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Development of a novel cross-streaking method for isolation, confirmation, and enumeration of *Salmonella* from irrigation ponds

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ABSTRACT

The 2013 Produce Safety Rules in Food Safety Modernization Act (FSMA) require regular testing for generic *Escherichia coli* in agricultural water intended for pre-harvest contact with the edible portion of fresh produce. However, the use of fecal contamination indicators frequently does not correctly reflect distribution of foodborne pathogens such as *Salmonella enterica*, and ensuring food safety may require direct detection and enumeration of pathogens in agricultural settings. Herein we report the evaluation of different cost-effective methods for quantification, isolation, and confirmation of *Salmonella* in irrigation pond water and sediment samples. A most probably number (MPN) dual enrichment culture method was used in combination with differential and selective agars, XLT4 and CHROMagar™ Salmonella plus (CSP). The necessity for PCR confirmation was evaluated, and methods were compared by cost and performance measures (i.e., sensitivity, specificity, positive predictive value, and negative predictive value). Statistical analyses showed that using XLT4 as the initial selective agar to isolate *Salmonella* colonies improved recovery compared to CSP agar; however, PCR confirmation was required to avoid false positive results on either agar. Therefore, a novel cross-streaking method utilizing CHROMagar™ agar for individual colony confirmation of *Salmonella* presence/absence on XLT4 was developed. This method classifies the colony as positive if typical *Salmonella* appearance is observed on both agars. Statistical analysis showed that this method was as effective as PCR for species confirmation of pure individual strains isolated from enrichment cultures (sensitivity = 0.99, specificity = 1.00, relative to PCR). This method offers a cost-effective alternative to PCR that would increase the capacity and sensitivity of *Salmonella* evaluation.

1. Introduction

Salmonella is the most common bacterial foodborne pathogen in the United States, averaging 145 outbreaks, 3,913 illnesses, 403 hospitalizations, and 6 deaths annually for 1998–2008 (Gould et al., 2013). Salmonellosis is increasingly associated with produce contamination (Gould et al., 2011; Hanning et al., 2009). Specifically, irrigation water has been investigated as a potential source of pre-harvest contamination, and one of the most significant *Salmonella* outbreaks related to fresh produce was caused by Jalapeño and Serrano peppers that were contaminated by irrigation water (Behravesh et al., 2011; Mody et al., 2011). Presumably, aquatic systems become contaminated with *Salmonella* through the introduction of fecal material of infected animals (Ijabadeniyi et al., 2011; Pachepsky et al., 2011; Plusquellec et al., 1994). Once present, the pathogen may become established in these environments. For example, contaminated aquaculture ponds contribute to seafood-borne infections (Plusquellec et al., 1994). Rivers and ponds provide natural habitat for a variety of wildlife, such as reptiles, amphibians, and birds, which are all known to harbor *Salmonella* and therefore may also serve as reservoirs for this pathogen (Gorski et al., 2013; Pfleger et al., 2003; Reche et al., 2003).

The new Produce Safety Rules in Food Safety Modernization Act (FSMA) has required regular testing for generic *Escherichia coli* in agricultural water that directly contacts with the edible part of fresh produce. However, fecal indicator bacteria may not provide reliable *Salmonella* estimates due to the greater resistance of this pathogen to the stressful conditions associated with environmental reservoirs relative to indicator organisms (Pianetti et al., 2004; Polo et al., 1998). Thus, time-efficient and cost-effective detection methods for *Salmonella* from irrigation pond samples are needed for risk assessment and

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potential monitoring. Due to the relatively low levels of *Salmonella* contamination in aquatic environments (Escartin et al., 2002; Madsen, 1994), methods for recovery and enumeration of *Salmonella* generally include a most probable number (MPN) enrichment combined with the use of selective agars in combination with molecular confirmation, such as the polymerase chain reaction (PCR) (Haley et al., 2009; Jenkins et al., 2008; McEgan et al., 2013; Rajabi et al., 2011).

In this study, an MPN assay using lactose broth as pre-enrichment and tetrathionate (TT) as secondary enrichment (Rajabi et al., 2011) was combined with different downstream methods for confirmation of *Salmonella*. Statistical analyses of these detection methods included sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), using PCR confirmation as the "gold standard". The results demonstrated the validity of a novel cross-streaking method for confirmation of *Salmonella* presence by using both Xylose-Lysine-Tergitol4 (XLT4) and CHROMagar™ Salmonella plus (CSP) agars. The cross-streaking method consists of initial isolation of presumptive *Salmonella* colonies on XLT4 agar from MPN enrichment, followed by cross-streaking to both XLT4 and CSP agars. A colony was classified as *Salmonella* positive by cross-streaking if both agars simultaneously exhibited colonies with typical *Salmonella* appearance, and results from the cross-streaking method showed 99.95% agreement with PCR ($n = 1640$ isolates) with only a single false negative strain.

