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Fresh Farm Vegetables as a Source of Virulent Drug Resistant *Salmonella enterica*

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A B S T R A C T

Plants are well known as vectors for transmission of human enteric pathogens and exaggerate the outbreak events. *Salmonella enterica*, rod shaped, facultative anaerobic bacteria interferes with the microbial safety of raw vegetables. This study explored three sources: Village fields, Supermarkets and Street Vendors to ensure the microbial risk among seven raw vegetables (N=725). Water samples of the hand pumps sourced from village fields showed total coliforms >10ml^{-1} (75.6-98.5%) and faecal coliforms >10ml^{-1} (59.6-91.2%). The highest Aerobic Mesophilic count (6.66 log cfu g^{-1}) in spinach samples and highest *Salmonella enterica* count (5.09 log cfu g^{-1}) in tomato samples procured from vendors was observed. Resistance to Lyncosamides, Tetracycline, Imipenem, Metronidazole and Cloxacillin has been observed. Twenty isolates were found in high Multiple Antibiotic Resistance (MAR) index range 0.36-0.8. Polymerase Chain Reaction (PCR) analysis has revealed the presence of sipB (100%), sopB (100%), spiA (100%), invA (100%) except hilA gene which was found absent in all isolates. The in-vivo expression of these virulence genes and resistance to multiple antimicrobials can potentially threat the healthy being of consumers.

Introduction

Salmonellosis is a disease caused by *Salmonella* spp. which governs its transmission through contaminated water, vegetables, meat and meat products (Schleker *et al.*, 2015).

Salmonella was reported to be present in lettuce, spinach, tomato, radish (De oliveira *et al.*, 2011; Sant'Ana *et al.*, 2012). Recent multistate outbreak of *Salmonella* infection was declared by CDC in 2015 affecting a total of 907 persons. According to CDC 2016 report, *Salmonella* contaminated cucumbers imported from Mexico were the likely source of outbreak. Belonging to *Enterobacteriacea* family, *Salmonella* is well known for its intracellular nature. The recognition that the *Salmonella enterica* serovar *typhi* is host adapted pathogen is well established (Gopinath *et al.*, 2012). The plant edible regions and resident epiphytic bacteria harbour the colonization of *Salmonella* during stress and increase its fitness (Guo *et al.*, 2001; Delbeke *et al.*, 2015; Potnis *et al.*, 2015).

Epidemiological studies have shown that India has increased number of Multi Drug Resistant (MDR) *Salmonella* strains (Ochiai *et al.*, 2008). Resistance to traditional

antibiotics like ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole (Sood *et al.,*1999) against salmonella infections has led to switching for new antibiotics like fluoroquinolones and cephalosporins which are also being ineffective (Hasan *et al.,* 2008).

The targeting of virulence genes in the indigenous isolates of *Salmonella* may reflect the risk of illness channelled through raw consumption of vegetables. Various chromosomal species specific virulence gene such as *invA*, *hilA*, *sipA*, *sipB* and *sopB* are important elements in pathogenesis. The protein InvA is essential for epithelial invasion (Galán and Curtiss, 1990). The *hilA* gene product regulates the expression of invasion genes encoded on *Salmonella* pathogenicity island 1 (SPI1) and is important virulence determinant in *Salmonella* pathogenesis (Bajaj *et al.,* 1996). *Salmonella* invasion protein SipA aids the internalization of bacteria by binding and polymerising the host actin (Lilic *et al.,* 2003). Cell invasion protein SipB is required for cell to enter in host plasma membrane and induction of apoptosis by activating caspase-1 (Hersh *et al.,* 1999). *Salmonella* virulence mechanism involves delivery of effector protein sopB (inositol polyphosphate phophastase) that act into the host cytoplasm through type III secretion system (Galan and Zhou, 2000).

According to the regulations laid by Bureau of Indian Standard IS (5887:1999) for *Salmonella*, absence in 25gram of food sample is mandatory to keep it safe.

The present study focussed on the phenotypic profile based on biochemical and antibiotic susceptibility of indigenous *Salmonella enterica* isolates from fresh vegetables from three sources and distribution of its virulence genes so as to put forward proper sanitation strategy to obstruct the spread of food borne illness.

