IJPSR (2020), Volume 11, Issue 5 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 16 January 2020; received in revised form, 28 March 2020; accepted, 31 March 2020; published 01 May 2020

PREVALENCE OF EXTENDED-SPECTRUM β-LACTAMASE-PRODUCING *SALMONELLA* ON GREEN LEAFY VEGETABLES FROM A STREET VENDOR

Bhuvaneswari Manivel [*1] and P. Angalaparameswari [2]

Department of Chemistry and Biosciences [1], SASTRA Deemed to be University, SRC, Kumbakonam - 621001, Tamil Nadu, India.
Shrimati Indira Gandhi College [2], Tiruchirappalli - 620002, Tamil Nadu, India.

Keywords:

Salmonella, Food borne illness, Street vendor, ESBL Producers, Contamination

Correspondence to Author:
Dr. Bhuvaneswari Manivel

Assistant Professor, Department of Chemistry and Biosciences, SASTRA Deemed to be University, SRC, Kumbakonam - 621001, Tamil Nadu, India.

E-mail: bhunz2010@gmail.com

ABSTRACT: Consumption of fresh leafy vegetables by a street vendor cause salmonellosis is a major public health problem worldwide. The aim of the study to investigate total microbial count and the occurrence of ESBL producer and antimicrobial resistance pattern of *Salmonella* from fresh leafy greens. In this sample Salmonella spp. Population ranged from 40.33 to 35.33 × 10^2 CFU/g of fresh samples. The highest *Salmonella* spp. The population was observed in 40.33 × 10^2 CFU/g in green leaf, followed by Coriander and Ponnankanikirrai (red). Among the 10 isolates, five (50%) isolates namely NS-1, 2, 4, 5, and 7, could be ESBL producers by double-disc synergy test. From the 5 *Salmonella* spp., 4 isolates (88.89%) were resistance to cefuroxime and cefixime, 3 isolates (77.78%) resistant to ceftazidime and cefdinir, 2 isolates (33.33%) resistant to amoxiclav ceftriaxone and co-trimoxazole, and 1 isolate (11.11%) resistant to cefixime, amikacin, and chloramphenicol. Citric acid eradicated the *Salmonella* spp. averagely 100% at 3% concentration while 9.77 (0.5%) to 93% (2.5% concentration) from control. Acetic acid (3 %) decreased cell density by 100%, followed by acetic acid and Lactic acid. Among the 5 isolates, NS-1, NS-2, NS-4, NS-5, and NS-7 isolates were highly susceptible to antimicrobial agents. The data presented here indicate that street vendor green leaf were the potential reservoirs of *Salmonella* infection. This recommends good hygienic practices are mandatory to prevent the spread of disease. In future newer methods were adopted to reduce the risk of contamination.

INTRODUCTION: Food is of paramount importance to the sustenance of human health. However, the preparations of food typically end in unintended contamination.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.11(5).2451-61

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.11(5).2451-61

In developing countries, a wide variety of foods, which include vegetables, confectionary meat, and meat products and poultry, are usually prepared by food vendors.

Street-vendor foods provide a source of inexpensive, convenient, and often nutritious food for urban and rural poor; and a chance for self-employment and the opportunity to develop business skills with low capital investment. In general, seller food is familiarly associated with the

junk food, snacks, and street food; it is illustrious by its native flavor and by being purchased on the road, with or while not getting into any building. In spite of the numerous advantages offered by street foods, there are several health hazards associated with this sector of the economy. The problems associated with the methods of consumption of vendor foods considerably arise from traditional processing and packaging, improper handling temperature, poor personal hygiene of food handlers.

Biological contamination and foodborne illnesses are a worldwide health concern since about 1.8 million deaths worldwide have been attributed to the presence of *Escherichia coli*, *Salmonella* or *Shigella*. A variety of bacteria, parasites, and the virus can cause serious health problems after ingesting contaminated food or raw food products; these cases are known as foodborne illnesses (FBA). The main pathogens associated with food-related disease outbreaks are *Escherichia coli* O157: H7 and *Salmonella* spp. Among the main foodborne illnesses are diarrheal conditions such as gastroenteritis, typhoid fever, and shigellosis, among others. Diarrheal diseases alone can cause over 2.2 million human death globally every year [1].

In Europe, the presence of enteric bacteria in contemporary was answerable for a minimum of 87% of outbreaks by foodborne diseases. In 2006, were reported at least 1,267 cases by the presence of *Shigella* remains the leading cause with 33.4% above *Salmonella* was given a total of 5.2%. According to the Foodborne Disease Active Surveillance (FDAs) in 2012, predominant pathogenic infections were due to *Campylobacter* and also by *Salmonella*.

Of these three, *Salmonella* was the cause of about 33 deaths [2]. Recently as December 2012, it was reported that an outbreak caused by the presence of enteric bacteria associated with the consumption of organic spinach (Spinaciaoleracea) was over, leaving at least a total of 33 people infected in 5 states of the United States. These events are some of the many events that occur in the presence of plant-pathogenic bacteria resulting in adverse effects on human health and therefore require attention and continuous monitoring, especially concerning raw foods [3].

Fresh vegetables are fundamental components of the human diet, and there is considerable evidence of the health and nutritional benefits associated with the consumption of fresh vegetables [4]. Vegetables can become contaminated with microorganisms capable of causing human diseases while Bacteria such as *Clostridium botulinum*, *Bacillus cereus* and *Listeria monocytogenes* capable of causing disease, while *Salmonella*, *Shigella*, *Escherichia coli* and *Campylobacter* which reside in the intestinal tracts of humans and animals, are more likely to contaminate raw vegetables through contact with feces, sewage, untreated irrigation water or surface water. There is documented evidence of raw vegetables harboring potential food-borne pathogens, which can become contaminated while growing or during harvesting, post-harvest, handling, or distribution [5].

Leafy green vegetables are some of the most beneficial and healthy foods for human consumption. However, their raw state presents a greater risk of contamination by microorganisms. Other factors have the effect of contamination like the kind of vegetable, soil management, and handling once harvest**.** Today many of these vegetable products are cultivated applying alternative agricultural methods such as hydroponics techniques [3].

This study focuses on predicting suitable biological contaminants, leading to taking appropriate measures for the future. This study highlights the amount of disease-causing microbes found on the leaves of green vegetables [6].