2. Materials and methods

2.1. Sample collection from irrigation ponds

Evaluation of detection methods was conducted from November 2010 to September 2011, and consisted of samples collected from 10 irrigation ponds on farms within a broad region of the upper Suwannee River Watershed in southern Georgia, as described previously (Gu et al., 2013a, 2013b). Two water samples (10 L) and two wet sediment samples (1 L collected using benthic dredge, WILDCO® Fieldmaster® Mighty Grab II Dredge) were collected monthly from each pond. Collection vessels were sanitized with 10% bleach and rinsed with sterile water before sampling. All samples were stored on ice packs and transported to the lab for refrigeration. Microbiological analyses were begun within 24 hours of collection.

2.2. Salmonella most probable number (MPN)

As summarized in Fig. 1, *Salmonella* was enumerated by an MPN protocol using three dilutions of triplicate enrichment cultures, with some small modifications of our previous protocols (Rajabi et al., 2011). Aliquots of 500 ml, 100 ml, and 10 ml of water or 100 g, 10 g, and 1 g of sediment (sediment was allowed to settle and water decanted) were inoculated into equal volumes of double strength (2×) lactose broth (Fisher Scientific Inc.), except 1 g of sediment was inoculated into 10 ml of 1× lactose broth. A total of 147 water samples and 147 sediment samples were examined, and thus, there were a total of 294 MPN samples (i.e., 2646 TT cultures from a 3 tube × 3 dilution MPN). Type strain *Salmonella enterica* Typhimurium LT2 (ABC Research Laboratories, Gainesville, FL) was used as a positive control for enrichment cultures and PCR analysis. Inoculated lactose broth cultures were incubated at 37 °C for 24 hours. One milliliter of lactose broth was transferred to 9 ml of TT broth (Fisher Scientific Inc.) with 20 ml/L of iodine potassium solution (Sigma-Aldrich) for 24 hours at 37 °C.

2.3. Media for isolation and confirmation of Salmonella in MPN enrichment

Following enrichment in broth culture, presumptive *Salmonella* colonies were isolated on selective and differential agars, Xylose-Lysine-Tergitol 4 (XLT4, Remel, USA) and CHROMagar Salmonella Plus (CSP, CHROMagar Microbiology, Paris, France). XLT4 is typically used to recover *Salmonella* spp. (excluding *Salmonella* serotype Typhi) from food

