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Evaluation and comparison of rapid methods for the detection of *Salmonella* in naturally contaminated pine nuts using different pre enrichment media

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ABSTRACT

Foodborne outbreaks, involving pine nuts and peanut butter, illustrate the need to rapidly detect *Salmonella* in low moisture foods. However, the current *Bacteriological Analytical Manual* (BAM) culture method for *Salmonella*, using lactose broth (LB) as a pre enrichment medium, has not reliably supported real-time quantitative PCR (qPCR) assays for certain foods. We evaluated two qPCR assays in LB and four other pre enrichment media: buffered peptone water (BPW), modified BPW (mBPW), Universal Pre enrichment broth (UPB), and BAX® MP media to detect *Salmonella* in naturally-contaminated pine nuts (2011 outbreak). A four-way comparison among culture method, Pathatrix® Auto, VIDAS® Easy SLM, and qPCR was conducted. Automated DNA extraction techniques were compared with manual extraction methods (boiling or InstaGene™). There were no significant differences ($P > 0.05$) among the five pre enrichment media for pine nuts using the culture method. While both qPCR assays produced significantly ($P \leq 0.05$) higher false negatives in 24 h pre enriched LB than in the other four media, they were as sensitive as the culture method in BPW, mBPW, UPB, and BAX media. The VIDAS Easy and qPCR were equivalent; Pathatrix was the least effective method. The Automatic PrepSEQ™ DNA extraction, using 1000 μL of pre enrichment, was as effective as manual extraction methods.

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1. Introduction

Outbreaks of foodborne illness traced to pine nuts and peanut butter illustrate the need for rapid and reliable detection of *Salmonella* contamination in low moisture and high fat foods. During October and November of 2011, a multistate outbreak of human *Salmonella* Enteritidis infections was linked to Turkish pine nuts purchased from bulk bins at a grocery store chain (CDC, 2011). A total of 43 individuals from five states were infected with the outbreak strain of *S.* Enteritidis (CDC, 2011). This was the first reported outbreak of *Salmonella* in pine nuts, although a number of salmonellosis outbreaks have been associated with the consumption of nuts, nut products, and other low-moisture foods including peanuts, peanut butter, raw almonds, pistachios, chocolate, cereals, paprika chips, powdered infant formula, and dry seasonings (CDC, 2004, 2007, 2009, 2011, 2012; Lehmacher et al., 1995; Werber et al., 2005; Jourdan et al., 2008). Notably, contaminated peanut butter was the cause of two large *Salmonella* outbreaks in the US, sickening 1157 people in 47 states in 2006 and 2007 and 529 people in 43 states from 2008 to 2009 (CDC, 2007, 2009).

The infectious dose of *Salmonella* from low-moisture foods is reported to be very low. The *Salmonella* contamination level in an outbreak involving contaminated paprika on potato chips was 0.04–0.05 CFU/g (Lehmacher et al., 1995), and 1.5 MPN/g in an unopened jar of peanut butter associated with the 2006–2007 outbreak (Zink, 2008). In the toasted oat cereal outbreak, the contamination level of *Salmonella* was 0.042–0.427 MPN/g we found. These low infectious doses indicate that *Salmonella* does not need to proliferate in low-moisture foods in order to cause human illness: even a few *Salmonella* cells are sufficient to colonize the

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lower gastrointestinal tract and produce clinical symptoms (Waterman and Small, 1998). Fats in some foods may protect *Salmonella* against the acidic conditions of the stomach, increasing the likelihood of illness despite the low number of viable organisms consumed (D'Aoust, 1977; D'Aoust and Maurer, 2007).

Low contamination levels make the detection of *Salmonella* in low moisture foods difficult and time consuming. Although the BAM *Salmonella* culture method is able to detect 1 CFU/25 g sample, this process requires a minimum of 4 days, and the incubation techniques meant to raise the number or organisms above detection thresholds often promote overgrowth of background microflora. Two rapid detection methods, the VIDAS® Easy SLM (bioMérieux, Hazelwood, MO) (VIDAS Easy) and qPCR assays, have been reported to be effective screening tools for the detection of human pathogens from food samples (Temelli et al., 2011; Jasson et al., 2011; Cheng et al., 2009; Elizaquível et al., 2009).

The VIDAS Easy was recently approved as an AOAC Official Method (2011.03) (Crowley et al., 2011), able to detect 0.2–2 CFU/25 g sample and provide a presumptive result in 2 days (Crowley et al., 2011). It is a more practical candidate for the FDA's high-throughput laboratories than its predecessor, the VIDAS *Salmonella* SLM test kit, because it reduces both the number of subculture steps and the number of plating agars necessary to detect and isolate *Salmonella* from foods.

Techniques based on qPCR could be even more specific and efficient screening tools than the VIDAS assay (Cheng et al., 2009; Elizaquível et al., 2009). Several *invA* gene or *ttr* gene-based qPCR assays for the detection of *Salmonella* in food products or animal feces have been described; however, these have not been tested on low moisture foods (Malorny et al., 2004, 2009; Mäde et al., 2004; Elizaquível et al., 2009; González-Escalona et al., 2009; Tomás et al., 2009; Anderson et al., 2011). The FDA has developed an *invA* gene-based qPCR assay to detect *Salmonella* in foods (Cheng et al., 2008, 2009). It is currently used in the FDA's mobile lab. More recently, a multiplex qPCR assay targeting the *invA* gene and *ttrRSBCA* locus has been developed by FDA to improve the specificity of detecting *Salmonella* in foods products (González-Escalona et al., 2012). However, the limit of detection of qPCR (10^3–10^4 CFU/ml) presents a critical threshold: pre enrichment media must support the growth of *Salmonella* above the limit of detection level despite the presence of large numbers of competitive microflora ($>10^6$ CFU/g).