Bacterial pathogens act as an important precursor to cause food-borne illnesses. The incidence and frequency of foodborne outbreaks caused by contaminated fresh vegetables are on the hike. *Salmonella* is one of the pathogens most frequently linked to the consumption of fruit and vegetables. A wide spectrum of produce vehicles has been associated with *Salmonella* infections [7]. The consumption of fresh fruit and vegetables might, therefore, pose a food safety risk because they are susceptible to contamination by fecal material on the farm [5].

In present times, bacterial biofilms have been more linked to food safety issues globally. The existence

of pathogenic organisms in biofilms has been linked to foodborne illness outbreaks in cantaloupe melons, apples, and leafy greens. Bacteria living within biofilms can exhibit 1000 times more resistance to antimicrobials than their planktonic peers. The proximity of these bacteria within the biofilm community enhances gene transfer, resulting in increased genetic diversity of antimicrobial resistance [8]. Once biofilm forms on fresh produce surface, they not only can cause cross-contamination to other food products or processing equipment surfaces in industry, they also result in a potent health hazard to consumers.

As the food is largely the vehicle for the transmission of *Salmonella* to humans, all data on contamination of the various food types should be available for analysis. As a result of the serious implications from the consumption of contaminated vegetables, this work aimed at investigating *Salmonella* contamination and also determines their antibiotic resistance pattern as well as the adherence and pathogenic status of *Salmonella* [9].

MATERIALS AND METHODS:

Chemicals and Glassware Used: The chemicals and reagents used in *in-vitro* experiments were of AR and LR grade obtained from SD fine, Hi-media and sterile distilled water were used throughout the investigation. Glasswares used in the present investigation were thoroughly washed, dried and then sterilized at 180 °C for one hour in a hot air oven.

Media Sterilization: The media used in the present investigation were prepared as per recommendation and sterilized in pressure at 15lb for 15 min in an autoclave.

Collection and Processing of Samples: Five samples of fresh leafy greens and fresh poultry meat were collected from open markets in Namakkal and transported to the laboratory and processed within 24 h. Representative sections of the sample were taken using sterile utensils and placed in sterile plastic containers. The sample was taken and serially diluted up to 10^{-5} with sterile peptone broth.

Enumeration of Bacterial Load and *Salmonella* spp.: [10] Isolation and enumeration of bacteria were done on selective and non-selective media such as nutrient agar for the total viable count (TVC) and Salmonella-shigella Agar (SS Agar) for *Salmonella*. Plates were made in triplicates inappropriate media. For bacterial enumeration spread plate was used to determine the number of colony-forming units (CFU). For calculation range per plate is split by sample volume and expressed as CFU/g.

Maintenance of Culture: The single isolated colonies obtained from SS agar plates were sub-cultured on Nutrient agar slants and incubated at 37 ºC for 24 h and then stored at 4 °C for further analysis.

Preliminary Examination of Culture:

Gram's Staining: Gram's staining was carried out as per Hacker's modification and observed cell morphology and arrangements. The isolates were identified by the colony characteristics growth on the SS agar. Motility was tested by the hanging drop method. Motility was observed under the microscope (I0 x eyepiece and 45x ocular).

Extended-Spectrum β-lactamase - Producing *Salmonella* spp. by Double-Disc Synergy Test (DDS): [11] A disc of amoxiclav (20 µg Amoxycillin and 10 µg clavulanic acid) was placed on the center of the Muller-Hinton agar (Himedia) plate which was previously inoculated with isolates of *Salmonella* sp. Each disc of ceftazidime (30 µg), cefotaxime (30 µg) and ceftriaxone (30 µg) was placed around the amoxiclav disc 20 mm apart and incubated for 24 h at 37 °C. A clear extension of the edge of the inhibition zone of any of the antibiotics towards the disc containing amoxiclav was interpreted as positive for ESBL production.

Antibiotic Sensitivity Pattern of ESBL producing *Salmonella* spp: The susceptibility of the ESBL producing bacteria was determined by the Kirby-Bauer disk diffusion method according to the Clinical Laboratory Standards Institute guidelines [12]. The discs were Amoxyclav 30 mcg, Ceftazidime 30 mcg, Cefotaxime 30 mcg, Ceftriaxone 30 mcg, Amikacin 30 mcg, Chloramphenicol 25 mcg, Cefuroxime 30 mcg, Co- Trimoxazole 30 mcg, Cefixime 5 mcg and Cefdinir 5mcg. The nutrient broth was inoculated and incubated at 28 °C for 48 h. Then antibiotic discs were placed on Mueller-Hinton agar plates aseptically, at least 24 mm apart. The plates were incubated at 28 °C for 24 h. The zone of inhibition was interpreted according to the antibiotic disc.

Evaluation of Antibacterial Activity in Chemical Food Preservatives:

Preparation of Stock Solutions: The stock solutions of organic acids such as citric acid (1% w/v, *i.e.*, 0.1 g chemical preservative dissolved in enough sterile distilled water to make the final volume 10 ml), acetic acid and lactic acid (1% v/v, *i.e.*, 0.1 ml chemical preservative dissolved in enough sterile distilled water to make the final volume 10 ml) were prepared [13].

Antibacterial Activity by Agar Well Diffusion Method: In the agar, well diffusion method, PCA plates were inoculated with 100µl of each chicken associated bacterium adjusted to standardized inoculums (1.5×10^8 CFU/ml). 8 mm wells were made into agar plates containing the bacterial inoculums. 100 µl volume of the organic acid was poured into a well of inoculated plates. Sterilized distilled water was used as a control. The plates were incubated for 24 h at 37 °C [14]. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was determined if the zone of inhibition was higher than 8 mm. The mean and standard deviation of the diameter of inhibition zones were calculated.

Minimal Inhibitory Concentration (MIC) of Organic Acid: Minimal Inhibitory Concentration (MIC) of organic acids tests were carried out by Bauer 1966 method with modification. The stock solutions of the acetic acid, citric acid, and lactic acid were diluted, and 0.75 µl was added to each tube in the range of 5 to 0.0046% was obtained. The double-strength nutrient broth and inoculum were added and incubated at 37 °C for 24 h. MIC was detected by TTC (Triphenyl Tetrazolium Chloride, Loba). The colorless tetrazolium salt was reduced to a red-colored product by microbial activity. MIC was defined as the lowest concentration of acetic acid and citric acid that inhibited visible growth, as indicated by the TTC staining.

