

PCR detection of *Salmonella* spp. in Fresh Vegetables and Feed

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Abstract

Salmonella have been some of the most prevalent reported etiological agents in fresh-produce-associated outbreaks of both human and animal infections. The current study aims to develop a suitable rapid and simple PCR-based protocol for routine analysis of *Salmonella* spp. in vegetables and forages. Fifty-two (32 vegetable and 20 forage) samples collected from different farms in Zarqa, Jordan, where recycled wastewater from Khirbet Al-Samra Wastewater Treatment Plant serves as the source of irrigation water were examined by standard microbiological techniques (SMT). The PCR assay for the detection of *Salmonella* species in the collected field samples revealed the same positive samples directly from the vegetables and forages which gave positive results by SMT. Thus PCR technique is rapid, time saving and applicable to detect *Salmonella* spp. directly from vegetable and forage samples. The combination of a routine PCR test in conjunction with SMT could be effective in providing a more accurate profile of the prevalence of *Salmonella* in fresh vegetable and forage related samples.

Keywords: Forage, *nviA*, Polymerase chain reaction, *Salmonella* spp., Vegetable

1. Introduction

Salmonellae have been some of the most ever-present reported etiological agents in fresh-produce-associated outbreaks of both human and animal infections in recent years. Different species of the bacterial genus *Salmonella* cause an astounding variety of diseases in both human and animal hosts. These range from asymptomatic persistence through gastric infections to potentially fatal systemic disease such as typhoid fever, with symptoms include sudden fever, headache, diarrhea, stomach cramps, nausea and sometimes vomiting. Symptoms can occur from 6 to 72 hours after becoming infected. Some species of this bacterium can infect a wide variety of hosts, while others are very specific to the host (Keusch, 2002). *Salmonella* has been linked with consumption of contaminated foods of animal origin, meat, eggs, poultry as well as dairy products. Constant variations in dietary habits and agronomic practices, the increase in the consumption of vegetables that are consumed raw along with increased importation of fresh produce supposedly contribute to increase in the number of outbreaks associated with vegetables and animal feed of vegetable origin (Altekruse, Cohen, & Swerdlow, 1997). *Salmonella* outbreaks have been linked to tomatoes (Centers for Disease Control and Prevention, 1993), seed sprouts (Mahon et al., 1997; O'Mahony et al., 1990), watermelons (Blostein, 1991; Centers for Disease Control and Prevention, 1979; Centers for Disease Control and Prevention, 1979), and animal feeds (Gooding & Choudary, 1999; Rexach, Dilasser, & Fach, 1994). Disposal of treated wastewater for agricultural irrigation simultaneously solves water shortage problems and reduces potential environmental contamination. In Jordan, it is not allowed to irrigate vegetables with treated wastewater. According to the Jordanian standards (893/2006), it could be used for irrigating forages, industrial crops, cut flowers and for Golf courses and we should apply some management practices according to the standards. However, subject to distribution of waterborne disease, associated with treated wastewater reuse, a risk assessment of pathogen hazards, subject to diverse wastewater qualities utilization, as well as different irrigation technologies, is require. Consumption of fresh vegetables and animal feed of vegetable origin was epidemiologically linked to many cases of *Salmonella* infection. Contamination of fresh produce with *Salmonella* may occur at any point along the farm-to-table continuum, and *Salmonella* probably occur

intermittently at low levels together with the diverse natural flora. The established culture-based methods used to detect *Salmonella* in vegetable and animal feed of vegetable origin are laborious, time-consuming, and often not specific enough. The standard methods used today for analyzing *Salmonella* involve pre-enrichment in buffered peptone water (BPW), selective enrichment, plating on selective agar, and subsequent identification by biochemical tests (Bennett et al., 1998). The whole procedure takes at least mostly seven days to complete. Thus, rapid and sensitive methods for detecting *Salmonella* are in great demand in order to assure produce safety. One of the most promising methods for detecting *Salmonella* is based on the PCR, which combines simplicity with specificity and sensitivity for detecting the pathogens in food and feed. Several PCR assays have been developed by targeting various *Salmonella* genes, such as *inv-A* (Rahnet et al., 1992; Wang, Blais, & Yamazaki, 1995), 16S rRNA (Iida et al., 1993), *agf-A* (Doran et al., 1993), and *via-B* (Hashimoto et al., 1995), and virulence-associated plasmids (Mahon & Lax, 1993; Rexach, Dilasser, & Fach, 1994). These PCR assays are used mainly for detecting *Salmonella* in poultry, meat and milk samples (Bennett et al., 1998; Bennett et al., 1998; Chen et al., 1997; Kimura et al., 1999). Few of the assays have been used to detect the pathogens in fresh product. Therefore, the aim of this study was to develop a rapid and simple PCR-based protocol suitable for routine analysis of *Salmonella* spp. in vegetables and animal feed of vegetable origin.

