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Potential risks of endophytic *Salmonella sp.* contamination associated with raw salad vegetables and their drug-resistant pattern

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Abstract

Raw salad vegetables are considerable ingredients of vital nourishment but have been associated with relevant days with a flourishing threat of food poisoning from bacterial pathogens for instance Salmonella sp. Even though this is reflected in substantial known health trouble, extremely little is recognized regarding the behavior performance of Salmonella sp. with raw salad vegetables. The contemporary effort estimates the microbiological excellence of raw salad vegetables utilized in Surat city, Gujarat, India. A sum of 300 samples of 5 discrete largely consumed raw salad vegetables (Tomato N=60, Cucumber N=60, Cabbage N=60, Spinach N=60, Carrot N=60) from local vegetable markets were investigated for inclusive endophytic Salmonella sp.in relations of aerobic counts after removing surface microflora. Salmonella sp. has been detected 11.3 % of the total samples. Most Salmonella sp. isolated from above raw salad vegetables observed to have multidrug resistance. The investigation statistics reveal that raw salad vegetables may promote the durability of Salmonella sp. within raw salad vegetables. Endophytic Salmonella cells from salad vegetables could not be removed by simple washing and intensely highlight the significance of confirming the microbiological safety of raw salad vegetables.

Keywords: *Salmonella typhi* fitness, Plant contamination, Multidrug resistance, Immune response, Food safety.

Introduction

In the previous years, raw salad vegetables have been involved in abundant foodborne sicknesses across the globe, involving India[1-3]. The latest statistical evaluations of foodborne epidemics in the U.S. and the European Union have revealed that *Salmonella sp.* were the highest familiar bacterial pathogens associated among the fresh yield epidemics[4]. Fresh raw vegetables frequently are eaten without cooking or negligible additional processing is a vital constituent of the nourishing intake in humans[5]. Conversely, an emergent alarm has been developed regarding the welfare of raw vegetables expected to their involvement with *Salmonella sp*[.6,7]. Moreover, uninterrupted escalation in the numeral of foodborne epidemics associated with the ingestion of raw fresh vegetables has additionally elevated issues whether plants are factual substitute non-animal hosts for *Salmonella sp.*, or whether they are solely grounds where these pathogens magnificently inhabit and endure[8,9].

Recently, The fresh yield industry was involved in three independent multi-state epidemics concomitant to contaminated lettuce from Arizona and California of the U.S. (The United States) in 2018[10]. The three leafy green epidemics were cited in 294 sicknesses and six deaths across the U.S.[11-13]. From 1973 to 2012, leafy greens have covered more than half of the fresh yield - concomitant epidemics narrated in the U.S.[14]. Whereas threat managing approaches and governing necessities were established in return to produce-associated epidemics.

Predictably, plants are not contemplated as hosts *Salmonella sp.*; these bacteria repeatedly concomitant among mammalian or further animal hosts[8]. Furthermore, the latest outcomes have also revealed that *Salmonella sp.* colonize upon the exterior and innermost parts of the plants in equally epiphytic and endophytic existence, correspondingly[15-17]. Studies have indicated that *Salmonella sp.* needs an adjustment interval earlier being capable to flourish and endure in the plants, throughout the trauma influence of severe ecological circumstances on their epiphytic existence[18,19]. Additionally, to endure in severe environmental circumstances, it is recommended that *Salmonella sp.* could spread to the interior tissues of the plants to develop a virtual defense versus severe circumstances existing on the plant exterior and thus attain or improve competencies to colonize in plants and improve resistance to antimicrobial actions[20,21]. This provokes numerous alarms concerning the appliance, phenotypic behaviors, and virulence of *Salmonella sp.* concomitant with the non-animal accommodate i.e. plant.

Salmonella sp.: Species explicitly *Salmonella enterica* and *Salmonella bongori* are included in the genus *Salmonella* [22](appraised by Su and Chiu, 2007). *Salmonella enterica* is the most expected pathogen which is accountable for causing gastroenteritis, which can be further divided into various serovars[23]. It is the pathogen highest repeatedly associated with the ingestion of raw fruit and vegetables [24]. *S. enterica* serovars can inhabit seeds[25,26]., sprouted seeds [27], leaves [28,29], and fruit [30-31] of a range of plant species.