Molecular Ribotyping of ns-2: Molecular ribotyping of the selected strain was carried out using the partial sequence of 16S rRNA.

Isolation of Genomic DNA: [15] Mid – log phase culture of the bacteria (40 ml) was centrifuged (Remi Instruments Pvt., Ltd, Mumbai) at 5000 rpm for 10 min at 4 °C. The supernatant was discarded, and the cell pellet was dissolved in 8.75 ml of TE buffer and to that 50 µl of proteinase K (10 mg/ml) and 1 ml of 10% Sodium Dodecyl Sulphate (SDS) was added, mixed gently, and incubated at 37 °C for 1 h. Then an equal volume of the phenol-chloroform mixture (1:1) was added, kept for 10 minutes at 4 °C and centrifuged at 10,000 rpm for 10 min at 4 °C. Then the aqueous phase was taken to that 0.1 ml of 5M Sodium acetate (pH 5.2), and 20 ml of isopropanol was added. The precipitated DNA was gently collected washed with 70% ethanol. Then, the DNA was dissolved in 1 ml of TE buffer.

Agarose Gel Electrophoresis: [15] The agarose gel electrophoresis (Genei, Bangalore) was carried out in a horizontal submarine electrophoresis unit. 30 ml of agarose gel 1.0% (w/v) was prepared for electrophoresis using a 1X TBE buffer. The gel was allowed to solidify, and 5 µl of the DNA was mixed with 2 µl of gel loading dye and then loaded on to the gel and electrophoresis was carried out at 80 V for 1 h. 100 bp DNA Ladder (Bangalore Genei) was used as the marker. The gel was stained with a 0.5 mg/ml Ethidium bromide and viewed on a UV transilluminator, and then the image was captured with the help of the Gel Doc System (Bio-Rad).

Ribotyping using 16S rRNA: Ribotyping was victimized using universal primer combine for 16S rDNA. A portion of the 16S rRNA gene was amplified from the genomic DNA. The sequence of forward (16SF) and reverse (16SR) primers used for amplifying 16S rDNA were obtained from Sigma, India and are as follows: [16]

16 SF -ITS1F -5’ GAGGATGCATCACACTGGAA 3’

16 SR -ITS2R - 5’ CCGGCTTATTCTGTCGGTAA 3’

Polymerase Chain Reaction (PCR): PCR was performed in a thermal cycler (Genei, Bangalore), according to Kumar *et al.*, 2002, with some modification under the following standardized conditions.

DNA Sequencing: Nucleotide sequences of the PCR amplicon were determined by using DNA Sequencer in the Vellore Institute of Technology, Vellore. The partial sequence of the 16S rRNA gene obtained was submitted to Genbank through

BankIt programme, at National Center for biotechnological Information (NCBI) site (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank). The identity of the sequences was determined by comparison with the sequences available in the database using BLAST software.

Phylogenetic Tree Construction: The phylogenetic tree was constructed using the neighbor-joining method implemented in CLUSTAL W. Tree was constructed using the nucleotide evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. The tree was visualized using the CLUSTAL W N-J tree.

RESULTS: Five samples of fresh leafy greens were purchased from open markets in the Namakkal bus stand as regular customers.

Estimation of Microorganisms of Fresh Leafy Greens: The bacterial population fresh leafy greens were estimated. Ranges of the microbial count of fresh leafy greens were 45.02 to 81.33 × 10^5 CFU/g. Among the different leafy greens, a statistical difference was found in bacterial load ($p>0.05$). The highest bacterial population was 81.33 and 66.33 × 10^5 CFU/g in Coriander and Sirukirai respectively which bacterial population was on par. Mint and Arakkirai showed the next bacterial population load which ranges from 51.67 and 45.02 × 10^5 CFU/g respectively.

The bacterial load of the samples (Sirukirrai, Mint, Arakkirai, and Red leaf were 66.33, 51.67, 45.02 and 38.33 × 10^5 CFU/g respectively which was on par. The least bacterial load was found in Ponnan-gannikirai (Red) (38.33 × 10^5 CFU/g).

Enumeration of *Salmonella* spp.: *Salmonella* spp. was enumerated and tabulated in **Table 1**. In SS agar plates, *Salmonella* spp. produced colorless, usually with black center colonies, which considered as *Salmonella* population in the samples. *Salmonella* spp. were tabulated in **Table 1**. *Salmonella* spp. population ranged from 21.33 to 40.33 × 10^2 CFU/g of fresh samples. The highest population was observed in 40.33 × 10^2 CFU/g in Arakkirai, followed by Coriander (35.33 × 10^2 CFU/g), green leaf (34.33 × 10^2 CFU/g) and Ponnangannikirai (Red) (30.67 × 10^2 CFU/g). Only 5 samples contaminated with *Salmonella* spp.

Morphological and Biochemical Characterization of *Salmonella* spp: The individual colorless, usually with black center colonies from SS agar, were subcultured in nutrient agar slants. The isolates were observed as gram-negative and motile. The catalase and methyl red showed positive results, and oxidase, Indole, Voges-Proskauer, citrate utilization, and urease showed a negative result. Based on the above mention colony morphology in SS agar and biochemical test, all isolates identified as *Salmonella* spp. and which were designated as NS-1 to NS- 10.

Extended-Spectrum β-lactamase - Producing *Salmonella* spp: The extended-spectrum beta-lactamase-producing *Salmonella* spp. were observed and recorded in **Table 2**. Among the 10 isolates, five (50%) isolates namely NS-1, 2, 4, 5 and 7 could be ESBL producers by double-disc synergy test. The isolates were used for further studies.

Antibiotic Sensitivity Pattern of ESBL Producing *Salmonella* spp: The results of the antibiotic susceptibility test for 10 antimicrobial agents are shown in **Table 3** and **Fig. 5**. From the 5 *Salmonella* spp.. The 4 isolates (66.67%) were sensitive to amikacin and chloramphenicol, 3 isolates (88.89%) were susceptible to co-trimoxazole, 2 isolates (88.89%) showed susceptible to amoxiclav, 2 isolates (88.89%) were sensitive to cefotaxime while 1 isolate (11.11%) was susceptible to ceftazidime and ceftriaxone.