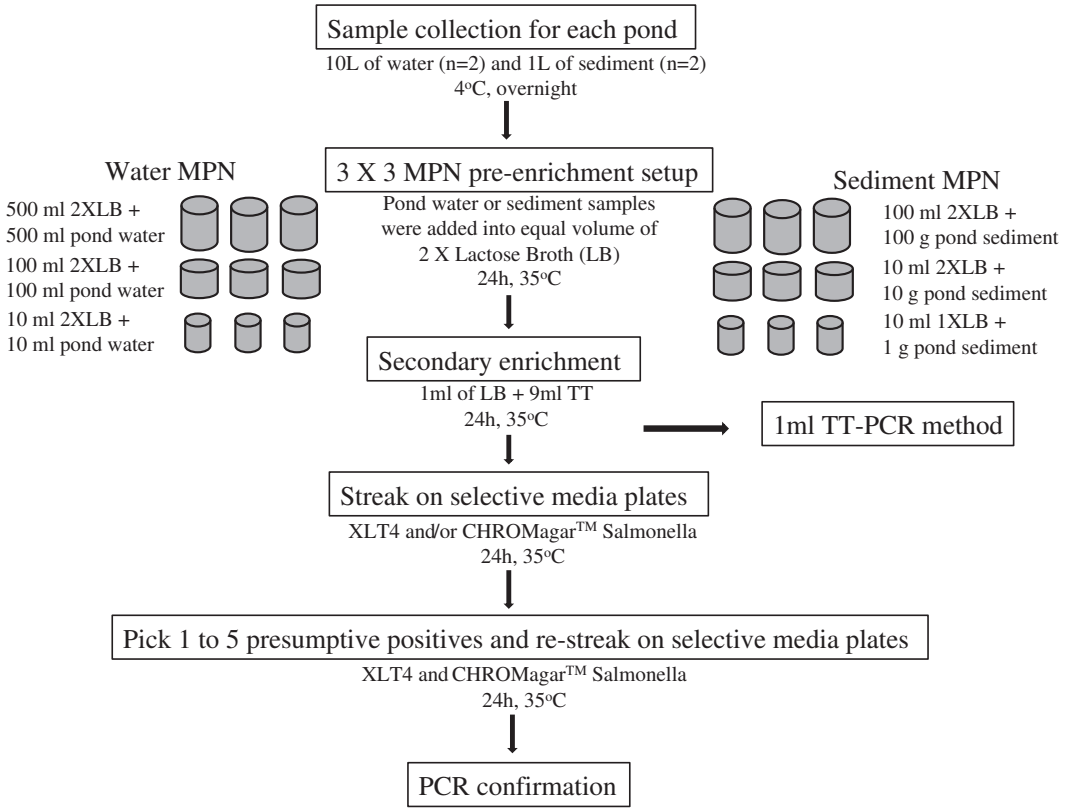

Fig. 1. Flowchart diagram for most probable number (MPN) enumeration, isolation and identification of *Salmonella*. TT = tetrathionate broth; XLT4 = xylose lysine desoxicholate agar with tergitol 4; LB = Lactose Broth.

and environmental samples. Xylose provides the carbon source while lysine utilizes the ability of *Salmonella* to decarboxylate lysine and to produce an alkaline pH environment. Tergitol 4 serves as a selective agent to reduce gram-positive flora and non-*Salmonella* enteric gram-negative bacilli (XLT4 Product Information). Typical appearance of *Salmonella* on this agar is an isolated red colony with a black center that may present a yellow halo with longer incubation. CSP is a selective chromogenic medium used to detect and isolate *Salmonella* spp. including lactose positive *Salmonella* (CSP product information). Typical appearance of *Salmonella* on CSP is an isolated mauve colony, while other members of the same Enterobacteriaceae family appear colorless (*E. coli*, *Proteus*) or blue (Coliforms). The major advantage of CSP compared to CHROMagar *Salmonella* (CAS), an earlier version of chromogenic medium produced by the same company, is the isolation of lactose positive *Salmonella*, primarily from poultry sources. Therefore, CSP is recommended over CAS for food and environmental samples (CSP product information). The performance of XLT4, CAS, and CSP in terms of typical colony appearance and the number of false positives and false negatives identified by the manufacturer, as well as by other sources, are listed in Table 1. In our experiments, CSP rather than CAS, was chosen because the water and sediment samples from environmental sources were assumed to have diverse populations that may include lactose negative strains and serotypes that are not detected by CAS.

The efficacy of six different downstream detection methods for confirmation of *Salmonella* in the TT broth from the MPN enrichment protocol described above was examined. Specifically, these detection methods are as follows: (1) XLT4-CSP-PCR method was considered the gold standard, and colonies were isolated from TT broth on two selective agars, XLT4 and CSP, followed by PCR for individual isolated colony confirmation from one or both agars; (2) TT-PCR was an attempt at more rapid detection using PCR of the DNA extracted directly from TT broth without colony isolation; (3) XLT4-CSP used the criteria for *Salmonella* positive samples based on isolation of typical colonies on either XLT4 and/or CSP agar but without PCR confirmation; (4) XLT4-PCR only considered XLT4 presumptive positive colonies followed by PCR confirmation for individual colony; (5) XLT4 used XLT4 but without PCR confirmation for individual colonies; (6) CSP-PCR used isolation on CSP followed by PCR for individual colony confirmation; and (7) CSP used CSP without PCR confirmation.

2.4. Isolation of Salmonella on selective agars

An aliquot of each tube of TT culture (1 ml) was transferred into a new centrifuge tube and stored at −20 °C for subsequent DNA extraction and PCR. The remaining TT culture was used to isolate individual colonies on XLT4 or CSP agars. All XLT4 and CSP plates were used within 3 weeks of preparation and divided into two equal sections to test two TT samples/plate by streaking each half for isolation of *Salmonella*. All plates were incubated at 37 °C for 24 hours. The XLT4 plates were also examined at 20 hours. Up to 5 presumptive positive colonies with typical *Salmonella* appearance were re-streaked onto 1/6–1/8 of a plate using both XLT4 and/or CSP in order to obtain a good isolated colony and incubated for 24 hours at 37 °C. Colony appearance was recorded and the most isolated colony with typical *Salmonella* appearance from XLT4 and/or CSP agars were inoculated into 10 ml Luria-Bertani (LuB) broth overnight for further PCR confirmation. Presumptive positive strains were also spread on LuB agar plates, incubated overnight, and harvested for frozen storage (−80 °C) in LuB with 50% glycerol.

