Journal of Microbiological Methods 101 (2014) 86–92

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

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

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ARTICLE INFO

Article history:
Received 24 March 2014
Accepted 24 March 2014
Available online 13 April 2014

Keywords:
Salmonella
Cross-streaking
XLT4
CHROMagar™ *Salmonella* Plus
Irrigation ponds
PCR

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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http://dx.doi.org/10.1016/j.mimet.2014.03.012
0167-7012/

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

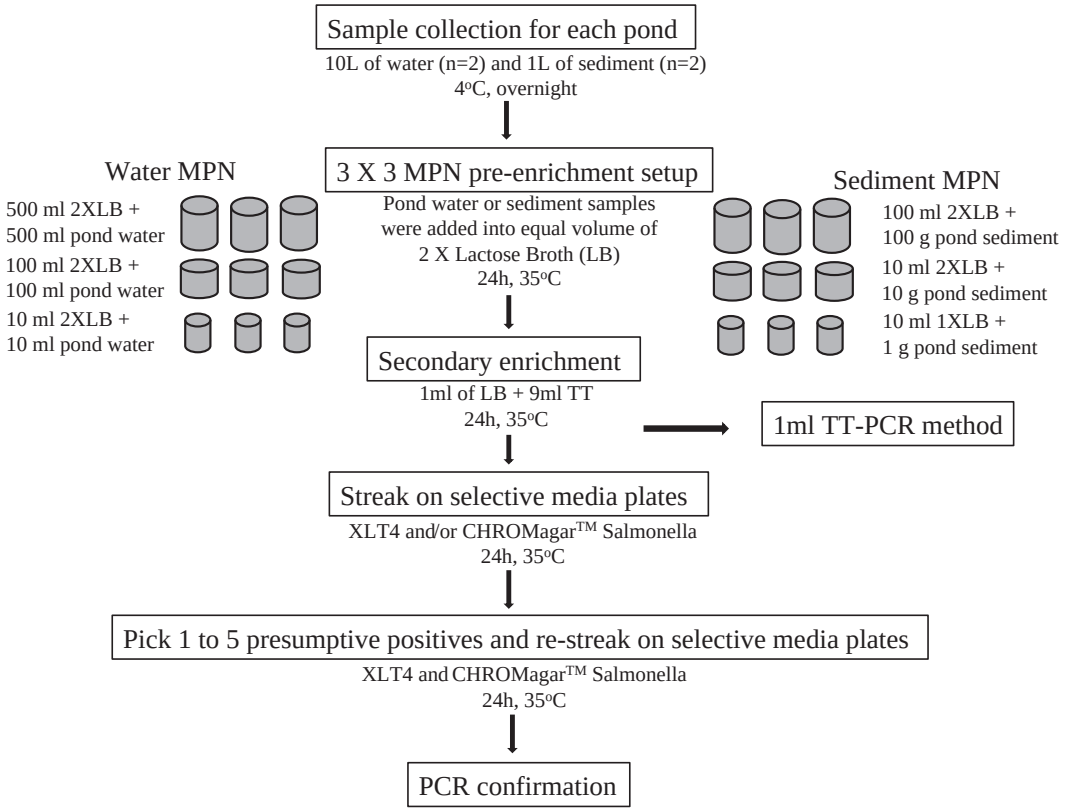

Fig. 1. Flowchart diagram for most probable number (MPN) enumeration, isolation and identification of *Salmonella*. TT = tetrathionate broth; XLT4 = xylose lysine desoxicholate agar with tergitol 4; LB = Lactose Broth.