Potential risks of plant contamination: Abundant analysis has explored the probable risks of contamination in the resource sequence together during pre-cultivation and post-cultivation stages. Throughout the stages, harmful inhabitants can launch their habitat and get nurtured on cultivating yield. The hazard was capable of increased within subsequent yield whichever through promoting straight contamination or through the propagation of surviving harmful inhabitants throughout handling and post- cultivation managing measures.

The principal cause of contamination in the area is mostly found to be water after too many studies. Potential causes are overspill from adjacent cattle feedstuffs and watering from an infected water supply. The hazard concomitated by employing water from a variety of resources that diverge in the microbiological attribute for watering of yield has been evaluated and the requisite for superior policies documented[32,33]. This proposes an inferior possibility of spreading pathogens from unhygienic water throughout drip sprinkling of water against a superior possibility through overhead sprinkler routines. However, watering is not the merely narrated itinerary of contamination concomitant to water.

The consumption of water in post-cultivation handling has similarly performed a task. An outburst of illnesses *amongst S.*Newport was concomitant to the ingestion of mangoes remedied with a procedure comprising boiling water targeted at avoiding the introduction of fruit flies[34]. Pathogens may be relocated to the ecosystem by the remedy of inefficiently composted or raw cattle composts or sewage[35-38]. The feces of wild animals may similarly be a resource[39,40]. In laboratory circumstances, infected flies have been revealed to the straight transmission of bacteria to plant leaves or fruits[41-43]. Post harvesting practices, extending from storage and sluicing to slicing, furthermore probable sources of contamination[44]. Incise exteriors of leaves are a precise recipient for harmful microbes such as *Salmonella sp.*, which indicates precise colonization concerning them[45].

Materials and methods

List of Materials and reagents: i. Ice-bag, ii. Sterile plastic zipper bags, iii. Sterile plastic water collection bottles, iv. Sterile disposable gloves, v. 2% sodium hypochlorite, vi. Antibiotics, vii. Buffered Peptone Water (BPW), viii. Rappaport-Vassiliadis (RV) broth, ix. Tetrathionate (TT) broth, x. Hektoen Enteric Agar (HEA), xi. Xylose Lysine Deoxycholate (XLD) agar, xii. Triple Sugar Iron (TSI) agar, xiii. Mueller-Hinton agar (MHA), xiv. Gram staining reagents, xv. Hi25™ Enterobacteriaceae Identification Kit, xvi. Glasswares.

Study area: Surat city, recognized as the diamond and textile capital of India, is located in the state of Gujarat, India (Figure-1). The region is densely occupied with various cultural people. Surat is growing and introducing salad vegetables from adjacent regions and states.

Sample collection: A sum of 300 samples of raw salad vegetables [each of n=60 Tomato(A), Cucumber(B), Cabbage(C), Spinach(D), Carrot (E)] were collected from December 2018 to November 2019 from the regional vegetable market. Samples were accumulated at random times and from random retailers of a random area of the Surat city (Katargam, Varachha, Amroli, Olpad, Adajan, Kamrej) (Figure- 2,3,4,5,6). Some of the samples were collected from regional farmers and others from random retailers.

During sample collection details of cultivation, transportation, and source origin is collected from the retailer by using data collection form. Whenever sample was collected directly from farmers, from each farm supporting samples such as irrigation water (Figure-7) and soil was also analyzed to find out the exact route of contamination. The samples were transferred rapidly to the laboratory by using an icebox and were proceeding immediately 5-6 hours after gathering. The individual vegetable sample was kept in an individual sterile plastic zipper bag and marked as a specific ID number and with the collection date.