2.5. PCR confirmation assay

In order to detect *Salmonella* presence using PCR, DNA was extracted using a boiling method whereby bacteria were collected from 1 ml of overnight TT or LuB culture by centrifugation at 10,000 × g for 1 min. Cells were re-suspended in 200 μl phosphate-buffered saline, boiled at 100 °C for 7 min using a heat block, and centrifuged again at 10,000 × g for 1 min. Supernatants (150 μl) were transferred to new tubes and stored in a freezer at −20 °C for further use. Primer pairs were based on the *invA* and *invE* genes of *Salmonella* (Stone et al., 1994), and nucleotide sequences are 5′-TGCCTACAAGCATGAAATGG-3′ (*invE*) and 5′-AAAC TGGACCACGGTGACAA-3′ (*invA*). DNA extract (1 μl) was mixed with 2.5 μl of 10× buffer (5 PRIME), 400 nM of each deoxinucleotide triphosphate (dNTP, Invitrogen), 400 nM of each primer (Sigma-Aldrich), and 0.5 μl of *taq* polymerase (5 PRIME) in a final volume of 25 μl. The PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of DNA amplification under the following conditions: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s with a final extension at 72 °C for 5 min. The PCR products were analyzed by gel electrophoresis in a 0.5× Tris-acetate-EDTA (TAE, Fisher Scientific Inc.) buffer. Each PCR product (9 μl) was combined with 1 μl of 10× loading dye (Qiagen) and run on 1.5% agarose gel (Promega) containing 0.5 μg/ml ethidium bromide (EtBr, Fisher Scientific Inc.) for 40 min at 90 V/cm. The positive *Salmonella* band is 457 base pairs based on comparison to standard DNA molecular weight markers (50 bp DNA Ladder from New England Biolabs).

2.6. Analysis of performance of detection methods

As described in previous studies (Carrique-Mas et al., 2009; Hyeon et al., 2012; Soria et al., 2012), a variety of performance criteria are usually calculated to describe the performance of binary detection methods. These performance measures are defined as follows: (1) sensitivity (the true positive rate) is the (conditional) probability to classify a unit as positive given that the unit is positive, (2) specificity (the true negative rate) is the (conditional) probability to classify a unit as negative given that the unit is negative, (3) positive predictive value (the positive precision rate) is the (conditional) probability that the unit is positive given that it is classified as positive, and (4) negative predictive value (the negative precision rate) is the (conditional) probability that the unit is negative given that it is classified as negative. Often, other performance measures are also reported, including false positive rate (1 − sensitivity), false negative rate (1 − specificity), false discovery rate (1 − positive predictive value), and false acceptance rate (1 − negative predictive value). In

Table 1
Comparison of *Salmonella* isolation agars.

Medium	Typical *Salmonella* appearance	False positive species	False negative (*Salmonella* Serotypes)	References
XLT4	H_2S-positive strains appear blackor black-centered with a yellow periphery	*Citrobacter freundii*	Typhi Arizonae Senftenberg	Schonenbrucher et al., 2008 Manufacturer's protocol
CHORMagar *Salmonella* Plus (CSP)	Mauve colonies	Some *Pseudomonas aeruginosa* Some *E. coli*	Dublin	CHROMagar *Salmonella* Plus Product Information
CHROMagar *Salmonella* (CAS)	Mauve colonies	*Candida albicans* *P. aeruginosa* *Aeromonas hydrophila* Some *E. coli*	Not identified	Gaillot et al., 1999 CHROMagar *Salmonella* Product Information Maddocks et al., 2002

order to calculate these measures, a "gold standard" is needed as a baseline or common basis of comparison, which would ideally be an infallible detection method. The XLT4-CSP-PCR method is a commonly used and scientifically sound method and was considered as the gold standard in these analyses. The XLT4-CSP-PCR method classifies a unit (i.e., a TT sample) as positive if it contains colonies with typical *Salmonella* appearance on either agar and these isolate(s) are later confirmed as *Salmonella* positive using PCR; otherwise the unit is classified as negative. The estimates of these performance measures are usually based on the *confusion matrix* of actual and predicted group membership of the classified units which has the following values (entries): TP is the number of true positive units (in our case, the number of XLT4-CSP-PCR positive units that were classified as positive by the detection test), TN is the number of true negative units, FP is the number of false positive units, and FN is the number of false negative units. The estimates of the performance measures are calculated as follows: sensitivity = TP/(TP + FN), specificity = TN/(TN + FP), positive predictive value = TP/(TP + FP), and negative predictive value = TN/(TN + FN).