Effect of Anti-microbial Agents against *Salmonella* spp. on Leafy Greens: All the anti-microbial agents showed a maximum reduction in average microbial density at 3% concentration as compared to the microbial density of control **Table 5**. Acetic acid decreased the cell density from averagely 5.48 (0.5%) to 67.07% (3% concentration) from control **Table 4**. The NS-5 isolates highly controlled (84.46%) by acetic acid at 3% concentration, followed by NS-7 and1 (75.32%), NS-6 (61.22%), and NS-2 (56.09%). Citric acid destroyed the *Salmonella* spp. averagely 16.45 (0.5%) to 84.29% (3% concentration) from control **Table 5**. The NS-1 isolates highly controlled (84.46%) by acetic acid at 3% concentration, followed by NS-5 and 6 (75.32%), NS-7 (61.22%), and NS-2 (56.09%). Lactic acid

(3%) decreased cell density 100%, followed by citric acid and acetic acid when compared with the other 2 antimicrobial agents. Among the 5 isolates, NS-5 and NS-2 isolate highly susceptible to antimicrobial agents when compared with other isolates such as NS-7, NS-6, and NS-1.

Molecular Ribotyping of NS-2: Molecular ribotyping of the selected strain was carried out using the partial sequence of 16S rRNA sequencing and a phylogenetic tree was constructed.

TABLE 1: DETERMINATION OF THE *SALMONELLA* COUNT FROM LEAFY GREENS OF OPEN MARKETS

S. no.	Name of the Leafy greens	Average Total *Salmonella* counts $\times 10^1$	SD*
1	Sirukirai	34.33	2.5166
2	Arakkirai	40.33	3.3472
3	Ponnangannikirai (Red)	30.67	1.5275
4	Mint	21.33	2.6457
5	Coriander	35.33	1.5275

* SD- Standard deviation

TABLE 2: DETECTION OF EXTENDED SPECTRUM β-LACTAMASE PRODUCING *SALMONELLA spp*.

S. no.	Name of the isolates	Zone of inhibition (Diameter in mm)			
		Amoxyclav 30mcg	Ceftazidime 30mcg	Cefotaxime 30mcg	Ceftriaxone 30mcg
1	NS-1	19	-	21	13
2	NS-2	13	15	19	24
3	NS-3	-	12	23	25
4	NS-4	13	-	19	14
5	NS-5	19	-	20	15
6	NS-6	-	-	12	17
7	NS-7	17	20	16	15
8	NS-8	-	-	15	16
9	NS-9	-	-	1	19
10	NS-10	20	-	18	19

\- No Zone

TABLE 3: ANTIBIOTIC RESISTANT PATTERN OF EXTENDED SPECTRUM β-LACTAMASE PRODUCING *SALMONELLA* spp

S. no.	Name of the isolates	Zone of inhibition (Diameter in mm)									
		Amoxiclav 30mcg	Ceftazidime 30mcg	Cefotaxime 30mcg	Ceftriaxone 30mcg	Amikacin 30mcg	Chloramphenicol 25mcg	Cefuroxime 30mcg	Co-trimoxazole 30mcg	Cefixime 5mcg	Cefdinir 5mcg
1	NS-1	19 (S)	-(R)	21 (IM)	13(R)	23 (S)	22 (S)	12 (R)	23 (S)	19 (S)	18 (IM)
2	NS-2	13 (R)	15 (IM)	19(IM)	24 (S)	-(R)	35(S)	-(R)	15 (IM)	-(R)	11(R)
3	NS-4	13 (R)	-(R)	19(IM)	14 (IM)	30(S)	28(S)	-(R)	12 (IM)	-(R)	10(R)
4	NS-5	19 (S)	-(R)	20(IM)	15(IM)	24(S)	20(S)	8(R)	22(S)	8(R)	19 (IM)
5	NS-7	17 (IM)	20(S)	16(IM)	15(IM)	32(S)	29(S)	-(R)	-(R)	-(R)	10(R)

R- Resistant; IM- Intermediate; S- Sensitive

TABLE 4: ANTIBACTERIAL ACTIVITY OF ORGANIC ACID AGAINST ESBL PRODUCING *SALMONELLA* SPP. BY AGAR WELL DIFFUSION METHOD

S. no.	Organic acids	The diameter of the inhibition zone (mm)				
		NS-1	NS-2	NS-5	NS-6	NS-7
1	Acetic acid (1%)	19	16	20	18	19
2	Citric acid (1%)	16	12	15	15	14
3	Lactic acid (1%)	11	10	12	11	11

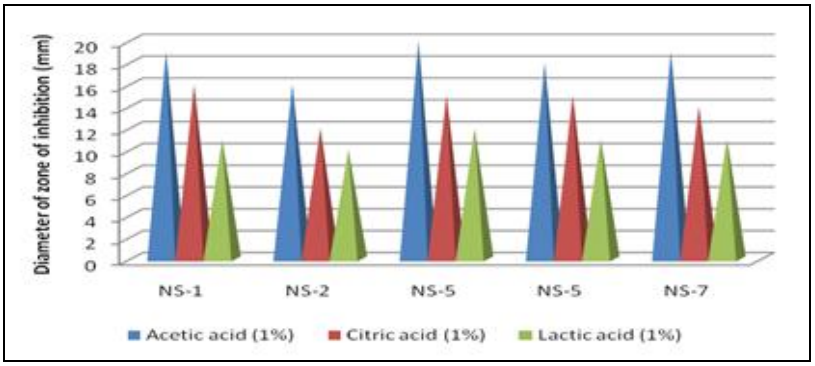
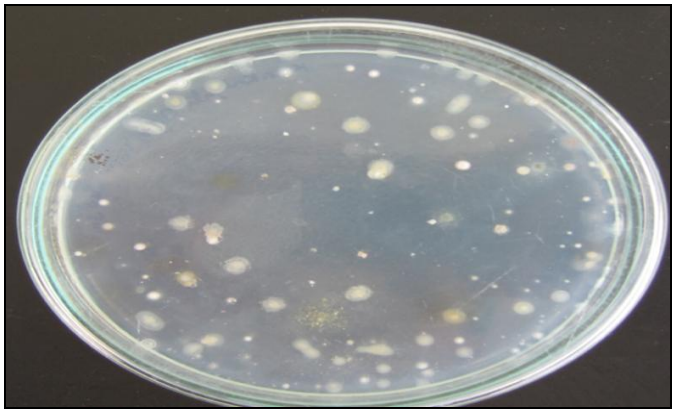