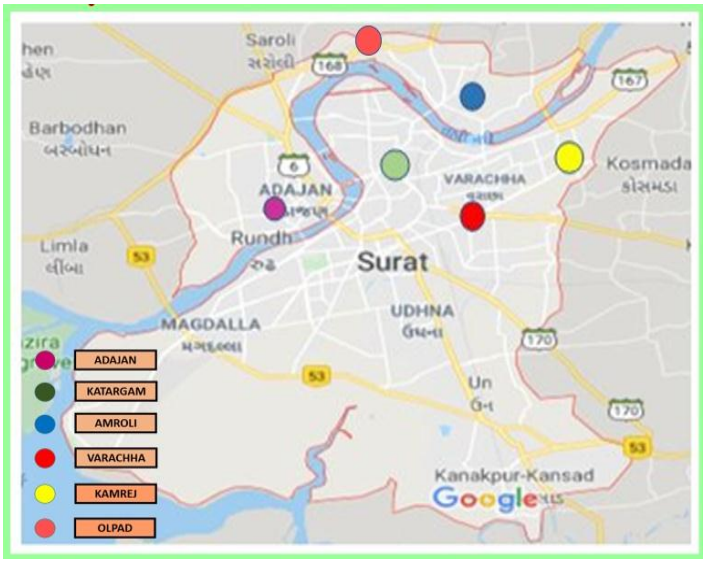

Figure-1: Study area shows different regions of Surat.

Microbiological analysis: Assumed endophytic strains of *Salmonella sp.* were acquired from the fragments of the exterior-sterilized raw salad vegetable samples employing earlier explained protocol[17]. In concise, individually salad vegetable sample exterior was sterilized using 2% sodium hypochlorite for 30 seconds at room temperature. Each sample was washed for three spells with sterile distilled water to entirely eradicate the decontaminators. 25 grams of each sample was evaluated aseptically, mixed by blending in 225 ml of BPW followed by overnight incubation at 37 ± 1°C for pre-enrichment of the sample, and methods explained below were further operated for the detection and confirmation of *Salmonella sp.*