To measure the agreement between the other assays and the XLT4-CSP-PCR method, we calculated Cohen's kappa coefficient (Petersen and Wachmann, 1998). The kappa coefficient is given by $K = \frac{Pr(A) - Pr(E)}{1 - Pr(E)}$, where Pr(A) is the probability that the two detection tests agree with each other and Pr(E) is the probability that the two detection tests agree by chance. The kappa coefficient takes values between 0 and 1, and higher values of kappa show stronger agreement between the two methods. Note that the agreement between two detection tests is calculated irrespective of whether one of the two tests is a gold standard. When we interpret the kappa values, we adopt the same interpretation as Soria et al. (2012). Specifically, a kappa value between 0.80 and 1.00 shows excellent agreement, between 0.61 and 0.80 shows good agreement, between 0.41 to 0.60 shows fair agreement, between 0.21 to 0.4 shows slight agreement, while a kappa value between 0.01 and 0.20 shows poor agreement. In addition, we also perform McNemar's test of homogeneity of positive detection rates of the two methods. The McNemar's test is given by $\chi^2 = \frac{(b-c)^2}{b+c}$, where b and c are the number of discordant results of the two detection tests. Specifically, b is the number of samples classified as positive by the first method and negative by the second method and c is the number of samples classified as negative by the first method and classified as positive by the second method. The reference distribution of McNemar's test is the chi-squared distribution with one degree of freedom. All statistical analyses were performed in R (R core Team, 2013, http://www.r-project.org). We used the contributed R package *bdpv* (Schaarschmidt, 2012, http://cran.r-project.org/web/packages/bdpv/bdpv.pdf) to estimate and to construct confidence intervals for the performance measures, and the contributed package *irr* (Gamer et al., 2012) to compute the kappa coefficient and to test its significance. McNemar's test is available in the *stat* package of the R statistical language.

2.7. Analysis of performance of the cross-streaking method

The cross-streaking method is a novel detection method that was developed from the results of the evaluation of the methods described above and is proposed as an alternative to the application of PCR for confirmation of colony identification. Samples from TT broth were initially streaked to XLT4, and presumptive positive colonies were subsequently re-streaked to CPS agar. The cross-streaking method classifies a colony as positive if it exhibits typical *Salmonella* appearance on both XLT4 and CSP agars and it classifies a colony as negative if it does not. In order to ensure isolation of a single colony from primary XLT4 agar isolation, colonies were also re-streaked to XLT4. The PCR assay was considered to be the gold standard when we calculated the performance measures of the cross-streaking method. In our statistical analysis, we compared the results for bacterial isolates exhibiting presumptive positive colonies on at least one selective agar and confirmed as *Salmonella* positive by PCR in this study ($n = 1349$) and also 30 isolates that were confirmed positive *Salmonella* strains from other aquatic environmental sources (Rajabi et al., 2011). The remaining 261 colonies were non-*Salmonella* isolates as determined by PCR. As described above, sensitivity, specificity, positive predictive value, and negative predictive value were calculated to evaluate the performance of the cross-streaking method. The strength of agreement was again calculated using the kappa coefficient and McNemar's test of agreement was performed to test the equality of the positive detection rates of the cross-streaking and PCR detection methods.

2.8. Assessment of material cost

Material cost was calculated based on precise reagent consumption including lactose broth, tetrathionate broth, XLT4 agar, CSP agar, PCR reagents, gel electrophoresis reagents, and approximate costs of other disposable materials, such as conical tubes, wood applicators, 1.5 ml centrifuge tubes, pipette tips total expenses per MPN for each detection method were calculated in US dollars.

3. Results and discussion

3.1. Evaluation of performance of TT_PCR for rapid detection of Salmonella

Table 2 shows the confusion matrix of TT_PCR compared with XLT4-CSP-PCR ($n = 1602$). The values of the confusion matrix of actual and predicted group membership for the TT_PCR were: TN = 1493, FP = 10, FN = 64, and TP = 35, respectively. These results indicated that the XLT4-CSP-PCR method had a higher positive detection rate (6.1%) than TT_PCR (3.0%). Indeed, as shown in Table 3, evaluation of these results using XLT4-CSP-PCR as a gold standard was confirmed by the McNemar's test, which is highly significant ($\chi^2 = 37.96$, $P < 0.0001$). Furthermore, Cohen's kappa statistic is 0.47, which shows these two detection methods are only in a fair agreement. Evaluation of the performance of TT_PCR also showed that TT_PCR attained high specificity (0.99) and high negative predictive value (0.96) but had a low sensitivity (0.35) and a low positive predictive value (0.78). These data showed that pre-screening of MPN enrichment samples by TT_PCR did not yield results that were consistent with XLT4-CSP-PCR. Overall, TT_PCR is two times faster, less labor intensive, and less expensive (see estimate below) than XLT4-CSP-PCR; however, its sensitivity (of 0.35) is small compared to XLT4-CSP-PCR. Moreover, *Salmonella* strains are not recovered using the TT_PCR test and are not available for further molecular characterization. Decreased positive detection rate of TT-PCR compared to XLT4-CSP-PCR may result from inhibition of PCR by compounds in TT DNA extract. The performance of TT_PCR might be increased by using more refined DNA extraction methods (Klerks et al., 2006) or by using more sensitive PCR assays (e.g., with lower detection limits such as nested PCR) (Klerks et al., 2004). Considering the higher positive detection rates and the availability for further molecular typing for isolated strains, we consider XLT4-CSP-PCR as a better method in this circumstance.