FIG. 1: ANTIBACTERIAL ACTIVITY OF ORGANIC ACID AGAINST ESBL PRODUCING *SALMONELLA* SPP. BY AGAR WELL DIFFUSION METHOD

TABLE 5: MINIMUM INHIBITORY CONCENTRATION (MIC) OF VARIOUS ORGANIC ACIDS ON ESBL PRODUCING *SALMONELLA* SP. NS-2

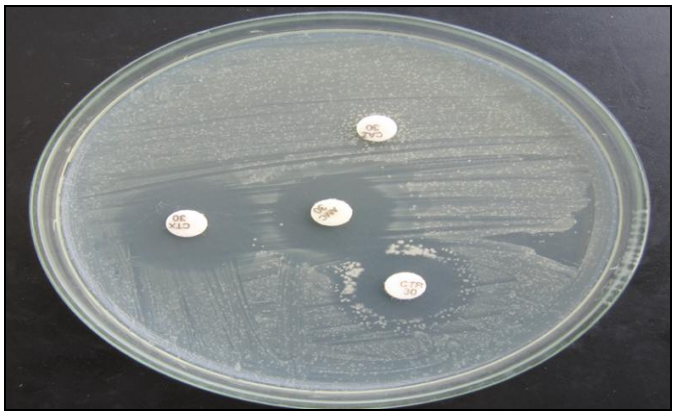
S. no.	Acid concentration (%)	Acetic acid	Citric acid	Lactic acid
1	5	-	-	-
2	2.5	-	-	-
3	1.25	-	-	+
4	0.6	-	+	+
5	0.3	+	+	+
6	0.15	+	+	+
7	0.075	+	+	+
8	0.038	+	+	+
9	0.019	+	+	+
10	0.0093	+	+	+
11	0.0046	+	+	+
12	CONTROL	+	+	+

+growth; - no growth

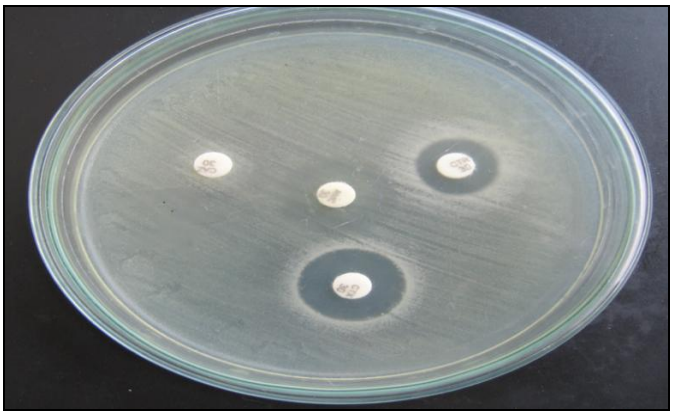


TOTAL COUNT OF BACTERIA

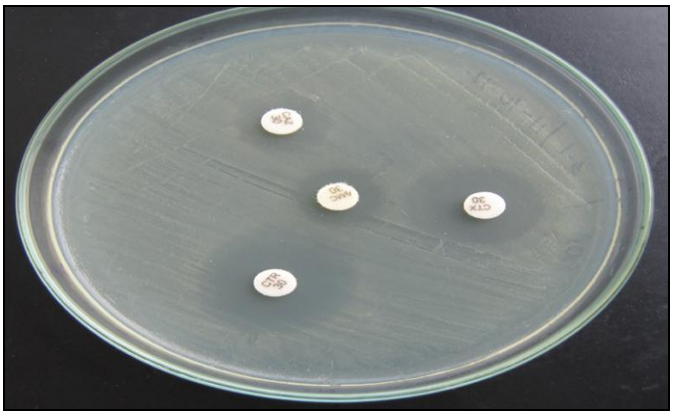
TOTAL COUNT OF *SALMONELLA* SP.



ESBL PRODUCING SALMONELLA SP



NON-ESBL PRODUCING *SALMONELLA* SP



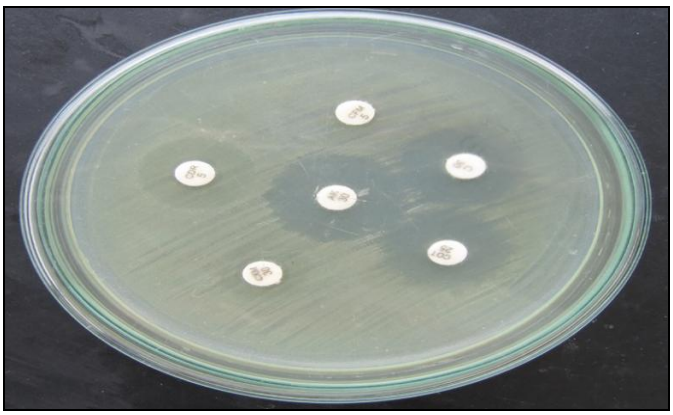


PLATE 1: ANTIBIOTIC SENSITIVE PATTERN OF ESBL PRODUCING *SALMONELLA* SP.

16s rRNA Based Identification and Phylogenetic Relationship: The multidrug-resistant *Salmonella* AS-13 was selected based on the antibiotic-resistant patterns. *Salmonella* AS-13 genomic DNA was isolated and PCR amplified with 16S rDNA. Electrophoretic analysis of PCR products obtained from the amplification of 16S rDNA genes confirmed that full length (1207bp) genes were amplified for *Salmonella* AS-13 **Fig. 2**. The Molecular Weight was 366348.00 Daltons in single-stranded and 734901.00 Daltons in double-stranded. The G+C content was 55.51%, and A+T content was 44.49% while 306 number of adenine with 25.35 mol %, 284 number of cytosine with 23.53 mol %, 386 number of guanine with 31.98 mol % and 231 number of thymine with 19.14 mol % were found **Fig. 2**.

