yersinia* isolates (81% at 6-hr and 75% at 24-hr, vs. 19% to 56% for other broths and subculture times, $p < 0.001$) (**Figure 3(a)**).

3.2. Heat Injured Stools

We also examined the potential consequences of extreme heat conditions that may result during transport. Generally, heat injured stool recovery was poor compared to non-heat injured with a higher limit of detection across organisms. A total of 59 stool specimens were setup and analyzed. Dilutions from 10^5 to 10^2 CFU/mL were inoculated and subcultured. The organism stool distribution was as follows: *Campylobacter* (n = 20), *Salmonella* (n = 16), *Shigella* (n = 13) and *Yersinia* (n = 10). Briefly, recovery was lower for direct inoculation and after 24-hour enrichment for heat injured stools compared to non-heat injured stool specimens. At each enrichment subculture, every organism except *Salmonella* had significantly lower isolation than non-heat injured samples ($p < 0.05$). Media and enrichment broth isolation trends were similar to non-injured stool data (**Figures 1(b)-3(b)** and **Table 2**). There was significant overgrowth of commensal flora on the heat injured 24-hour enrichment stool cultures which did not allow for identification of any *Campylobacter* isolates.

4. Discussion

In this study, our first step was to develop a broad-use enrichment for the cultivation of *Campylobacter*, *Salmonella*, *Shigella*, and *Yersina* termed Enteric Bacteria Enrichment (EBE) broth. Due in-part to the variety of growth requirements and incubation conditions unique to GI bacterial pathogens there is no routine multi-pathogen broth in clinical microbiology [2]. EBE broth was developed for incubation at 37˚C with a mineral-oil overlay. Although, inconsistent with PHL role, we decided to use 37˚C as our standard incubation temperature to establish a baseline for broth performance as if an unknown specimen was received at a sentinel laboratory for stool culture. The oil layer did not pose a recovery issue for the other facultative anaerobes [21]. In preliminary experiments, we observed no difference in *Campylobacter* isolation with the mineral oil overlay and incubation in a microaerophilic container (data not shown). Although, this testing was not exhaustive, and incubation at 42˚C or the addition of supplemental antibiotics such as polymyxin B could increase recovery, this issue was not investigated [22].

Salmonella and *Shigella* are common organisms submitted to PHL for con-

firmation and characterization. We compared organism recovery of two gold-standard agars (HEK and XLD) with a recently released chromogenic agar for *Salmonella* and *Shigella* (SS NoPro). XLD performed the best for *Salmonella*, and *Shigella* isolation was similar across media types except for SS NoPro after 24-hour enrichment. XLD agar has been documented as superior to other gold-standard broad-use enteric agars for recovery of these organisms [15] [23] [24]. For *Salmonella* and *Shigella* enrichment, SB and EBE were comparable. Optimal agar/broth combinations varied for *Salmonella* and *Shigella*. SB/XLD was the best combination for *Salmonella* while SB and EBE coupled with SS No-Pro were better for *Shigella* strains. *Shigella* stools were evaluated a magnitude higher than the rest of the spiked specimens due to an early indication that recovery was poor at lower dilutions.

CIN agar is routinely used in clinical diagnostics for *Yersinia* but there may have been a benefit to inoculating *Yersinia* strains to HEK and XLD for a comparison of broad-media use as well [24] [25]. Gold-standard cold enrichment even performed on selective media, is time-consuming and labor intensive requiring multiple steps and is poorly suited for recovery of all *Yersinia* strains [9]. Nevertheless, incubation of EBE broth at 37˚C may not allow for suitable *Yersinia* detection since the optimal enrichment temperature is maximally 25˚C - 30˚C. Most enterobacteria will outgrow *Yersinia* at the higher incubation temperature leading to underestimation.

This study is the first to our knowledge to evaluate enteric bacterial pathogen isolation from stool using a broad-use laboratory developed enrichment broth with standardized incubation conditions. Agar media supporting multiple enteric pathogens has been developed and evaluated for use with stool specimens [15] [18] [24]. The focus of enteric enrichment broth development has been mainly aimed at the recovery of a particular pathogen or simultaneous enrichment of multiple bacteria from food products [12] [20] [26] [27] [28] [29]. A universal pre-enrichment broth (UPB) was developed to enhance injured Gram-positive and Gram-negative enteric pathogen recover in food [29] [30]. UPB is non-selective making it difficult for use with specimens that have high levels of commensal flora. Multi-pathogen enrichment broths have been reported for isolation of organisms from food. For instance, the Selective Enrichment Broth (SEL) was formulated to allow the simultaneous growth of *Salmonella enterica*, *E. coli* O157:H7 and *Listeria monocytogenes* [12] [28]. Xiao *et al.* (2010) developed an enrichment broth to support simultaneous growth of *Salmonella*, *Vibrio parahaemolyticus*, and *Vibrio cholerae* [26]. The most promising multiplex enrichment broth effort was the development of the SSSLE broth supporting *Salmonella*, *Staphylococcus aureus*, *Shigella flexneri*, *Listeria monocytogenes*, and *E. coli* O157:H7 recovery from beef and pork [27]. Our study contained a wider variance of enteric bacterial strains and was conducted with human stool specimens opposed to food matrices.

There are some limitations to this study. Artificial stool specimens and heat

stress conditions were contrived based on previous studies comparing enteric organism enrichment and isolation methods [28] [31] [32] [33] [34]. Routinely, stool specimens prior to PHL receipt would be stored by the sentinel laboratory before shipping or transportation to the PHL for processing decreasing bacterial concentration within the sample. This study did not account for storage or transport time prior to agar media or enrichment broth inoculation, thus recovery rates may be over-estimates of actual PHL observations. Organism distribution was skewed towards *Campylobacter* and *Salmonella*, although this is consistent with identifications seen clinically in the region. Initial EBE broth development plans included *Vibrio* and Shiga-toxin producing *Escherichia coli* O157 but no clinical isolates were available at our medical center for testing. Additionally, the workflow for Shiga-toxin producing *E. coli* confirmation can be labor and resource intensive including the reliance on a screening immunoassay prior to plating and isolate identification [35]. Future directions should include a clinical or public health laboratory based study involving true patient specimens and a larger more inclusive sampling. A comparison of EBE broth to standard practices can be more thoroughly conducted and optimal culture conditions can be established.

5. Conclusion

In conclusion, these findings have important implications related to bacterial enteric disease laboratory surveillance. With increased burden of pathogen isolation at PHL, procedural labor and resource costs strain laboratory infrastructure increases the need to provide efficient isolation methods. This study formulated an enrichment broth, EBE, which allowed the concurrent growth of multiple GI pathogens with standardized incubation. Our results suggest that there may only be utility for enrichment of CIDT positive *Salmonella* stool specimens. The data also indicate how difficult it is to successfully formulate a broth able to support a variety of bacterial GI pathogens. Based on our study data, it may be beneficial for PHL to eliminate direct inoculation of CIDT positive *Salmonella* specimens only pursuing enrichment and subsequent subculturing at a specific time-point for optimal fiscal and isolation balance. Enrichment of other GI bacterial pathogens may lack adequate recovery to balance associated labor and resources costs. Further exploration of enhancing culture yields and cost analyses for CIDT positive specimens is warranted.

Conflicts of Interest

The views expressed in this manuscript are those of the author(s) and do not reflect the official policy or position of the Department of the Army, Department of Defense, or the US Government. There are no conflicts of interest to disclose.

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