Antibiotic Susceptibility of *Salmonella* spp Isolated from Fresh Leafy Vegetables Samples by Using Culture and Polymerase Chain Reaction Methods

S. E. Haramain, S. O. Yagoub, and A. A. Osman

ABSTRACT

Background: Microbial contamination continues to be one of the leading risks to food safety. Contaminated leafy green vegetables are the primary cause of infection among children, elderly, and immunocompromised people. The purposes of this work were to isolate and identify of *Salmonella* spp. in fresh leafy vegetables collected from Jeddah Central Market, Jeddah district, western area, kingdom of Saudi Arabia, estimated of the number and percentage of isolated *Salmonella* spp and determined the antimicrobial susceptibility of the isolated *Salmonella* spp.

Methods: Five-hundred samples were examined for the presence of *Salmonella* spp, by using standard microbiological and biochemical tests. Further, detection of *Salmonella* spp. was done by PCR with the primers targeting *invA* gene, a key factor for entry of *Salmonella* into epithelial cells. Susceptibility of the isolated *Salmonella* spp was done toward thirteen different antibiotics.

Results: The percentage of isolation of *Salmonella* spp was 1.2 % (06/500). It was isolated as (0.40%, 02/500) from Basil, (0.20%, 01/500) from Spinach, Rocket, Parsley and Chards. Two isolates (2/6, 33.3%) showed positive *Salmonella* *invA* gene (244 bp). All isolated *Salmonella* showed resistance to Cephalexin (30 µg/disc), Metronidazole (5 µg/disc) and Methicillin (5 µg/disc).

Keywords: *Salmonella* spp, Food borne pathogens, Antimicrobial susceptibility, *Salmonella* *invA* gene.

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I. INTRODUCTION

The necessity for fresh fruits and vegetables caused an expansion of the market share for minimally processed vegetables. Foodborne diseases are causing by nearly 250 pathogens including bacteria, viruses, and parasitic organisms [1]. *Salmonella* spp. are the most frequently reported cause of foodborne illness in both humans and animals [2]. Bacteria other than *Salmonella* spp. were reported such as *Staphylococcus aureus*, *Clostridium perfringens*, *Campylobacter* spp. *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Bacillus cereus*, and Enteropathogenic *Escherichia coli* which with *Salmonella* spp. are causing more than 90 % of food poisoning [3]. There has been an expansion in the consumption of leafy green vegetables (LGV) during the past years due to public awareness of the welfare of healthy nutrition, however, latest outbreaks of salmonellosis which had been proved to be due to consumption of contaminated LGV raised a public alarm regarding the safety of freshly consumed leafy green vegetables (FCLGV) [4]. As mentioned by [4] *Salmonella* is one of the most common causes of foodborne diarrheic disease outbreaks in Latin America and other regions of the world. As reported by [5] there are four *Salmonella* infections: *Salmonella enterica* serovar *typhimurium* cause gastroenteritis; *Salmonella typhimurium* and *Salmonella enteritidis* cause

bacteremia, osteomyelitis and reactive arthritis, while *Salmonella typhi* and *Salmonella paratyphi* cause enteric fever. As reported by [6] the incidence of typhoid fever caused by *Salmonella typhi* continues to increase with the incidence of 350-810 cases per 100,000 populations with a mortality rate of 2%. Most of human infections are derived from the use of contaminated foods, especially those of animal origin [7].

Salmonella species are becoming multidrug resistant to antimicrobials, thus making it difficult to treat. In the last two decades, there has been an increasing concern in the world for the emergence of multidrug resistant phenotypes among *Salmonella* serovar, in particular, *Salmonella enterica* serovar *Typhimurium* [8] and *S. enterica* serovar *Newport* [9]. *Salmonella typhimurium* DT104 was found to be resistant to at least five antibiotics included ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline, these resisted *Salmonella* spp caused severe infections and deaths in animals and humans globally [8]. Otherwise, controlling *Salmonella* infection became challenging due to its high tolerance to environmental stresses, widespread distribution, multiple drug resistance, and adaptability [10]. Excessive and improper uses of antibiotics are the main factor attributed to increasing of antibiotic resistant bacteria. The same authors added that the antibiotic resistant bacteria will survive and continue to

multiply through several mechanisms which allow them to survive antibiotic treatments. Traditional microbiological culture plating techniques for determining the pathogens are not sensitive and time consuming. Thus, PCR have been used as a rapid, specific, and sensitive method [11]. PCR assays have been used in specific detection of food-borne bacterial pathogens for more than 20 years ago [12]. Also, as part of quality control system of food safety [13]. Detection of *Salmonella* using PCR can be done in a short time with high accuracy so that appropriate handling can be done on food [14]. The *invA* gene from *Salmonella* contains unique DNA sequences and is proven to be a PCR target gene suitable for *Salmonella* detection [15].