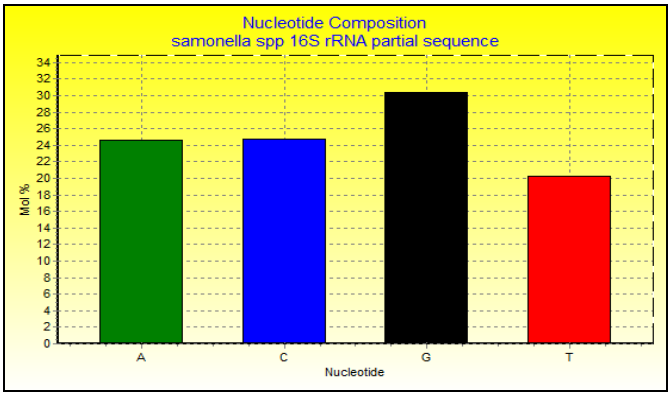


FIG. 2: NUCLEOTIDE MOLECULAR PERCENTAGE OF *S. TYPHIMURIUM* NS-2 16S rRNA PARTIAL SEQUENCE

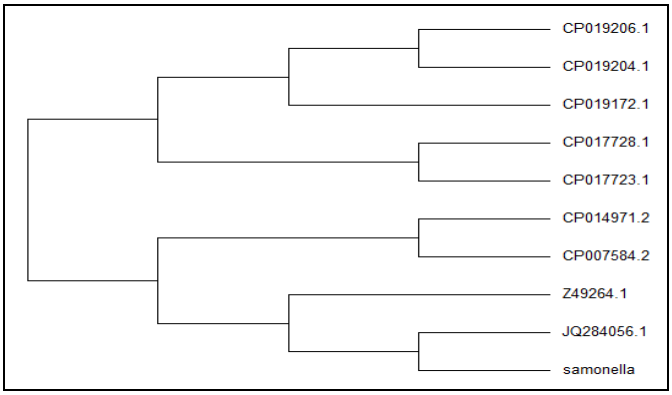


FIG. 3: PHYLOGENETIC TREE BASED ON THE WEIGHTED NEIGHBOR-JOINING METHOD FOR THE *S. TYPHIMURIUM* NS-2 16S rRNA, PARTIAL SEQUENCE

The amplified product was sequenced, and the sequence of DNA fragment was compared to the sequences available in GenBank, NCBI. Sequence analysis of these isolates was also performed using the BLAST (Blastn) search tool (http://www.ncbi. nlm. nih.gov) available on the NCBI homepage. The *Salmonella* AS-13 strains used in the study exhibited 96 to 98% sequence similarity to the *Salmonella* available in the NCBI database. These sequence data have been deposited in the GenBank (Submission number: SUB2484782), as detailed below in **Fig. 3**.

The phylogenetic tree generated by a weighted neighbor-joining **Fig. 3** methods revealed the evolutionary relationship of the strain AS-13 to a group of Salmonella. Thus, this strain was designated as Salmonella AS-13.

DISCUSSION: Fresh and fresh-cut leafy green vegetables are nutrient-rich foods with high levels of minerals, vitamins, and phytochemicals. Fresh fruit and vegetables are important components of a healthy and balanced diet; their consumption is encouraged in many countries by government health agencies to protect against a range of illnesses such as cancers and cardiovascular diseases. However, Vegetables have been associated with outbreaks of foodborne disease in many countries. Organisms involved include bacteria, viruses, and parasites. Raw vegetables can harbor many microorganisms, which may be dispersed over the plant or appear as microcolonies embedded in the plant tissue [18]. Vegetables are highly exposed to microbial contamination through contact with soil, dust, and water and by handling at harvest or during postharvest processing. They, therefore, harbor a diverse range of micro-organisms, including plant and human pathogens [19].

Some coliforms, including *Salmonellae*, *Shigellae*, and enteropathogenic *Escherichia coli*, are notable enteric pathogens. *Escherichia coli* O157: H7 and *Salmonella* spp. are the most dangerous foodborne bacterial pathogens in terms of human health and disease [20]. The microbial load and the presence of the bacterial pathogens in foods are a good indication of the food quality and the potential health risk they pose to consumers [21].

Various serotypes of *Salmonella* spp. have been reportedly responsible for foodborne epidemics in various countries, emphasizing the importance of the pathogen as a food safety concern. However, reports on the spreading of *Salmonella* from fish [22] as well as reports indicating that raw vegetables may harbor potential *Salmonella* [23] are increasing.

The spreading of multidrug-resistant phenotypes has also been increasingly described among *Salmonella* serovars worldwide in many reports [24, 25]. A contributing factor in the development of resistance stems from the use of antimicrobials in human medicine, veterinary medicine, animal husbandry, as well as agricultural and aquaculture practices [26]. These routine practices are important factors in the emergence of antibiotic-resistant bacteria that subsequently can be transferred to humans through the food chain.

Fresh leafy greens in Namakkal are typically sold at open markets which usually sold unwrapped condition at the roadside and near sewage channel, so the present investigation was carried out to study the occurrence, ESBL producing and antimicrobial resistance pattern of *Salmonella* from fresh leafy greens collected from open markets in Namakkal, Tamil Nadu. The total microbial counts were also determined to evaluate the microbiological quality of the products [27].

The majority of microorganisms associated with raw vegetables are gram-negative organisms tend to dominate the bacterial population. Many pathogenic and nonpathogenic bacterial species normally present on the surface of fresh produce. Microbial counts are within the range of 10^1-10^9 CFU/g, varying with fruit and vegetable type. The aerobic plate count ranged from 6.7 to 9.2 log10 CFU/g in vegetables [28]. In the present study, the mesophilic bacteria counts of fresh leafy greens were 81.33 to 66.33 × 10^5 CFU/g.

Many vegetables present nearly ideal conditions for the survival and growth of many types of microorganisms. The internal tissues are nutrient-rich, and many, especially vegetables, have a pH near neutrality. Their structure is comprised mainly of the polysaccharides cellulose, hemicellulose, and pectin. Some spoilage microbes are capable of colonizing and creating lesions on healthy, undamaged plant tissue [29]. The high microbial contamination observed in the fruits and vegetables in this study may be a reflection of storage, handling, transporting, and pre-washing conditions.