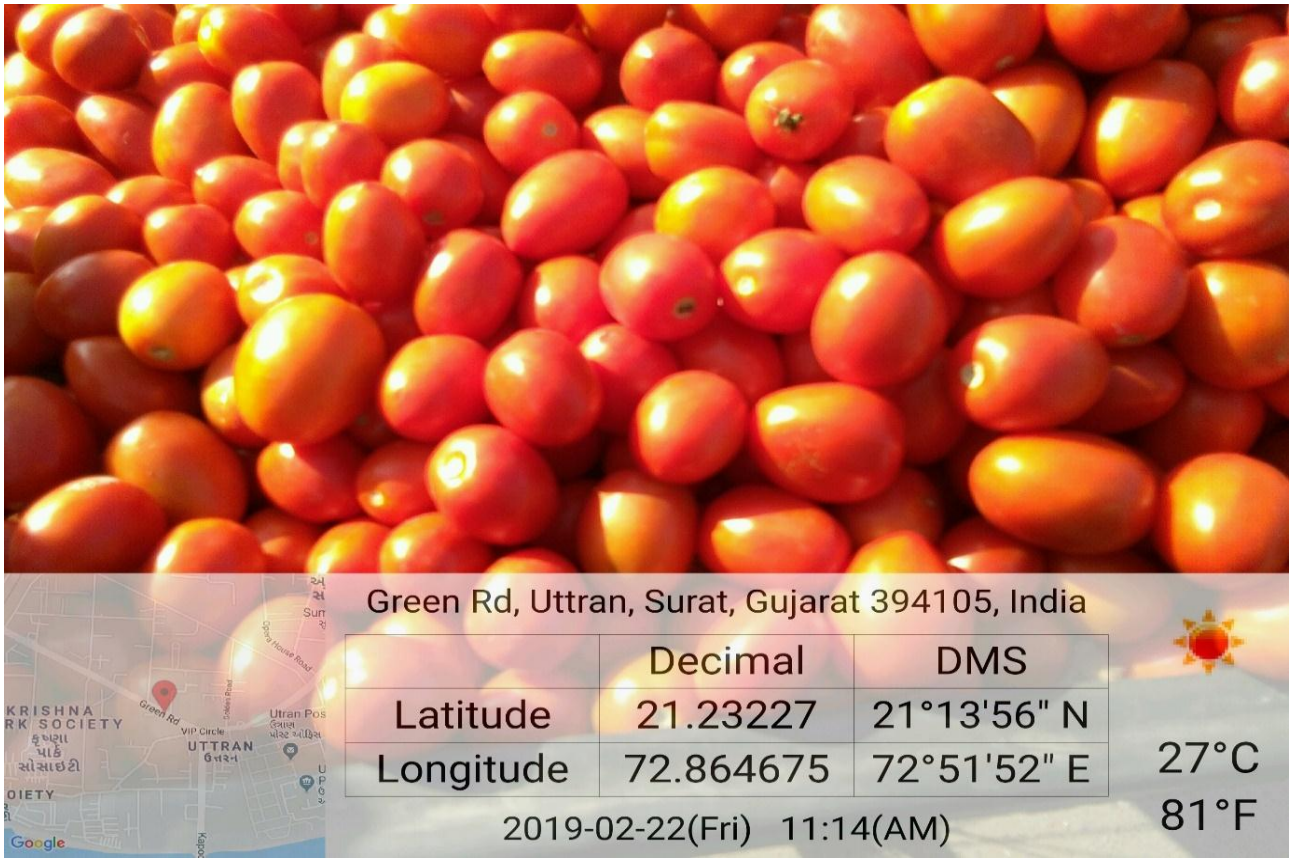

Figure-2: Sample A collection site.

Figure-3: Sample B collection site.

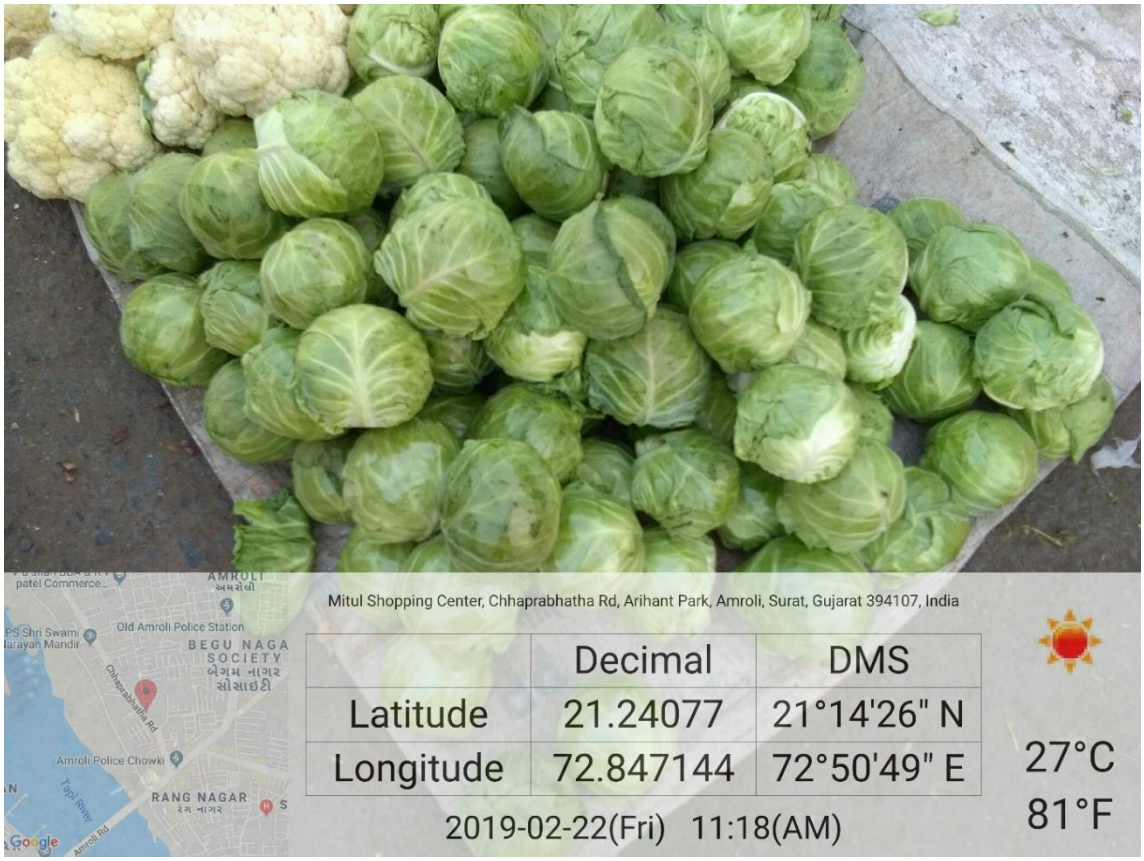

Figure-4: Sample C collection site

Figure-5: Sample D collection site.