3.2. Comparison of XLT4 and CHROMagar™ Salmonella plus for recovery and confirmation of Salmonella from TT broth

Evaluation of TT broth samples ($n = 2646$) from various water and sediment sources showed differences in performance of XLT4 and CSP as initial isolation agars (Table 3). Using isolation on two selective

Table 2
Confusion matrix for XLT4-CSP-PCR vs. TT-PCR ($n = 1602$ TT tubes) for confirmation of *Salmonella* in TT broth ($n = 1602$ tubes).

	XLT4-CSP-PCR positive	XLT4-CSP-PCR negative
TT_PCR positive	35	10
TT_PCR negative	64	1493

Table 3
Evaluation of MPN confirmation methods based on XLT4-CSP-PCR method as gold standard.

Method[a]	Performance and agreement of detection methods[b]					
	Sensitivity	Specificity	PPV	NPV	Kappa	McNemar's test
TT_PCR[b]	0.35 (0.26, 0.45)	0.99 (0.99, 1.00)	0.78 (0.64, 0.87)	0.96 (0.95, 0.96)	0.47 [0.00]	37.96 [0.00]
XLT4-CSP	1.00 (0.98, 1.00)	0.85 (0.83, 0.86)	0.38 (0.36, 0.40)	1.00 (1.00, 1.00)	0.49 [0.00]	372.00 [0.00]
XLT4	0.95 (0.91, 0.98)	0.93 (0.92, 0.94)	0.52 (0.52, 0.59)	0.99 (0.99, 1.00)	0.66 [0.00]	143.82 [0.00]
CSP	0.80 (0.75, 0.85)	0.90 (0.89, 0.91)	0.44 (0.41, 0.47)	0.98 (0.97, 0.98)	0.51 [0.00]	126.68 [0.00]
XLT4-PCR	0.95 (0.92, 0.98)	1.00 (1.00, 1.00)	0.99 (0.97, 1.00)	1.00 (0.99, 1.00)	0.97 [0.00]	9.09 [0.00]
CSP-PCR	0.80 (0.74, 0.85)	1.00 (1.00, 1.00)	0.99 (0.96, 1.00)	0.98 (0.98, 0.99)	0.88 [0.00]	44.02 [0.00]

PPV = positive predictive value, and NPV = negative predictive value. Values in round brackets indicate 95% confidence intervals for the corresponding parameters and value in [] indicates the p value of the corresponding test.
[a] Methods are described in Section 2.
[b] Performance measures are described in text and use the XLT4-CSP-PCR method as gold standard.

agars followed by PCR confirmation (XLT4-CSP-PCR method) as the gold standard, the performance measures for using both agars without PCR (XLT4-CSP) or for using XLT4 vs. CSP agar alone with (XLT4-PCR, CSP-PCR) or without PCR confirmation (XLT4, CSP) were calculated in terms of their sensitivity, specificity, positive predictive value, and negative predictive value. A good detection method should have high values on all these four performance measures. Although XLT4-CSP, XLT4, and CSP had high sensitivities (ranging from 0.80 to 1.00), high specificities (ranging from 0.85 to 0.93), and high negative predictive values (from 0.98 to 1.00), they were not considered ideal methods because the positive predictive values were all quite low (ranging from 0.38 to 0.52). This result implied that these detection methods have large false discovery rates (ranging from 0.48 to 0.62). Therefore, further PCR confirmation for each presumptive colony was critical, since using any type of agar without PCR confirmation may result in a high number of false positive colonies. While both XLT4-PCR and CSP-PCR methods had a specificity of 1.00 and a positive predictive value of 0.99, the XLT4-PCR method had a significantly higher sensitivity (0.95) compared to CSP-PCR (0.80) and a higher negative predictive value (1.00) than CSP-PCR (0.98). Cohen's kappa coefficients for XLT4-PCR and CSP-PCR compared to XLT4-CSP-PCR were 0.97 and 0.88, respectively, which showed that these tests were in strong agreement with the gold standard. On the other hand, McNemar's test shows that the positive detection rates for XLT4-PCR and CSP-PCR are significantly different from those of the gold standard (XLT4-PCR: $\chi^2 = 9.09$, $P < 0.001$; CSP-PCR: $\chi^2 = 44.02$, $P < 0.001$). Although the end results from using only one selective agar for initial isolation were significantly different from XLT4-CSP-PCR, the values of sensitivity, specificity, positive predictive value, and negative predictive value were all within a good range. Taking into consideration their relative performance, we concluded either XLT4-PCR or CSP-PCR could be used as alternatives to the gold standard.

