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 *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.

**I. INTRODUCTION**

The necessity for fresh fruits and vegetables caused an expansion of the market share for minimally processed vegetables. Foodborne diseases are caused 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 Entero-pathogenic *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 safety of fresh vegetables, especially those of animal origin [3]. *Salmonella enterica* species is the most common pathogen causing 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 *typhi* cause typhoid fever, while *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 (Rambach™ 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 invA1 (244 bp) gene carried out with Salm3 5′GCTGGCGCGGAAACGGCGAAG-3′ and Salm4 5′TCCCGGAGATTTCCATT-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, Cephalixin 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 (3.03%) 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).

| Food Item | No. of samples collected | N0 and % of isolated Salmonella 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%)                            |
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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 Cephalaxin (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), Norflaxocin (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.

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 lane4: 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 present 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

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%)|
| Cephalaxin      | 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%)|
| Gentamicin      | 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%)|
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 (5 µg) and Moxicillin (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 (58.3%;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 leafy vegetable 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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