Figure-6: Sample E collection site.

Figure-7: Sewage water directly used as irrigation water in a cultivation farm

Detection and isolation of Salmonella sp.: Detection and isolation were done with the help of the ISO 6579-1:2017 E suggested process. In concise, 1 and 0.1 mL of uniform pre-enriched peptone water have inoculated into tetrathionate (TT) broth with Novobiocin (Himedia) and Rappaport–Vassiliadis (RV) broth (Himedia), correspondingly. The pre-enriched broth was incubated for 24 ± 2 hr, correspondingly, at 37 ± 2°C (for TT broth) and 42 ± 2°C (for RV broth). The assured cultures were streaked onto Mac Conkey's agar plate (Himedia) at 37 ± 1°C for 24 hr., and XLD agar plate (Himedia) at 37 ± 2°C for 24 hr. The plates were inspected morphologically for the existence of distinctive *Salmonella sp.* colonies (Figure- 8,9). When plates exhibit, more than one colony was transported to HE agar plates (Figure-10) and TSI agar slant (Figure-11), followed by 25 biochemical tests using the Hi25™ Enterobacteriaceae Identification Kit for the generic identification of *Salmonella sp*.

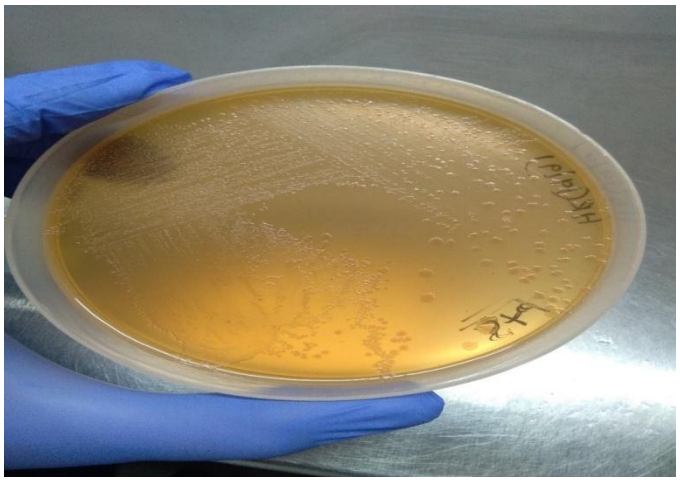

Figure-8: Colony characteristics observed on Mac Conkey's agar plate (Himedia).

Figure-9: Colony characteristics observed on the XLD agar plate (Himedia).

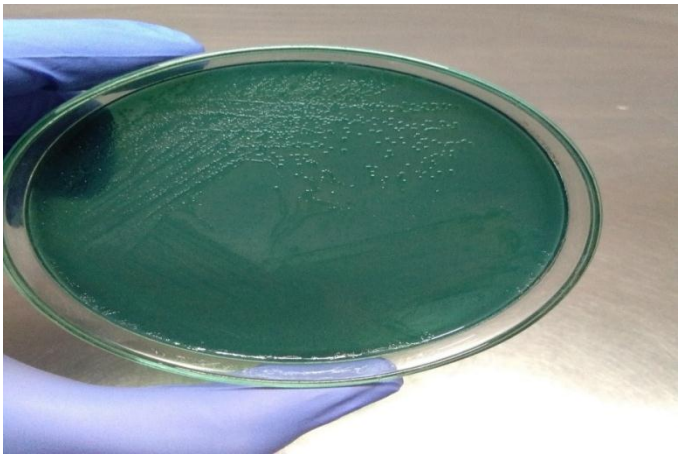

Figure-10: Colony characteristics observed on the HE agar plate (Himedia).