Kroupitski *et al*., [30] reported *Salmonella spp*. were found on 4% of the leafy vegetables, and 20% of the bean sprouts. In the present study, *Salmonella* spp. population ranged from 40.33 to 35.33 × 10^2 CFU/g of fresh samples. The highest population was observed in 40.33 × 10^2 CFU/g in Arakkirai, followed by Coriander, (35.33 × 10^2 CFU/g), green leaf (34.33 × 10^2 CFU/g) and Ponnakannikkirai (30.67 × 10^2 CFU/g). Only 4 (40%) samples contaminated with *Salmonella* spp. The potential source of *Salmonella* contamination is likely due to poor water quality, fecal contamination, poor sanitary conditions, or poor distribution and handling practices.

ESBL-producing Enterobacteriaceae have been related to most of the recently documented foodborne outbreaks which are associated with microbial contamination. In the present study, among the 10 isolates, five (60%) isolates namely NS-1, 2, 4, 5, and 7, could be ESBL producers by double-disc synergy test. ESBL producing bacteria may be acquired via contaminated food, it is important to implement a rational antibiotics policy during food production and to monitor the occurrence of resistant bacteria in food like meat, fruit, and vegetables [31].

A high level of antibiotic resistance is often related to the member of the Extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae [32]. Takkinen *et al*. [33] reported that about 0.5% of *Salmonella* serovars Typhi and Paratyphi obtained from patient's blood in Nepal were capable of producing ESBL. In the present study, from the 5 *Salmonella* spp., 4 isolates (88.89%) were resistance to cefuroxime and cefixime, 3 isolates (77.78%) resistant to ceftazidime and cefdinir, 2 isolates (33.33%) resistant to amoxiclav and ceftriaxone, co-trimoxazole, and 1 isolate (11.11 %) resistant to cefixime, amikacin, and chloramphenicol. These results agree with that of Zheng *et al*., [31] who demonstrated the sensitivity of *Salmonella* isolates towards chloramphenicol, neomycin, kanamycin, streptomycin, and cotrimoxazole.

Organic acids exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria, viruses, and fungi but are not sporicidal. They are, however, known to inhibit sporulation and spore germination, but this effect is reversible. In the present study, Acetic acid obliterated the *Salmonella* spp. averagely 6.68 (0.5%) to 85.39% (3% concentration) from control.

The 100% controlled at 3% concentration in isolates NS-5 and 78.79% in NS-7 and NS-1, 72.24 % in NS-6, 59.34% and 58.11% in Ns-2. The antibacterial activity of Acetic acid is mainly due to dehydration of protein and the enzyme to deactivate and prevent bacteria growing [34].

Lactic acid possesses antimicrobial activity and has been demonstrated to control postharvest decays of fruits efficiently. Post-harvest applications with Lactic acid have been used to delay aging or ripening, consequently reducing post-harvest decay and controlling many diseases in fruits and vegetables [35].

In the present study, lactic acid eliminated the *Salmonella* spp. averagely 6.12 (0.5%) to 75.00% (3% concentration) from control. The NS-5 and NS-2 isolates highly controlled (92.94%) by lactic acid at 3% concentration, followed by NS-6, NS-7, and NS-1.

Citric acid prevents toxin production by most bacterial pathogens, inhibits the growth of *Listeria monocytogenes,* and owns its inhibitory action due to chelation by the anion. The type of micro-organism, inoculum load, and environmental conditions influence conditions for the growth of pathogens in juice. Other factors are the pH of the juice, temperature of storage, and water activity (aw) [36]. In the present study, Citric acid eradicated the *Salmonella* spp. averagely 100% at 3% concentration while 9.77 (0.5%) to 93 % (2.5% concentration) from control. The 100% controlled at 2.5% concentration in isolates NS-1, 6, 5 7 and 2.

Lactic acid (3%) decreased cell density by 100%, followed by Citric acid and Acetic acid. Among the 5 isolates, NS-2, NS-7, NS-5, NS-4, and NS-1 isolate highly susceptible to antimicrobial agents when compared with another isolate. The variation of resistance and susceptibility of *Salmonella* isolates might be due to the richness and evenness of biological communities that reflect selective pressures that shape diversity within communities. The outcomes of this research will certainly shed new light on the diversity of *Salmonella* strains and their environmental [37].

The study suggests that fresh leafy vegetables harbor a high number of contaminants and *Salmonella* spp. in the local market samples; hence they are more prone to spoilage, making it necessary to process them before consumption. Thus the use of citric acid, lactic acid, and acetic acid (as rinsing agent) in vegetables for reduction of microorganisms can play an important role in food processing and cooking.

CONCLUSION: In conclusion, the data presented here indicate that Street vendor's green leaves are reservoirs of antibiotic-resistant *Salmonella* infection. There is potential for these antibiotic-resistant bacteria to be transferred to humans through contaminated street vendor green leaf *Salmonella* not only poses a serious threat to public health but also causes huge economic losses by generating mortality and morbidity to the human population. Multidrug resistance of *Salmonella* is a public health problem, and there is an urgent need to reinforce the surveillance of the use of antibiotics by farmers, veterinarians, and physicians. Therefore, the continued development of methods to reduce the risk of foodborne pathogens in poultry is critical.

ACKNOWLEDGEMENT: The authors gratefully acknowledge the Faculty and research scholars of Microbiology, SASTRA University, Kumbakonam, for the constant support throughout their research.

CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

REFERENCES:

1. World Health Organization. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015; 255. http:// apps.who.int/iris/bitstream/10665/199350/1/9789241565165_eng.pdf?ua=1.
2. CDC: Preliminary Foodnet Data on the Incidence of Pathogens Transmitted Commonly Through Food, 10 States, 2006. Atlanta, GA: Centers for Disease Control and Prevention 2013 http://www.cdc.gov/mmwr/preview/mmwrhtml/
3. Abadías M, Usal J, Anguera M, Solsona C and Vinas I: Microbiological quality of fresh, minimally-processed fruit and vegetables, and sprouts from retail establishments. International Journal of Food Microbiology 2008; 123: 121-29.
4. Barrantes K and Achi R: Calidadmicrobiológica y análisis de patógenos (Shigella y Salmonella) en Lechuga. Revista de la Sociedad Venezolana de Microbiología 2011; 31: 1-36.
5. Bardet A: Organic products, and irrigation water transfer of human pathogens to vegetables. Infos-Ctifl 2007; 49-52.
6. Critzer FJ and Doyle MP: Microbial ecology of foodborne pathogens associated with produce. Current Opinion in Biotechnology 2010; 21(2): 125-30.