The efficiency of the agars without assuming that the XLT4-CSP-PCR is the gold standard was also assessed (Table 4). XLT4-CSP-PCR identified 229 positive samples, as confirmed by PCR, while XLT4-PCR and CSP-PCR identified 218 and 183 positive samples, respectively. These results indicated a loss in the positive detection totals when using either XLT4 or CSP as initial streaking agar, instead of using both agars. The values of "predicted positives before PCR confirmation" are the total predicted positive samples of XLT4-CSP, XLT4 and CSP, and the agar efficiency rate (%) is calculated by total predicted positives after PCR/total predicted positives before PCR × 100. This analysis was performed in order to evaluate the agar efficiency; specifically, if a streaking detection method without PCR confirmation had a sufficiently high agar efficiency rate, then that method would be preferable since labor expenses and cost of reagents associated with future individual colony confirmation would be decreased. As seen in Table 4, the agar efficiency rate for XLT4-PCR and CSP-PCR were 55 and 43%, respectively, and was even lower for the XLT4-CSP-PCR method (38%). Therefore, XLT4-PCR had the highest agar efficiency rate, lowest cost (described below), and lowest labor intensity due to fewer false positive results. Also, it was noted that an improved strategy might be to identify presumptive positive colonies on XLT4 plates at 20 rather than 24 hours of incubation, as suggested by the manufacturer's protocol, since an increase in the false positive rate for colonies after 20 hours of incubation was observed (data not shown).

Based on the environmental samples in this study, employing CSP agar for isolation of *Salmonella* from TT broth showed more false positives than applying the XLT4 agar. Although previous research indicated that the CAS agar had better sensitivity and specificity than other *Salmonella* selective agars, such as XLD (Xylose-Lysine-Desoxycholate) and HEA (Hektoen Enteric Agar) (Gaillot et al., 1999; Maddocks et al., 2002), to the best of our knowledge, this is the first paper that compares the performance of the CSP and XLT4 agars. The results presented herein showed that using XLT4 alone was a good a substitution for using the two agars (XLT4 and CSP) in conjunction with PCR because it was less labor intensive, had lower cost and showed a good performance in terms of sensitivity, specificity, positive predictive value, and negative predictive value when compared to the XLT4-CSP-PCR method.

Table 4
Comparison of the efficacy of XLT4-CSP-PCR vs. XLT4-PCR vs. CPS-PCR methods.

Parameters	Efficiency rate for detection methods[a]		
	XLT4-CSP-PCR	XLT4-PCR	CSP-PCR
Total predicted positives (before PCR confirmation)	603	394	418
Total predicted positives (confirmed by PCR)	229	218	183
Total predicted negatives	2417	2628	2463
Agar efficiency rate (%)[b]	38 %	55 %	43 %
Isolation efficiency	Lowest	Highest	Medium
Labor intensity	Highest	Lowest	Medium
Cost	Highest	Lowest	Medium

[a] Detection methods are described in Section 2.
[b] Agar efficiency rate (%) = total predicted positives after PCR/total predicted positives before PCR × 100%.

3.3. Evaluation of a novel cross-streaking method to replace PCR confirmation

Based on evaluation of experimental methods described above, a cross-streaking method was developed and represents a new discovery. Sequential isolation (cross-streaking) of typical colonies from the primary isolation agar (XLT4) to the alternate secondary agar (CSP) was highly predictive for colony identification by PCR. In this study, XLT4

Table 5
Confusion matrix for cross-streaking method vs. PCR for confirmation of individual *Salmonella* isolates (n = 1641).

	PCR positive	PCR negative
Cross-streaking positive	1379	0
Cross-streaking negative	1	261

Table 6
Comparison of cross-streaking method to PCR for confirmation of *Salmonella*.

Method	Performance and agreement of cross-streaking method with PCR results[a]					
	Sensitivity	Specificity	PPV	NPV	Kappa	McNemar's test
Cross-streaking	0.999 (0.996, 1.000)	1.000 (0.986, 1.000)	0.999 (0.994,1.000)	0.991 (0.966, 0.996)	0.998 [0]	0 [1]

Performance measures described in text, values in parenthesis ( ) indicate 95% confidence intervals for the corresponding parameters, and values in brackets [ ] indicate the p value of the corresponding test.
[a] Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), Kappa coefficient and McNemar's test statistic of the cross-streaking method, as compared to PCR ($n = 1641$).

