International Journal of Research in Pharmacy and Pharmaceutical Sciences
ISSN: 2455-698X; Impact Factor: RJIF 5.22
Received: 21-01-2019; Accepted: 23-02-2019
www.pharmacyjournal.in
Volume 4; Issue 2; March 2019; Page No. 105-108

Inv-A gene specific PCR for detection of *Salmonella spp.* in fresh vegetables and forages

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Abstract
Salmonella has been one of the most prevalent reported etiological agents in fresh-produce-associated outbreaks of both human and animal infections. The principal objective of this research study was to develop a suitable rapid and simple PCR-based protocol for routine analysis of *Salmonella spp.* in fresh vegetables and forages. Fifty-two (24 vegetables and 28 forages) samples collected from different farms in Zarqa, Jordan, where recycled wastewater from Khirbet Al-Samra Wastewater Treatment Plant serves as a source of irrigation water were examined by standard microbiological techniques (SMTs). The same samples were analyzed directly by PCR. The results for both methods (SMTs and PCR) were the same for *Salmonella spp.* The results proved that the PCR technique was applicable to detect *Salmonella spp.* in fresh vegetable and forage samples in less time and with high accuracy. The combination of both methods (PCR & SMTs) can give a more effective and more accurate profile of the prevalence of *Salmonella spp.* in fresh vegetable and forage related samples.

Keywords: forage, inv-A gene, polymerase chain reaction (PCR), *Salmonella spp.*, vegetable

1. Introduction

Salmonella has been one of the most ever-present reported causing agents in fresh-products in both human and animal infections spreading in recent years [7]. Different species of *Salmonella* cause an astounding variety of diseases in both hosts (human and animal). These diseases differ from gastric persistence infections (asymptomatic) to systemic diseases such as typhoid fever (with symptoms that need 6-72 hrs. after infection) including headache, sudden fever, diarrhea, stomach cramps, nausea, and sometimes vomiting. Some species of this bacteria can infect a wide variety of hosts, while others are particularly for a certain host [16]. Most presidents of *Salmonella* are in contaminated foods of animal origin such as meat, eggs, poultry, and dairy products [23]. Constant variations in dietary habits and agronomic practices and the increase in the consumption of vegetables that are consumed raw along with increased importation of fresh produce contribute to increase in the number of outbreaks associated with vegetables and animal feed of vegetable origin [1]. *Salmonella* outbreaks have been linked to tomatoes, seed sprouts [20, 25], watermelons [6, 8, 12], and animal feeds [11, 27]. 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 golf courses and we should apply some management practices according to the standards and considering that uses of treated wastewater for irrigation solve water shortage as well as environmental contamination. Consumption of fresh vegetables and animal feed of vegetable origin was conjugated by many infections caused by *Salmonella spp.* Contamination of fresh products by *Salmonella spp.* could be occurred in anytime along transferring them from farm-to-consumer. The traditional methods (depend on culture media) that used for *Salmonella* detection in vegetables and animal feed of vegetable origin are complicated, time and money-consuming, and also not specific enough. The standard methods that used recently for *Salmonella* detection need pre-enrichment of the sample in buffered peptone water (BPW), which is selective enrichment, followed by inoculation on selective agar base, looking for suspected colonies, subsequent identification and confirmation by biochemical tests [4]. The previous procedure takes many days to complete (could reach 6-7 days). In this case, there is an urgent need for faster, safer, and more sensitive methods to use for *Salmonella* detection. The PCR method is one of the most promising techniques, which combines safety, specificity, simplicity, time-saving, and sensitivity for detecting the pathogens in food and feed. Several PCR assay methods have been developed by targeting various *Salmonella* genes, such as *inv-A* [26, 30], 16S rRNA [18], *agf-A* [10], *via-B* [13], and virulence-associated plasmids [21, 27]. These PCR assays are used mainly for detecting *Salmonella* in milk, meat, and poultry samples [3, 4, 9, 17] while they are rare for pathogens in fresh product detection. Therefore, this research study was aimed to develop a simple and rapid PCR-based technique that is 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 2018, fifty-two (24 vegetables and 28 forages) 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 (Alfalfa and white corn as forages and cauliflower, tomato, and eggplant as vegetables) were placed under refrigerated conditions and transported to the laboratory for prompt processing and bacteriological examination.

2.2. SMTs 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 mins in 225 ml of buffered peptone water (BPW) and thereafter, under aerobic conditions (T 37°C for 20-24 hrs.) were incubated by selective enrichment of 0.1 in 10 ml of Rappaport-Vassilliadis (RV) broth. At 42°C for 18-24 hrs., 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 18-24 hrs. 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 (PCR-NS) test and 1 ml of the 37°C RV broth for the PCR-RV test.