2. Materials and Methods

2.1 Samples collection

In the summer of 2016, fifty-two (32 vegetable and 20 forage) samples were collected from different farms in Zarqa, Jordan, where the source of irrigation water was recycled wastewater from Khirbet Al-Samra Wastewater Treatment Plant. The entire samples (Alfaalfa and white corn as Forage type and Cauliflower, Tomato and Egg plant: as vegetables) were placed under refrigerated conditions and transported to the laboratory for prompt processing and bacteriological examination.

2.2 SMT for *Salmonella* identification and detection

SMTs for various *Salmonella* spp. detection were carried out in accordance with ISO 6579-1: 2017; Briefly, 25 g of vegetable and forage samples were subjected to homogenization in a stomacher for 1 - 2 min in 225 ml of buffered peptone water (BPW) and thereafter, under aerobic conditions (T 37°C for 20 to 24 h) were incubated by selective enrichment of 0.1 in 10 ml of Rappaport-Vassiliadis (RV) broth. At 42°C for 18 to 24 h, the RV broth was incubated, subcultured onto Xylose Lysine Desoxycholate (XLD) agar, Hektoen Enteric agar and Salmonella Chromogenic Agar and then re-incubated at 37°C for 18 to 24 h. All media were provided by Oxoid, (Basingstoke, UK). Hypothetical positive colonies (non-lactose fermentative with appropriate colony morphology) were biochemically, morphologically and serologically identified through slide agglutination test with polyvalent and monovalent somatic (O), virulent (Vi) as well as tube agglutination test for flageller (H) antigens (Defco Laboratories, Detroit, Michigan, USA) in parallel with positive control ATCC 14028s. 1 ml of BPW already incubated at 37°C was set aside for the PCR-Non Selective test (PCR-NS) and 1 ml of the 37°C RV broth for the PCR-RV test.

2.3 DNA extraction

The cultures (Buffered Peptone Water and Rappaport-Vassiliadis) that stayed overnight were subjected to centrifugation at 5000 rpm for 3 min and the supernatant were carefully decanted. The bacteria pellets were thrice washed using phosphate buffer saline pH 7.2, which was resuspended in 400 µL tris-EDTA buffer (pH 8.0) and heated at 100°C in water bath for 30 min. Thereafter, it was allowed to cool at room temperature and underwent centrifugation for 10 min at 14,000 rpm. 5 µL aliquot of the supernatant was used as model DNA in the PCR.

2.4 PCR primers, DNA amplification and detection

A 26-bp forward primer (5'GTG AAA TTA TCG CCA CGT TCG GGC AA3') and a 22-bp reverse primer (5'TCA TCG CAC CGT CAA AGG AAC C3') (Moussa et al., 2010), targeting the *inv-A* *Salmonella* spp. gene, were utilized in PCR to achieve a 284-bp product. Amplification was conducted in 25 µl total volume containing 1.0 µM each primer, 0.2 mM each dNTP, 1.5 mM MgCl₂, 0.5 U *Taq* DNA polymerase, 1 X PCR buffer and 5 µl template. A negative control comprising similar reaction mixture but without the DNA template was added in all of the experiment. And a positive control (ATCC 14028s) is used.

An initial denaturation for 3 min at 95°C was accompanied by 35 denaturation cycles at 95°C for 10 s, annealing at 64°C for 15 s and extension at 72°C for 5 s. Finally, an additional extension was achieved for 10 min at 72°C. An aliquot of 10-µl of each of the PCR product underwent electrophoresis on a 1.0% agarose gel for 1.0 h at 100

V, stained in ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) for 10 min, and then visualized and photographed under ultra-violet (UV) illumination.