7. Fett WF: Naturally occurring biofilms on alfalfa and other types of sprouts. J Food Prot 2000; 63: 625-32.
8. Crepet A, Albert I, Dervin C and Carlin F: Estimation of microbial contamination of food from prevalence and concentration data: application to Listeria monocytogenes in fresh vegetables. Appl Env Microb 2007; 73: 250-58.
9. John L: An introduction to bacterial identification-general principles 2001. http://www.jlindquist.net.
10. Tortora G: Microbiology, Benjamin Publishing Co. Inc., NewYork, NY, USA, 5th edition, 1995.
11. Bauer AW, Kirby WMM, Sherries JC and Turck M: Antibiotic susceptibility testing. Am. J. Pathol 1966; 45: 493-96.
12. Rahman MS: Handbook of food preservation. CRC Press, Boca Raton, FL: 2007.
13. EUCAST: European Committee on Antimicrobial Susceptibility Testing breakpoint table for interpretation of MICs and zone diameters 2017; http://www.sfm - microbiologie.org/UserFiles/files/casfm/CASFMV1_0_MARS_2017.pdf.
14. Sambrook J and Russell DW: "Molecular cloning: A laboratory manual. cold spring harbor laboratory press, 2001.
15. Johnson TR and Case CL: Laboratory Experiments in Microbiology, Pearson Education, San Francisco, California, USA 8th edition 2007.
16. Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ: Basic local alignment search tool. J Mol Biol 1990; 5(3): 403-10.
17. Doyle M and Erickson M: Summer meeting 2007 – the problems with fresh produce: an overview. J Appl Microbiol 2008; 105: 317-30.
18. Moller Nielsen E, Skov MN, Madsen JJ, Lodal J, Bochner Jespersen J and Baggesen DL: Verocytotoxin-producing Escherichia coli in wild birds and rodents close to farms. Appl Environ Microbiol 2004; 70: 6944-47.
19. Lynch M: *Escherichia coli* O157: H7 outbreaks due to raw leafy green vegetables. Memorandum by the US Center for Disease Control and Prevention 2007; 1: 3.
20. Rangel JM, Sparling PH, Crowe C, Griffin PM and Swerdlow DL: Epidemiology of *Escherichia coli* O157: H7 outbreaks, United States, 1982–2002. Emerg Infect Dis 2005; 11: 603-09.
21. Centers for Disease Control and Prevention. Multistate outbreaks of salmonella serotype Poona infections associated with eating cantaloupe from Mexico - the United States and Canada, 2000-2002. Morb Mortal Wkly Rep 2011; 51: 1044-47.
22. Rane S: Street Vended Food in Developing World: Hazard. Indian Journal of Microbiology 2011; 100-06.
23. Lapidotand A and Yaron S: Transfer of Salmonella serovar Typhimurium from contaminated irrigation water to parsley is dependent on curli and cellulose, the biofilm matrix components. J of Food Pro 2009; 72(3): 618-23.
24. Barro N, Bello A, Itsiembou Y, Savadogo A and Ou-Ouattara CA: Street-vendor foods improvement: Contamination mechanisms and application of food safety objective strategy: Critical review. Pakistan Journal of Nutrition 2007; 6: 1-10.
25. Sivaplasingham S, Friedman CR, Cohen L and Tauxe RV: Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J Food Prot 2004; 67: 2342-53.
26. Guchi B and Ashenafi M: Microbial load, prevalence and antibiograms of salmonella and shigella in lettuce and green peppers, Ethiopian Journal of Health Sciences 2010; 20(1): 43-47.
27. Semenov AV, van Overbeek L, Bruggen V and Ariana HC: Percolation and survival of Escherichia coli O157: H7 and Salmonella serovar Typhimurium in soil amended with contaminated dairy manure or slurry. Applied and Environmental Microbiology 2009; 75(10): 3206-15.
28. Delaquis P, Bach S and Dinu LD: The behavior of *Escherichia coli* O157: H7 in leafy vegetables. J Food Prot 2007; 70: 1966-74.
29. Kroupitski Y, Pinto R, Belausov E and Sela S: Distribution of *Salmonella typhimurium* in romaine lettuce leaves. Food Microbiology 2011; 28: 990-7.
30. Zheng J: Colonization and Internalization of Salmonella enteric in tomato plants. Applied and Environmental Microbiology 2013. DOI:10.1128/AEM.03704---1.
31. Takkinen J, Nakari UM, Johansson T, Niskanen T, Siitonen A and Kuusi MA: Nationwide outbreak of multiresistant *Salmonella typhimurium* var. Copenhagen DT104B infection in Finland due to contaminated lettuce from Spain, Eurosurveillance Weekly 10, 2005. http://www.eurosurveillance.org/ew/2005/050630.asp.
32. Avery LM, Killham K and Jones DL: Survival of *E. coli* O157: H7 in organic wastes destined for land application. J Appl Microbiol 2005; 98: 814-22.
33. Sapers G, Gorny J and Yousef AE: Salmonella contamination of fruits and vegetables. In: Microbiology fruits and vegetables. CRC Press, London 2005; 126.
34. Cardoso, Ribeiro MO, Dos AR and Santos LR: Antibiotic resistance in *Salmonella enteritidis* isolated from broiler carcasses. Brazilian Journal of Microbiology 2006; 37(3): 368-71.
35. Donkor ES, Nortey T, Opitan A, Day N and Akyeh ML: Antimicrobial susceptibility of *Salmonella typhi* and *Staphylococcus aureus* isolates and the effect of some media on susceptibility testing results. The Internet Journal of Microbiology 2008; 4(2): 1-5.
36. FDA 2008: National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS). Retail meat annual report, 2006. FDA, Bethesda MD. http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM073302.pdf.

How to cite this article:
Manivel B and Angalaparameswari P: Prevalence of extended-spectrum β-lactamase-producing salmonella on green leafy vegetables from a street vendor. Int J Pharm Sci & Res 2020; 11(5): 2451-61. doi: 10.13040/IJPSR.0975-8232.11(5).2451-61.