2.3. DNA extraction

The cultures (BPW and RV) that stayed overnight were subjected to centrifugation at 5000 rpm for 3 mins, and the supernatant was 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 a water bath for 30 mins. After that, it was allowed to cool at room temperature and underwent centrifugation for 10 mins at 14,000 rpm. 5 µL aliquot of the supernatant was used as a 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'), 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 $MgCl_2$, 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 mins 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 mins 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 µg ml^{-1}) for 10 mins, and then visualized and photographed under ultra-violet (UV) illumination.

3. Results

The standard microbiological techniques showed only one positive isolate of *Salmonella spp.* (1.9%) out of 52 examined vegetable and forage samples (Table 1). The specificity of the oligonucleotide primers as well as typing of the recovered *Salmonella spp.* from SMTs were carried out by testing of all the recovered *Salmonella spp.* in addition to the standard positive and standard negative strains with PCR, using primer pairs targeting for the inv-A gene. All *Salmonella spp.* were positive for amplification of 284-bp fragments of the 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 spp.*, while lanes 3-20 showing no amplification. All the tested samples with standard microbiological techniques as well as the positive and negative control samples were tested by PCR using the same primer pairs after selective enrichment in RV broth. All bacteriologically positive samples (100%) were positive by PCR and amplification of 284-bp fragments specific for the inv-A gene was observed. The negative control samples were negative for the PCR assay, and there is no amplification detected with the four primer pairs.

Table 1: Results of standard microbiological techniques (SMTs).

Sample name	Sample type	Sample number	Standard microbiological techniques	
			Positive sample	%
Alfalfa	Forage	14	0	0
Cauliflower	Vegetable	9	1	1.9
White corn	Forage	14	0	0
Tomato	Vegetable	10	0	0
Eggplant	Vegetable	5	0	0

Fig 1: Gel electrophoresis: Amplification of inv-A genes. (Lane 1 is the positive control, Lane 2 showing positive amplification of 284-bp fragments).

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 spp.* by regulatory agencies is still primarily based on standard microbiological techniques which may take up to 7 days to confirm the results [29]. The earlier the 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 [5]. The current study was done to investigate the incidence of different *Salmonella spp*. in fresh vegetables and forages from a local source; therefore, 52 samples collected from farms were examined by SMTs. 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 an inconsequential source of *Salmonella* foodborne pathogens [2, 15, 28]. The SMTs used in this study reported by ISO 6579-1:2017 was characterized by excellent 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 diseases in humans, are difficult and time-consuming, 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 RV broth and compare with the standard microbiological techniques. In the present study, the PCR produced a positive amplification of 284-bp fragments of the inv-A gene, specific for all members of *Salmonella spp.* (Figure 1). This result was parallel to those obtained by Oliveira *et al.*, 2002 [24] and Malorny *et al.*, 2003 [22], who reported that the primers, which target the inv-A gene, which is not carried by any other bacterial species [19], was able to identify all the examined *Salmonella spp.,* whereas all non-*Salmonella spp.* 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 antigens [14].

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

5. Conclusion

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

6. Conflicts of Interest

We hereby declare that there are no conflicts of interest regarding the publication of this research paper.

7. Acknowledgement

We convey many thanks and appreciation to the editors, and we would also like to express our gratitude to the entire staff at the Medical Sciences Laboratory, Faculty of Science, The University of Jordan, Amman, Jordan, who have been so helpful and cooperative in giving their support at all times to help us achieve our goal.