3. Results

The standard microbiological techniques revealed positive isolation of only one *Salmonella* spp. (1.9%) out of 52 examined vegetable and forage samples (Table 1).

The specificity of the oligonucleotid primers as well as typing of the recovered *Salmonella* spp. from SMT were carried out by testing of all the recovered *Salmonella* spp. in addition to the standard positive and standard negative strains with PCR, using a primer pairs targeting for *inv-A* gene. All *Salmonella* spp. were positive for amplification of 284 bp fragments of *inv-A* gene and all non *Salmonella* spp. were negative (Figure 1) Lane 1 is the positive control ,Lane 2 showing positive amplification of 284 bp fragments of *Salmonella* species, while lanes 3-20 showing no amplification. All the examined field samples with SMT as well as the negative control samples were tested by PCR using the same primer pairs after selective enrichment on RV broth. All bacteriologically positive samples (100%) were positive by PCR and amplification of 284 fragments specific for *inv-A* gene was observed. The negative control samples were negative for the PCR assay and no amplification could be detected with the four primer pairs.

Table 1. Results of standard microbiological techniques

Name of samples	Types of samples	Number of samples	Standard microbiological techniques	
			Total positive	%
Alfaalfa	Forage	14	0	0
Cauliflower	Vegetable	9	1	1.9
White corn	Forage	14	0	0
Tomato	Vegetable	10	0	0
Egg plant	Vegetable	5	0	0



Figure 1. Agarose gel electrophoresis showing amplification of *invA* genes

4. Discussion

Vegetables and forages are one of *Salmonella* reservoirs that can be transmitted to humans and animals through the food-chain. The detection of *Salmonella* species by regulatory agencies is still primarily based on standard microbiological techniques which may take up to 7 days to confirm the results (Stoneet al., 1994). The earlier a foodborne outbreak is alleged, the faster the source of the pathogen can be identified, and the sooner the public can recover confidence in the food supply (Stoneet al., 1994). The current study was aimed to investigate the incidence of different *Salmonella* spp. in fresh vegetables and forages from local source, therefore, 52 samples

collected from farms were examined by SMT. The results observed in Table 1 revealed a low incidence of *Salmonella* spp. isolation among both fresh vegetables and forages (1.9%).

These results indicated the health hazard of fresh vegetables and forages as a inconsequential source of *Salmonella* foodborne pathogens (Altekruse, Stern, & Swerdlow, 1999; Humphrey, 2002; Schlundt, 2002). The SMT used in these study reported by ISO 6579 (2002) was characterized by very good analytical parameters which allow the detection of low numbers of potentially stressed cells of various *Salmonella* spp. through the use of pre-enrichment in BPW followed by selective enrichment in RV broth and finally plated into three different *Salmonella* selective agars.

Traditional methods of identification of pathogens, which cause disease in humans, are time consuming and laborious although control of the infection depends increasingly on the availability of rapid and precise diagnostic tests for monitoring. Therefore, the present study was aimed to investigate the sensitivity of PCR protocol in conjunction with selective enrichment in Rappaport Vassiliadis broth and compared with standard microbiological techniques. In the present study, the PCR produced positive amplification of 284 bp fragments of *invA* gene (1.9%), specific for all members of *Salmonella* species (Figures 1). This result was parallel to those obtained by Oliveira et al. (2002) and Malorny et al. (2003), who reported that the primer, which target the *invA* gene, which is not carried by any other bacterial species (Lin et al., 2007), was able to identify all the examined *Salmonella species*, whereas all non *Salmonella species* gave negative results. Moreover, PCR has several advantages over the slide agglutination test with polyvalent antisera, because serogrouping is not possible when *Salmonella* isolates lack O- antigen (rough strain) or lack both O and H antigen (Lin et al., 2007).

In conclusion, the PCR assay obviously proved to be a highly specific, sensitive and time saving method for detecting *Salmonella*. *The combination of a routine PCR test in conjunction with SMT could be effective in providing a more accurate profile of the prevalence of Salmonella in fresh vegetable and forage related samples.*

5. Conclusion

The PCR assay obviously proved to be a highly specific, time saving and sensitive technique for *Salmonella* detection. *The combination of a routine PCR test along with SMT can effectively provide a more correct profile of Salmonella prevalence in fresh vegetable and forage related samples.*

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