II. MATERIAL AND METHODS

A. Samples Collection

A total of 500 leafy vegetable samples were collected from Vegetables and Fruits Jeddah Central Market, Jeddah district, western area- kingdom of Saudi Arabia. Samples obtained covering ten types of leafy green vegetables (Lettuce, Spanish, Rocket, Parsley, Mallow, Coriander, Portulaca, Lettuce, Dill, Basil and Chard). All samples were collected freshly at the morning and transported to the laboratory in sterile separate polyethylene bags (20 cm x 35cm).

B. Samples Preparation and *Salmonella* Identification

Damaged particles were removed from leafy green vegetable samples. Chopping board, knife, vegetable trays were wiped with 70% ethanol. Each sample were cut into small pieces (1 cm³) under aseptic conditions and then 25 grams of each leafy green vegetable sample were weighed and transferred into sterile Stomacher bags (tempo bags, Bio Merieux SA), 225 ml of sterile buffered peptone water (BPW) were added automatically by Delumat S (AES CHEMUNEX, Bio Merieux SA) devise (dilution system), All samples were blended in Stomacher 400 (AES CHEMUNEX, Bio Merieux SA) for 2 min.

Detection of *Salmonella* based on method 3.1.1:2007 [4], this method is based on primary enrichment (buffer peptone water, BPW), secondary selective enrichment (Rappoport-Vassiliadis Soy, RVS) and selective plating (RambachTM agar and XLD agar (Xylose lysine deoxycholate agar). Then, followed by confirmation based on method 3.1.5:2007 [4], using a range of biochemical tests.

C. Molecular Methods

1. DNA extraction from isolated *Salmonella* spp

Bacteria was cultured on nutrient agar and incubated for 24 hrs. at 37 °C. Extraction of DNA was performed by boiling for 10 min and centrifuged at 6000 rpm for 5min. The supernatant was used for amplification by PCR with *Salmonella* specific primers *invA*.

2. PCR: Specific primers used

2.1. Primers set and PCR amplification program

Salmonella specific primers, S139 and S141 [16] have respectively used, the following nucleotide sequence based on the *invA* gene of *Salmonella* 5' GTG AAA TTA TCG CCA CGT TCG GGC AA -3' and 5' TCATCG CAC CGT CAAAGG AAC C -3' which amplify a 389-bp fragment within the conserved *invA* gene sequence of *Salmonella* spp.

Detection of *invA* (244 bp) gene carried out with *Salm3* 5'GCTGCGCGCAACGGCGAAG-3' and *Salm4* 5'TCCCGGCAGAGTTCCCAT-3'. PCR reaction with these primers were carried out in a 50 µL amplification mixture containing 25 µL of PCR Master mix (Genei, Bangalore), 2 µL of each primer, 11 µL of molecular grade water and 10 µL of extraction for each isolate. Amplification was conducted in Master-gradient Thermo-cycler (Eppendorf). The cycles conditions were as follow: An initial incubation at 94 °C for 60 sec. Followed by 35 cycles of denaturation at 94 °C for 60 sec, annealing at 64 °C for 30 sec and elongation at 72 °C for 30 sec, followed by 7 min final extension period at 72 °C.

D. Antibiotics Susceptibility Test

1. Kirby-Bauer Method

The Clinical and Laboratory Standards Institute (CLSI), disc-diffusion method was used for antibiotic sensitivity testing [17]. Turbidity of the isolates was compared with 0.5 McFarland standard and each of the isolates was inoculated onto the surface of a sterile Muller and Hinton plates using a sterile swab in order to ensure even distribution of the inoculums, the plates were allowed to dry and antibiotic discs were placed on the surface of the plates. (Methicillin 5 µg, Cephalexin 30 µg, Metronidazole 5 µg, Ampicillin 10 µg, Amoxicillin 25µg, Chloramphenicol 30 µg, Gentamicin 10 µg, Streptomycin 10 µg, Amikacin 30 µg, Tetracycline 30 µg, Doxycycline 30 µg, Ciprofloxacin 5 µg and Norfloxacin 10 µg). After 30 minutes of applying the disc, the plates were inverted and incubated for 24 hours at 37 °C. The clear zone that developed around each disc was measured as the zones of inhibition in millimeter (mm) and calculated based on CLSI guidelines.