Materials and Methods

Study area and sampling

The Buddha Nallah, a natural stream of Sutlej, traversing through Ludhiana city of Punjab state has high Total Dissolved solids >1000 mg/L, chlorides up to 400 mg/L, Chemical Oxygen Demand >400 mg/L, Biochemical Oxygen Demand 52-195 mg/L, Most Potable Number upto 2400+ per 100ml. Villages along the Buddha Nallah are known for vegetables growers. They use irrigation pumps, river or streams for cultivation. Epidemiological surveillance study for the microbiological quality of fresh vegetables was routinely carried out during the vegetable of the season for the period of $2^{1/2}$ years (July 2013-December 2015).Water samples from the irrigation pumps were collected according to the standard method of BIS (IS-10500:1991).

A total of 420 samples of salad vegetables such as carrot, radish, cucumber, tomato, cabbage, spinach and long melon were procured from village fields along Buddha Nallah. In comparison, 75 samples from supermarkets and 230 samples from street vendors were also collected.

Enumeration of *Salmonella enterica*

The fresh vegetable, 25grams of sample was taken and washed with autoclaved water so as to omit any environmental contamination. Vegetable sample was chopped with the help of sterile knife into 2-3cm pieces and transferred to the 225ml water blank. It was shaken vigorously for uniformity and serial dilutions of the suspension were spread onto Salmonella-Shigella Agar Base (HiMedia Laboratories Pvt. Ltd., Mumbai) IS (5887:1999) plates in triplicates for each dilution and incubated at 37°C for 24-48 hours. Following incubation, all colonies on

dishes containing 30-300 colonies were counted per dilution and log cfu g^{-1} was calculated. The presumed colonies were verified by complete phenotypic characterisation.

Phenotypic characterization

The isolates were verified as *Salmonella* spp. by their size, shape, Gram's staining, simple staining, motility and colony morphology, biochemical tests, virulence based tests and further confirmed with molecular method. Antibiotic susceptibility was also evaluated using the Kirby–Bauer disc diffusion method on Mueller Hinton agar with 25 antibiotics belonging to 15 different classes; β-Lactams, Fluoroquinolones, Aminoglycoside, 1st Generation Cephalosporins, 2nd Generation Cephalosporins, 3rd Generation Cephalosporins, Chloramphenicol, Tetracycline, Metronidazole, Macrolide, Sulphonamides, Lyncosamides, Glycopeptides, Nitrofurans and Carbapenems.

Bacterial genome extraction

Genomic DNA was extracted from colonies that were identified as *Salmonella enterica* with the help of Invitrogen Easy DNA® Isolation Kit (Invitrogen Inc.) as per the manufacturer's protocol from selected bacterial strains grown in nutrient broth for overnight at optimal temperature. DNA was eluted into 100µl 1X TE buffer (Invitrogen Inc.). Quantity and quality of DNA was checked on TECAN 2000 Nanoquant Plate.

The DNA of all the samples was diluted to 25ng/µl by adding nuclease free water and stored at -20°C. Alternatively, DNA quality was checked on 0.8% agarose gel. A single sharp band of DNA signified high quality of DNA. DNA of each sample was diluted to 25ng per µl of nuclease free water (Promega Inc.) before the PCR assay.

PCR assay

Uniplex PCR was performed with reaction volume of 30 µl for each sample, that contained 1.5 mM of 25mM $Mgcl_2$ (Promega Inc.), 1X Go Taq TM buffer (Promega Inc.), 0.2 mM of dNTPs (Promega Inc.), 0.5µM of each primers (Table 1), 2U of GoTaqTM DNA polymerase (Promega Inc.) and 50 ng/µl of the DNA template. The final volume of the reaction was adjusted with Nuclease free Water (Promega Inc.).The PCR conditions consisted of a pre-incubation step for 5 min at 94°C, 35 cycles of denaturation at 94°C for 30sec, annealing step for 30sec (Table 1) with extention time of 40sec at 72°C and a termination step with a final extension for 7min at 72°C.