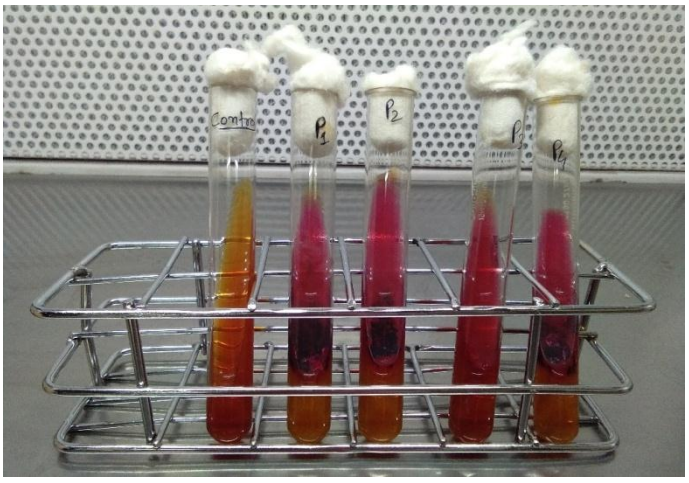

Figure-11: Growth characteristics of TSI agar slants shows H_2S and gas production.

Molecular characterization of *Salmonella sp.:* Molecular methods are valuable indications for the comprehensive categorization of Enteropathogenic *Salmonella sp.* Out of 34 isolates from the above study 2 isolates (DP1 AND DP3) subjected to molecular categorization and the rest, isolates are under process.

Antibiotic drug resistance testing and pattern study: Antibiotic drug resistance testing of *Salmonella sp.* was analyzed applying the disk diffusion method on MHA medium (Figure-12) corresponding to the CLSI 2012. Entirely 15 antibiotic drugs were tested against isolated *Salmonella sp*. (Table-2).

Results and discussion

Endophytic *Salmonella sp.* isolates from raw salad vegetables: Out of 300 raw salad vegetable samples, 34 (11.3%) samples were confirmed for endophytic *Salmonella sp.* where the prevalence ranged from 0.6% to 4.6% differing from the variety of raw salad vegetables (Table-1).

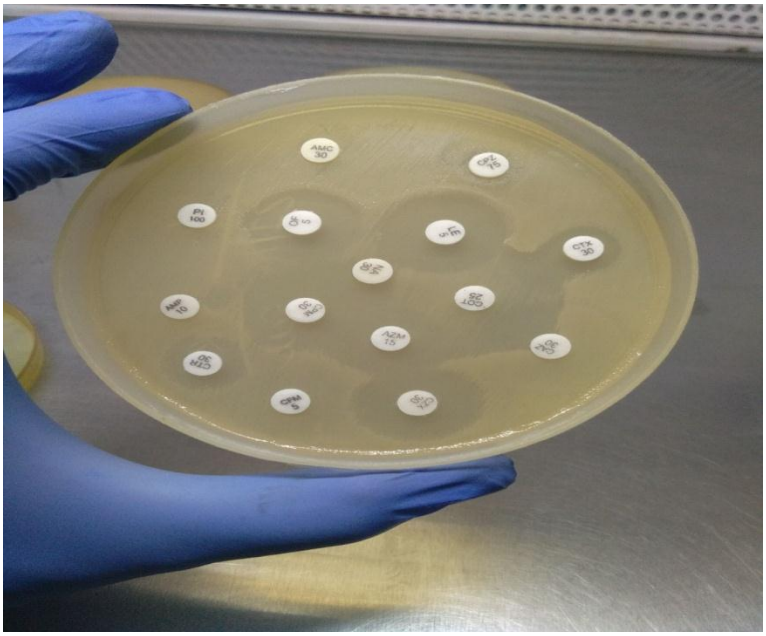

Figure-12: Drug resistance pattern of DP1 isolates on MHA medium plate.

Table-1: Outline of the prevalence of *Salmonella sp.* in raw salad vegetables.

Raw salad vegetable type(n)	% Prevalence* of *Salmonella sp.*	Number (%) of *Salmonella sp.* isolates	Isolated *Salmonella sp.* labeled as.
Tomato(A) n =60	2.6	8 (23.5)	AP1- AP8
Cucumber (B)n =60	2.0	6 (17.65)	BP1-BP6
Cabbage (C)n =60	1.3	4 (11.76)	CP1-CP4
Spinach (D)n =60	4.6	14 (41.17)	DP1 -DP14
Carrot (E)n =60	0.6	2 (5.88)	EP1-EP2
Total n= 300	11.3	34 (11.33)	-

*% prevalence = (total number of isolates /total number of samples analysed) X 100

16s rRNA sequence analysis: The following 16s rRNA sequence analysis was found in the selected isolated *Salmonella sp.*