Currently, the FDA's BAM *Salmonella* culture method uses LB as the pre enrichment medium for pine nuts. Most *Salmonella* cannot utilize lactose, but some background microflora (*Escherichia coli*, *Enterobacter* spp., and *Klebsiella* spp.), possibly present on pine nuts, use lactose to produce acid, causing the pH of the pre enrichment media to fall and thereby suppressing the growth of competitive microflora. Although *Salmonella* is fairly acid tolerant, its growth is also somewhat suppressed under acidic conditions; this is not problematic for detection via culture methods, since selective enrichment and selective/differential plating follow pre enrichment. However, such suppression can be a problem for qPCR methods where pre enrichment in LB alone may not be sufficient to raise the final levels of *Salmonella* above the 10^3 CFU/mL limit of detection for PCR assays.

One option for eliminating the 24 h selective enrichment procedure used in the culture method is the Pathatrix® Auto System (Life Technologies, Grand Island, NY), a flow-through immunocapture (FTI) device that uses immuno-magnetic separation (IMS) technology. In this system, antibody-coated paramagnetic particles selectively bind to and concentrate a target organism from its food matrix. Pathatrix has been used to efficiently isolate *Salmonella*, *E. coli* O157:H7, and *Shigella* from artificially-inoculated food samples (Warren et al., 2007; Fedio et al., 2011; Lau et al., 2012; Chen et al., 2014; Wall et al., 2014). This method has been certified by AOAC's Research Institute for *Salmonella* isolation (Certification number 090203B). Whether it can rapidly and reliably detect *Salmonella* in naturally-contaminated food samples has not been established.

To help determine which methods might provide rapid, sensitive, and reliable screenings or alternatives to the BAM culture method for isolating and detecting *Salmonella* contamination, this study compared the relative efficacies of five pre enrichment media (LB, BPW, mBPW, UPB, and BAX® MP media (Dupont, Wilmington, DE)) by evaluating the performance of qPCR, VIDAS Easy, and Pathatrix for the detection of *Salmonella* from naturally-contaminated pine nuts. Since automated systems, in combination with qPCR methods, are expected to support the FDA's high-throughput regulatory laboratories, we also compared the performance of two automated, magnetic-bead-based, DNA extraction systems: PrepSEQ™ nucleic acid extraction kit (Life Technologies, Grand Island, NY) and BioSprint 96 One-For-All Vet Kit (Qiagen, Germantown, MD) to two manual DNA extraction methods: boiling and InstaGene™ (Bio-Rad, Hercules, CA).

2. Materials and methods

2.1. Sample source

Naturally-contaminated Turkish pine nuts from the November 2011 outbreak were obtained from a state laboratory.

2.2. Evaluation of qPCR assays in five different pre enrichment media

2.2.1. Sample preparation

Twenty-five gram pine nuts per sample were aseptically transferred into individual, sterile Whirl-Pak™ filter bags (Fisher Scientific, Pittsburgh, PA) and soaked in 225 mL portions of LB, BPW, mBPW, UPB, and BAX broths. All pre enrichment mixtures were incubated for 24 ± 2 h at 35 ± 2 °C. The bags were folded over to form a secure, but not air-tight, closure during incubation. A total of 20 replicates for each pre enrichment medium were used for the detection of *Salmonella*.

2.2.2. Determination of contamination level

A 3-tube most probable number (MPN: 100, 10, 1.0, and 0.1 g) analysis was performed to determine the contamination level of pine nuts before pre enrichment. For each of three 100 g and 10 g levels, 100 g and 10 g pine nuts were added to 900 mL and 90 mL, respectively, of LB, BPW, mBPW, UPB and BAX broths in sterile Whirl-Pak™ filter bags. For each of three 1.0 g and 0.1 g levels, 1.0 g and 0.1 g of pine nuts were added in 9.0 mL and 0.9 mL, respectively, of LB, BPW, mBPW, UPB and BAX broths in sterile 16×150 mL snap cap test tubes. The pH values of LB were measured and adjusted, if necessary, to 6.8 ± 0.2 with 1 N NaOH, as described in the BAM *Salmonella* culture method (FDA, 2014). These samples were not blended. The MPN pre enrichments were incubated for 24 ± 2 h at 35 ± 2 °C.

2.2.3. Isolation of Salmonella from test portions

After sample preparation and pre enrichment, the BAM culture method was followed FDA, 2014). Pine nuts were treated as a low microbial load food (Total aerobic plate count found at 1.8×10^4 CFU/g). Aliquots of 1.0 and 0.1 mL from the incubated pre-enrichments were subcultured to 10 mL tetrathionate (TT) broth (BD) and to 10 mL Rappaport-Vassiliadis (RV) medium, respectively. TT broth was incubated for 24 ± 2 h at 35 ± 2 °C, and RV medium was incubated for 24 ± 2 h at 42 ± 0.2 °C. Each incubated selective enrichment broth was streaked to bismuth sulfite (BS; BD),

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