8. References

1. Altekruse SF, Cohen ML, Swerdlow DL. Emerging foodborne disease. Emerg. Infect. Dis. 1997; 3:285-293.
2. Altekruse SF, Stern NJ, Swerdlow DL. Compylobacter jejuni- An emerging foodborne pathogen. Emerging Infectious Diseases. 1999; 1:28-35.
3. Bailey JS. Detection of Salmonella cells within 24–26 hrs in poultry samples with the PCR BAX system. J. Food Prot. 1998; 61:792-795.
4. Bennett AR, Greenwood D, Tennant C, Banks JG, Betts RP. Rapid and definitive detection of Salmonella in foods by PCR. Lett. Appl. Microbiol. 1998; 26:437-441.
5. Bhagwat AA, Lauer W. Food borne outbreaks in raw produce can be prevented. Food quality. 2004; 11:62-63.
6. Blostein J. An outbreak of Salmonella javiana associated with consumption of watermelon. J. Environ. Health. 1991; 56:29-31.
7. Centers for Disease Control and Prevention. Multi-state outbreak of *Salmonella* serotype Montivideo infections. Centers for Disease Control and Prevention, Atlanta, Ga, Publication EPI-AID, 1993, 93-97.
8. Centers for Disease Control and Prevention. *Salmonella oranienburg* gastroenteritis associated with consumption of precut watermelons, Illinois. Morb. Mortal. Wkly Rep. 1979; 28:522-523.
9. Chen S, Yee A, Griffiths M, Larkin C, Yamashiro CT, Behari R, *et al.* The evaluation of a fluorogenic polymerase chain reaction assay for the detection of Salmonella species in food commodities. Int. J. Food Microbiol. 1997; 35:239-250.
10. Doran JL, Collinson SK, Burian J, Sarlos G, Todd ECD, Munro CK, *et al.* DNA-based diagnostic test for Salmonella species targeting agfA, the structural gene for thin aggregative fimbriae. J. Clin. Microbiol. 1993; 31:2263-2273.
11. Gooding CM, Choudary PV. Comparison of different primers for rapid detection of Salmonella using the polymerase chain reaction. *Mol. Cell. Probes.* 1999; 13:34-347.
12. Gayler GE, MacCready RA, Reardon JP, McKernan BF. An outbreak of salmonellosis traced to watermelon. *Public Health Rep.* 1955; 70:311-313.
13. Hashimoto Y, Itho Y, Fujinaga Y, Khan AQ, Sultana F, Miyake M, *et al.* Development of nested PCR based on the Via B sequence to detect Salmonella typhi. J. Clin. Microbiol. 1995; 33:775-777.
14. Hoorfar J, Baggesen DL, Porting PH. A PCR-based strategy for simple and rapid identification of rough presumptive Salmonella isolates. J. Microb. Method. 1999; 35:77-84.
15. Humphrey TJ. Contamination of eggs shell and contents with Salmonella entrica serovar Enteritides. In: Salmonella entrica serovar Enteritides in human and animals. Epidemiology, Pathogenesis, and Control. Iowa State University Press, Ames, IA, 2002, 183-192.
16. Keusch GT. Systemic gastro-intestinal infections: a clinical overview. In: Sussman, M. Ed., *Molecular Medical Microbiology*, San Diego, Academic Press, 2002, 1357-1363.
17. Kimura B, Kawasaki S, Fujii T, Kusunoki J, Itoh T, Flood SJA. Evaluation of TaqMan PCR assay for detecting Salmonella in raw meat and shrimp. J. Food Prot. 1999; 62:329-335.
18. Lida K, Abe A, Matsui H, Danbara H, Wakayama S, Kawahara K. Rapid and sensitive method for detection of Salmonella strains using a combination of polymerase chain reaction and reverse dot-blot hybridization. FEMS Microbiol. Lett. 1993; 114:167-172.
19. Lin CL, Chiu CH, Chu C, Huang YC, Lin TY, Ou JT. A multiplex chain reaction method for rapid identification of Citrobacter freundii and Salmonella species, including Salmonella Typhi. J. Microbiol. Immunol. Infect. 2007; 40:222-226.

20. Mahon BE, Pönkä A, Hall W, Komatsu K, Beuchat L, Dietrich S, Siitonen A, Cage G, *et al*. An international outbreak of Salmonella infections caused by alfalfa sprouts grown from contaminated seed. J. Infect. Dis. 1997; 175:876-882.
21. Mahon J, Lax A. A quantitative polymerase chain reaction method for the detection in avian faeces of salmonellas carrying the spvR gene. Epidemiol. Infect. 1993; 111:455-464.
22. Malorny B, Hoorfar J, Hugas M, Heuvelink A, Fach P, EllerbroekBunge C, *et al*. Interlaboratory diagnostic accuracy of a Salmonella specific PCR-based method. Int. J. Food Microbiol. 2003; 89:241-249.
23. Moussa IM, Gassem MA, Al Doss AA, Sadik MWA, Abdel Mawgood AL. Using molecular techniques for rapid detection of Salmonella serovars in frozen chicken and chicken products collected from Riyadh, Saudi Arabia. African Journal of Biotechnology. 2010; 9(5):612-619.
24. Oliveira SD, Santos LR, Schuch DMT, Silva AB, Salle CTP, Canal CW. Detection and identification of Salmonellas from poultry by PCR. Vet. Microbiol. 2002; 87:25-35.
25. O'Mahony M, Cowden J, Smyth B, Lynch D, Hall, M, Rowe B, Teare EL, *et al*. An outbreak of Salmonella saint-paul infection associated with bean sprouts. *Epidemiol. Infect.* 1990; 104:229-235.
26. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galan JE, Ginocchio C, *et al*. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes*. 1992; 6:271-279.
27. Rexach L, Dilasser F, Fach P. Polymerase chain reaction for Salmonella virulence-associated plasmid genes detection: a new tool in Salmonella epidemiology. *Epidemiol. Infect*. 1994; 112:33-43.
28. Schlundt J. New directions in foodborne disease prevention. Int. J. Food Microbiol. 2002; 78:3-17.
29. Stone GG, Oberst RD, Hays MP, McVey S, Chengappa MM. Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. J Clin. Microbiol. 1994; 32:1742-1749.
30. Wang H, Blais BW, Yamazaki H. Rapid confirmation of polymyxin-cloth enzyme immunoassay for group D salmonellae including *Salmonella enteritidis* in eggs by polymerase chain reaction. Food Control. 1995; 6:205-209.