Antibiotic drug resistance and pattern study: The antibiotic drug resistance study of *Salmonella sp.* was investigated against 15 commercially accessible antibiotic drugs (Table-2). The above experimental statistics show, considerable drug resistance in the isolated *Salmonella sp*. Out of all isolates drug resistance to AMC 30 (Amoxiclav) was most prevalent (35.29%). Resistance towards, CPZ, PI, CTX, NA, COT, AMP, CPM, CAZ, and CZX was observed in moderate to high proportions ranging from 5.88% to 29.41%. Out of all drug-resistant *Salmonella* isolates, four isolates (AP1,AP6 isolated from tomato (A) samples and DP1, DP3 isolated from spinach(D) samples) (11.76%) were obtained to be resistant to 5 or more antibiotic drugs.

Discussion: The above analysis was commenced to reveal the prevalence of *Salmonella sp.* from raw salad vegetables and their drug-resistant pattern along with finding out potential risks of endophytic contamination of raw salad vegetables at the Surat city of Gujarat, India.

In this investigation, the prevalence (11.3%) of *Salmonella* in raw salad vegetables is supported with former studies, where this pathogen was noticed at 4.0%[46,47]. One of the investigations performed in India on raw yield stated a much elevated (28.1%) occurrence of this pathogen[48]. However, surveys conducted in Spain and the Czech Republic stated a depleted prevalence from 0.1 to 0.3%[49,50]. The inconsistency in data may reveal the distinction in agricultural traditions and hygiene procedures[6]. In the present study, *Salmonella sp.* strains were recovered as endophytes from the inner tissues of surface-sterilized samples. This realizes the former conclusions that *Salmonella sp.* can

penetrate or colonize the deep tissues of plants[16,17] and colonize or endure to parts that are unreachable to sterilization action[51,52].

After the 16s rRNA sequencing process, BLAST analysis was performed with the existing 16srRNA bacterial sequence available in the nucleotide databases (NCBI). Sequencing and BLAST result revealed that the sample which was labeled as DP1 showed similarity with ***Salmonella bangori*** which showed sequence similarity with ***Salmonella bongori strain* NCTC 12419 16S ribosomal RNA** Accession number NR_074888.1 (Figure-13) and **DP3** showed similarity with ***Salmonella enterica*** which showed sequence similarity with ***Salmonella enterica subsp. enterica serovar Typhimurium strain* ATCC 13311 16S ribosomal RNA** Accession number NR_119108.1 (Figure-14). Results of the 16S rRNA BLAST analysis revealed that raw salad vegetable isolates belong to the *Salmonella* strain.