was selected over CSP for primary selection based on relative performance and cost of the agars. Typical colonies that were initially isolated from both agars were assessed for species identification by PCR (Table 5). All isolates that were positive on both agars were also confirmed as *Salmonella* positive by PCR ($n = 1611$), and using PCR as the gold standard, FP = 0. We also examined *Salmonella* isolates from a prior study (Rajabi et al., 2011), and only one isolate from the Suwannee River was found to be PCR positive, CSP positive, but negative on XLT4 i.e., FN = 1. The cross-streaking method was highly performant (with respect to the PCR confirmation), with a sensitivity of 0.99, specificity of 1.00, PPV of 1.00, and NPV of 0.99 (Table 6). The kappa coefficient of agreement between the cross-streaking and PCR assay was 0.99. No bacterial species were found to be false positive using both agars, which explains the high specificity of the cross-streaking assay. Also, cross-streaking was a more cost-effective and time-saving confirmation method compared to PCR or other traditional biochemical tests. However, there are some limitations of this assay as well. First, some *Salmonella* strains are H_2S negative, forming atypical pinkish colonies without a black center on XLT4, and would represent false negative strains using this method. Secondly, it is important to obtain truly isolated colonies so re-streaking to both XLT4 and CPS is recommended. Further studies are required to identify potential false positive and false negative results using this assay.

3.4. Assessment of material cost

The reagent and supply cost per MPN for each protocol (detection method) was calculated in US dollars (Table 7). The costs of reagents include lactose broth, tetrathionate base, iodine solution, XLT4 agar base, tergitol, CSP base, PCR and electrophoresis reagents, while the costs of other supplies include disposable materials, such as wood applicators, 15 ml/50 ml conical tubes, microcentrifuge tubes, pipette tips, and petri dishes. The cost per MPN is calculated based on 9 enrichment culture tubes. The cost of PCR varies depending on how many PCR reactions are needed for each method. Estimated averages of 9 PCR reactions per MPN for TT_PCR and 4 PCR reactions per MPN for all other detection methods were used for calculations. The most expensive test was XLT4-CSP-PCR (18.6 USD/MPN) since it used both selective agars as well as PCR reagents. Savings for the other tests depended on the details of the protocols. For example, the TT_PCR test cost about 13.5 USD/MPN and was approximately 27.4% less expensive than XLT4-CSP-PCR. The reduction in expenses for the TT_PCR test was the result of not using selective medium. The XLT4-PCR and CSP-PCR detection tests cost to 13 and 15.8 USD/MPN and were 30.1 and 17.9% less expensive than XLT4-CSP-PCR, respectively. This decrease in expenses was due to the reduction of one type of initial isolation agar. The cross-streaking method cost 11.74 USD/MPN and was the least expensive assay with a 37% cost decrease compared to the gold standard using two agars and PCR. Additional savings would be realized by eliminating the purchase of a thermocycler to perform the PCR.

Table 7
Assessment of material cost per MPN for different *Salmonella* detection assays.

Detection method[a]	Material Cost (USD) per MPN[b]						
	LB	TT[a]	XLT4	CSP	PCR	Other[c]	Total
XLT4-CSP-PCR	3.15	1.88	2.80	5.60	2.64	2.50	18.60
TT-PCR	3.15	1.88	0	0	5.94	2.50	13.50
XLT4-CSP	3.15	1.88	2.80	5.60	0	2.50	16.00
XLT4-PCR	3.15	1.88	2.80	0	2.64	2.50	13.00
XLT4	3.15	1.88	2.80	0	0	2.50	10.30
CSP-PCR	3.15	1.88	0	5.60	2.64	2.50	15.08
CSP	3.15	1.88	0	5.60	0	2.50	13.10
Cross-streaking	3.15	1.88	2.80	1.40	0	2.5	11.73

[a] Detection methods are described in Section 2 and Table 1.
[b] Materials include lactose broth (LB), tetrathionate broth (TT), XLT4 and CSP agars, and PCR reagents (including electrophoresis reagents) based on 9 reactions/MPN for TT-PCR and 4 reactions for the other assays. Agar costs are based on streaking ½ plates for initial isolation from TT broth and 1/8 plates for restreaking of colonies in the cross-streaking assay.
[c] Other materials include wood applicators, 15 ml/50 ml conical tubes, microcentrifuge tubes, pipette tips and petri dishes.

4. Conclusion

This study demonstrated that the XLT4-PCR method using XLT4 as initial *Salmonella* selective agar followed by PCR for colony confirmation was a cost effective and time saving method to isolate *Salmonella* from irrigation water and sediment samples. The exclusive use of less expensive XLT4 ($24/L) compared to CHROMagar ($55/L) for primary isolation significantly reduces the labor and reagents costs. Although some loss of recovery was seen using only XLT4, simplifying the assay and reducing costs would permit evaluation of more samples and could potentially improve recovery. Also, a novel cross-streaking detection method was proposed as an alternative to PCR confirmation of MPN for situations where PCR may not be available or practical. Further evaluation is required to identify potential false positive and false negative aspects of this method.

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