After amplification, the product was loaded onto the 1.5% agarose gel, visualized under UV light and photographed using SYNGENE gel documentation system with Gene snap software programme.

Results and Discussion

MPN index of irrigation water

Water for irrigation has utilized faecal coliform as an indicator of acceptable quality. It was observed that all vegetable samples growing areas (village fields) around Buddha nallah have contaminated ground water with high MPN index of >10/ml (1000/100ml) with highest positive percentage of 98.5 and 91.2 for cucumber and spinach respectively (Table 2). This value is above the desirable limit (1,000 MPN/100ml as recommended Indian environment ministry) and undesirable for irrigation purpose.

Water can predispose the coliform to the fields if the ground water is mixed with the sewage, runoff water in from immediate vicinity of livestock and use of untreated

manure into the fields. Owing to dissemination of pathogens into the field, greater risk may establish which aids reaching the pathogens to their threshold of virulence with increase in nutrient abundance.

Aerobic Mesophilic Count (AMC) of vegetables

AMC per gram for each vegetable among three sources showed no significant difference (P>0.05) in mean log cfu value (Table 2). Among three sources, the mean AMC for spinach was in range of 6.25-6.66 log cfu g^{-1}, tomato 4.51-5.71log cfu g^{-1}, carrot 4.94-5.84 log cfu g^{-1}, Radish 4.8-5.75 log cfu g^{-1}, Cucumber 5.15-5.92 log cfu g^{-1}, cabbage 4.07-5.24 log cfu g^{-1} and Long melon 4.96-6 log cfu g^{-1} (Table 2). Similar to the findings of the present study, aerobic plate count of raw vegetables were evaluated to be in range of 5-10 log cfu g^{-1} (Viswanathan and Kaur, 2001; Saddik *et al.,* 1985; Aycicek *et al.,* 2006; Jeddi *et al.,* 2014).

The highest count on spinach phyllosphere was due to its leafy structure, leaf features such as glandular trichomes and stomata where bacteria aggregate and propagate in the ambient temperature conditions (25-35°C). AMC is considered indispensible microbiological indicators for food quality as it reflects the exposure of the sample to any contamination, and, in general, the existence of favourable conditions for the multiplication of microorganisms. In our study, the AMC of all the vegetables from street vendors has mean count lower than previously reported by Viswanathan and Kaur (2001). This might be due to the fact that the washing of raw vegetable prior to microbiological analysis has eliminated the effect of bacteria in soil which is attached to the phyllosphere.

Among different sources, the highest AMC was found in vegetables from vendor whereas lowest count was of vegetables procured from supermarkets (Table 2). Seo *et al.,* (2010) reported AMC ranged between 2.0-9.7log cfu g^{-1}in 345 minimally processed vegetables from department store, supermarket, and restaurant. Carrot samples procured from wholesalers and retail stores had found with total plate count of 3.8–6.5log cfu g^{-1} and 7.8log cfu g^{-1} (Aycicek *et al.,* 2006; Abadias *et al.,* 2008). Mean AMC of supermarket vegetables (Table 2) was observed in agreement with Cardamone *et al.,* (2015) and Aycicek *et al.,* (2006). The lower AMC of supermarket vegetables as compared to village fields and vendors was due to the thorough washing of the vegetables and storage at low temperature. However, prolonged storage and inherent quality of the vegetable are the other determinants of microbial spoilage.

On comparison of village field samples with those which were vending, no significant value observed and it implies to the fact that majority of the vendors purchase vegetables from the nearby village fields from where easy marketing with low freight is possible.

This leads to the circulation of same microbial quality product throughout city and made negligible or little change due to poor handling and improper sanitation.

Occurrence of *Salmonella enterica*

Out of 420 vegetable samples collected from village fields, 243 (57.90%) were positive for *Salmonella enterica* which was higher than its percent occurrence in supermarket samples (38/75, 50.12%). The percent contamination increased to 73.48% in vendor samples.