III. RESULTS

In this study, *Salmonella* spp. from different LGV samples were isolated by standard culture methods, identified by biochemical testing and confirmed by PCR by using *Salmonella*-specific-genes. A total of 6 *Salmonella* spp. was isolated (6/500, 1.2 %) (Table I). These isolates were as follows: two out of 23 from Basil samples (8.70%), 1/33 (3.030%) from Spinach, 1/60 (1.70%) from Rocket, 1/82 (1.2%) from Parsley and 1/48 (2.1%) from Chads. However, *Salmonella* spp. was not detected in Mallow, Coriander, Portulaca, Lettuce and Dill (Table I). All the 6 i isolates (6/6,100%) were confirmed as *Salmonella* using the *salm3* and *salm4* primer set (Fig. 3). The highest percentage of the isolation of *Salmonella* spp was observed in samples that collected from Makkah area (3/6, 50%) (Table I and Fig. 1).

TABLE I: NUMBER AND PERCENTAGES OF *SALMONELLA* SPP. ISOLATED FROM LGV SAMPLES USING CHROMAGARTM MEDIA

Food Item	No. of samples collected	No.and % of isolated <i>salmonella</i> spp.
Basil	23	2(8.70%)
Chards	48	1(2.08%)
Spinach	33	1(3.03%)
Rocket	60	1(1.70%)
Parsley	82	1(1.20%)
Mallow	66	0(0.00%)
Coriander	60	0(0.00%)
Portulaca	52	0(0.00%)
Lettuce	52	0(0.00%)

Fig. 1. Number and percentage of isolated *Salmonella* spp. from different LGV samples.

A. Detection of *invA* gene of *Salmonella*

Detection of *Salmonella invA1* encoding gene done by using PCR tests as indicated in Fig. 3. Two isolates (2/6, 33.3%) showed positive *Salmonella invA* gene (244 bp), while, four samples did not show the target band.

B. Antibiotics Susceptibility of the Isolated *Salmonella* spp.

All the six *Salmonella* isolates 6/6 (100%) showed resistance to Cephalexin (30 µg), Metronidazole (5 µg) and Methicillin (5 µg). While 5/6 (83.3%) resistant to Ampicillin (10 µg), 3/6 (50%) were resistant to Amoxicillin (25 µg), and 1/6 (16.7%) resistant to Chloramphenicol (30 µg) (Table II). The resistance pattern of isolated *Salmonella* spp. was shown in Fig. 2. On the other hand, as shown in (Table II), all the 6 *Salmonella* isolates were sensitive to amikacin (30 µg), ciprofloxacin (5 µg), Doxycycline (30 µg), Gentamicin (10 µg), Norfloxacin (10 µg), Streptomycin (10 µg), Tetracycline (30 µg) followed by chloramphenicol (30 µg) (5 isolates/6, 83.3%), amoxicillin 25 µg (5/6, 83.3%).

Fig. 2. Antibiotics susceptibility of *Salmonella* spp. isolated from LGV.

TABLE II: ANTIBIOTICS SUSCEPTIBILITY OF *SALMONELLA* SPP. ISOLATED FROM LEAFY VEGETABLES SOLD ON JEDDAH CENTRAL MARKET

Antibiotics	Conc. mcg	R %	S %	I %
Methicillin	5 µg	6(100%)	0(00.0%)	0(00.0%)
Cephalexin	30 µg	6(100%)	0(00.0%)	0(00.0%)
Metronidazole	5 µg	6(100%)	0(00.0%)	0(00.0%)
Ampicillin	10 µg	5(83.3%)	0(00.0%)	1(16.7%)
Amoxicillin	25 µg	3(50.0%)	2(33.3%)	1(16.7%)
Chloramphenicol	30 µg	1(16.7%)	5(83.3%)	0(00.0%)
Gentamycin	10 µg	0(00.0%)	6(100%)	0(00.0%)
Streptomycin	10 µg	0(00.0%)	6(100%)	0(00.0%)
Amikacin	30 µg	0(00.0%)	6(100%)	0(00.0%)
Tetracycline	30 µg	0(00.0%)	6(100%)	0(00.0%)
Doxycycline	30 µg	0(00.0%)	6(100%)	0(00.0%)

Fig. 3. Image of PCR products running on 1.5% agarose gel for detection of *Salmonella* spp. using *salm3/4* primers. Lane (M): 100bp ladder; lane 1: positive control (387 bp); lane 2-4: PCR products (387 bp) of *salm3/4* from *Salmonella* spp. and lane 5: negative control.