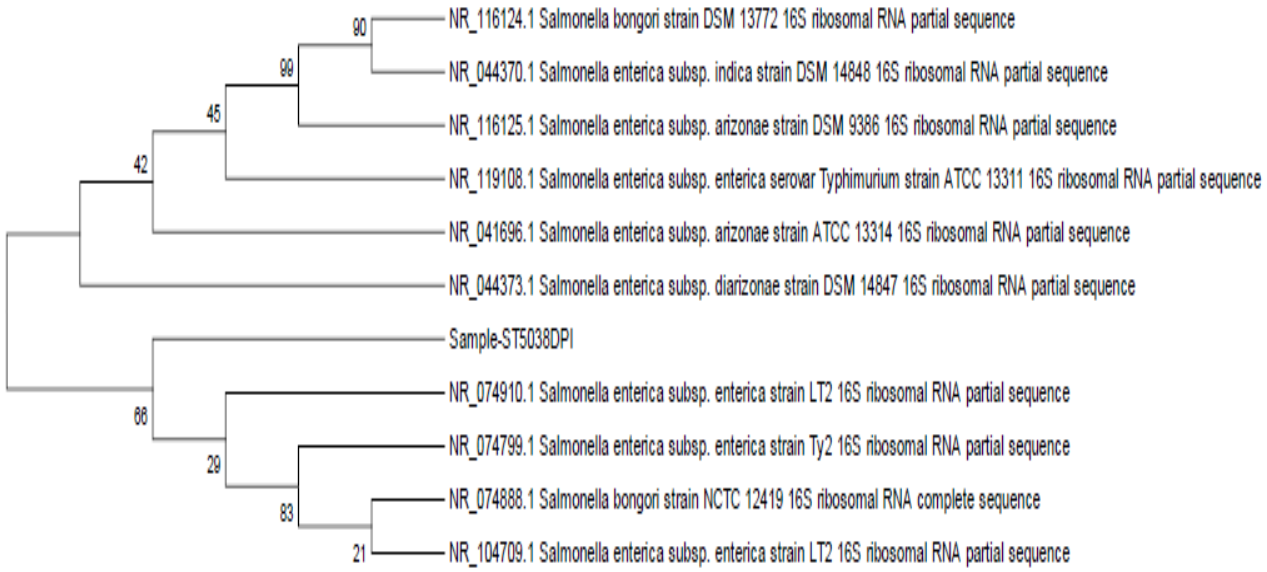

Figure-13: The evolutionary history was inferred using the Neighbour-Joining method and analyses were conducted in MEGA6.

Figure-14: The evolutionary history was inferred using the Neighbor-Joining method and analyses were conducted in MEGA6.

Table-2: Prevalence of antibiotic drug resistance in isolated *Salmonella sp.*

Antibiotics(HiMedia) CODES*	Amt(s)/Disc(μg)	Resistant *Salmonella sp. (n)*	Resistant *Salmonella sp. (%)*	Name of isolated resistant *Salmonella sp.*
AMC 30	30	12	35.29	AP1,AP6,AP8,BP3,BP5,CP1,CP4, DP1,DP3,DP7,DP10,DP11,DP14,EP2
AMP 10	10	10	29.41	AP1,AP3,AP6,AP7,BP3,BP5,CP1, DP1,DP3,DP7
CAZ 30	30	10	29.41	AP1,AP6,BP3,BP4,DP1,DP3,DP6 ,DP10,DP12,EP2
CPZ 75	75	8	23.52	AP1,AP6,BP3,CP4,DP1,DP3, DP4,DP11
CTX 30	30	8	23.52	AP1,AP6,BP3,CP1,DP1,DP3, DP7,EP1,
PI 100	100	4	11.76	AP1,DP1,DP3,DP7
NA 30	30	4	11.76	AP2,AP3,DP1,DP2
CPM 30	30	4	11.76	AP2,AP6,DP1,DP7
COT 25	25	2	5.88	DP1,EP1
CZX 30	30	2	5.88	CP1,DP3
OF 5	5	0	0	-
LE 5	5	0	0	-
AZM 15	15	0	0	-
CTR 30	30	0	0	-
CFM 5	5	0	0	-

*AMC 30= AMOXICLAV, CPZ 75= CEFOPERAZONE, PI 100 =PIPERACILLIN,OF 5 = OFLOXACIN,LE 5= LEVOFLOXACIN, CTX 30=CEFOTAXIME, NA 30=NALIDIXIC ACID, COT 25=C0TRIMAXAZOLE, AMP 10=AMPICILLIN, CPM 30=CEFEPIME, AZM 15=AZITHROMYCINE, CAZ 30=CEFTAZIDIME, CTR 30=CEFTRIAXONE, CFM 5=CEFIXIME, CZX 30= CEFTIZOXIME

Conclusion

The contemporary analysis showed the potential risks of *Salmonella sp*. contamination of raw salad vegetables collected from the local vegetable market and direct from the farms in the Surat city of India. Detection of endophytic *Salmonella sp*. from raw salad vegetables indicating the approval of noble agricultural methods and superior hygienic exercises is mandatory to reduce the contamination of raw salad vegetables. From the above, it is observed that out of all 5 salad vegetables spinach showed the highest % of prevalence (4.6%) that indicate it may support the growth of the *Salmonella sp.* Though, the incidence of pathogenic bacteria such as *Salmonella sp*. in raw salad vegetables must not be undervalued, principally for unprocessed utilization. The extremely potential risks of vegetable performing as agents of the harmful microbes might be cultivating the vegetables using infected soil or irrigation with contaminated water which support data of this study as soil and water samples discovered to be contaminated with *Salmonella sp*. throughout the study. From the above data, it can be concluded that these data are alarming towards agricultural processes and food safety, and indicate the need for rethinking and research about challenging the fitness of *Salmonella sp.* which show the variety of hosts vary from plants to animals.

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