Fig. 4. Image of PCR products running on 1.5% agarose gel for detection of virulence *invA1* gene among *Salmonella* spp. isolates. Lane M: 100bp ladder lane; Lane 1: positive control for *invA1* gene (244 bp); lane 2: PCR product (244 bp) of *invA1* gene; lane 3: negative control and lane 4: negative sample *invA1* gene.

IV. DISCUSSION

Antimicrobial resistance of pathogenic bacteria causes treatment failure, increased hospitalization, and increased risk of mortality [18]. *Salmonella* has been reported to be presence both in raw and processed foods worldwide. Food-borne salmonellosis is the most prevalent disease and major source of *Salmonella* spp. in humans [19]. As mentioned by [20] presence of *Salmonella* in both raw and processed foods has been reported worldwide. In developing countries, detection

of *Salmonella* spp. is difficult, so, molecular methods with the genotypic diversity of the genes are required [21].

Owing to the above, this study done to examine the occurrence, distribution and antimicrobial susceptibility of *Salmonella* spp isolated from selected LGV collected from Jeddah Central Market of Saudi Arabia. In this study we found that the LGV samples taken from Makkah area were the most contaminated ones as our results showed that three of the isolated *Salmonella* spp out of the 6 isolates were from samples that collected from Makkah area (3/6, 50%) and this might be due to use of polluted water, unhygienic preparation processes and/or use of contaminated containers for transportation. [22] reported that there were about 300 kg of vegetative-animal compost used as fertilizer annually in wells located near agricultural fields in Saudi Arabia, moreover, only 40% of Saudi Arabia's land is linked by sewage pipelines suggesting possibilities of contamination with sewage water and/or animal compost. The same authors also said that sanitary networks are not covering human's settlement areas; instead, they rely on local septic tanks. However, septic tanks are designed to release supernatant effluent contaminated with human sewage into the adjacent area and this could be a source of contamination. The factors influencing the increase in salmonellosis outbreaks due to vegetables are changes in agricultural practices and eating habits [23]. Contamination with enteric pathogenic bacteria from horticultural products might be the main reason of infection, and two possible contamination routes are the use of organic fertilizers of animal origin and irrigation of crops with wastewaters [24].

All the six *Salmonella* isolates (100%) showed resistance to Cephalexin (30 µg), Metronidazole (5µg) and Methicillin (5 µg). While 5/6 (83.3%) resistant to Ampicillin (10 µg), 3/6 (50%) were resistant to Amoxicillin (25 µg), and 1/6 (16.7%) resistant to Chloramphenicol (30 µg). this results agree with [25] who found that a variable resistant patterns was observed for tetracycline (58%:47%), ampicillin (55.5%:31.4%), erythromycin (58.1%:62.7%), streptomycin (64.5%:76.5%), cephalothin (35.5%:39.2%) against *Salmonella* isolated from cabbage and spinach respectively and agree with a study done by [26] which showed a high resistance of *Salmonella* spp isolated from lettuce samples toward amoxicillin/ clavulanic-acid or ampicillin.

In the present study all six *Salmonella* isolates (100%) showed sensitivity toward Amikacin (30 µg), Ciprofloxacin (5 µg), Doxycycline (30 µg), Gentamicin (10 µg), Norfloxacin (10µg), Streptomycin (10 µg), Tetracycline (30 µg) followed by chloramphenicol (30 µg) (5/6, 83.3%), amoxicillin 25 µg (5/6, 33.3%), this results agree with the findings of [25], who found that all *Salmonella* isolates that recovered from vegetables and subjected to antimicrobial reactions were sensitive toward the aminoglycoside and quinolones. Isolates from cabbage showed. 80 % susceptibility to ciprofloxacin and an average of 72% susceptibility was exhibited against gentamicin, isolates from spinach vegetable demonstrated excellent sensitivity toward chloramphenicol (94%).

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