Cucumber samples from vendors had shown highest percent contamination by *Salmonella enterica* (82.85%) followed by 66.66% in case of tomato and 78.12% in cucumber from

village fields (Fig. 1). Results in this study completely contradict 1.3% and 3.3% occurrence observed by Abadias *et al.,* (2008) and García-Villanova *et al.,* (1987) in fresh produce. Comparable percentage prevalence of Salmonella was observed by Salleh *et al.,* (2003) in vegetable samples.

The high incidence may attribute to environmental contamination sourced from animal manure applied to the fields, contaminated water used for irrigation, post-harvest poor handling of the vegetables and improper storage conditions.

The samples with vendors had shown even highest rate of *Salmonella* which might be due to the additive effect of constant sprinkling of the contaminated water over the vegetables for retaining their freshness. The high predisposition of pathogen into the vegetables after harvesting should be avoided and critical decontamination approaches should be adopted.

All vegetables samples tested were found to have >4 log cfu g^{-1} mean count. Count from village field samples range from 4.32 log cfu g^{-1} in spinach to 4.97 log cfu g^{-1} in carrot (Table 2).

Supermarket samples were also found to effect the growth of *Salmonella enterica* with mean count range of 4.02 log cfu g^{-1} in long melon to 4.61 log cfu g^{-1}.

Among all vegetable samples, *Salmonella enterica* count was highest recorded in tomato samples collected from street vendors (Table 2). The contamination is mainly through root uptake system and through natural openings on plant surfaces. *Salmonella* thrive better in the stem scar and cracks in tomato and their survival rates make the inactivation of the internalized pathogen more challenging (Cummings *et al.,* 2001).

Mean count of *S. enterica* in carrot, radish, tomato, cucumber and spinach among the village fields and street vendor differ significantly from supermarket samples ($p<0.05$). The low count of *Salmonella* in supermarket vegetables as compared to village fields and vendors was due to the thorough washing of the vegetables and storage at low temperature. However, prolonged storage and inherent quality of the vegetable are the other determinants of microbial spoilage.

The organic agricultural practises in India emphasize on the use of animal manure to the fields which can be a factor of introduction of food linked pathogens. Pre-treatment of this fertilizer prior to employment should be critically considered.

Table.1 List of primers used for targeting species specific virulent genes of *Salmonella enterica*

Gene	Primer sequence (5'→3')	Product size (bp)	Annealing temp.	Reference
invA	*F-GCCTGCCGGAAGTATTGTTA* *R-ACCGCCAGACAGTGGTAAAG*	280	54.5°C	Suo *et al.,* 2010
hilA	*F-ATTGCCGAGAAAATGTGGAA* *R-TCAGCCCATGCCGTATTTAT*	169	52°C	*-do-*
Spi A	F-CCAGGGGTCGTTAGTGTATTGCGTGAGATG R-CGCGTAACAAAGAACCCGTAGTGATGGATT	550	66.5°C	El-Allaoui *et al.,* 2013
Sip B	F-GGACGCCGCCCGGGAAAAACTCTC R-ACACTCCCGTCGCCGCCTTCACAA	875	66.5°C	*-do-*
Sop B	F-CGGACCGGCCAGCAACAAAACAAGAAGAAG R-TAGTGATGCCCGTTATGCGTGAGTGTATT	220	66.5°C	*-do-*

Table.2 Microbiological analysis of raw vegetables collected from three source

Vegetables	Source	Total coliforms[b] >10/ml	Faecal coliforms[b] >10/ml	Mean AMC[a] (log cfu g^{-1})	*Salmonella enterica* count[a] (log cfu g^{-1})
Carrot	Village field	90.1	68.1	5.55[d]	4.97[k]
	Supermarket	ND	ND	4.94[d]	4.54[l]
	Street vendors	ND	ND	5.84[d]	4.83[k]
Radish	Village field	86.5	70.1	5.44[e]	4.77[m]
	Supermarket	ND	ND	4.8[e]	4.38[n]
	Street vendors	ND	ND	5.75[e]	4.72[m]
Cucumber	Village field	98.5	74.6	5.92[f]	4.60[o]
	Supermarket	ND	ND	5.76[f]	4.07[p]
	Street vendors	ND	ND	5.15[f]	4.51[o]
Tomato	Village field	75.6	64.9	5.71[g]	4.95[q]
	Supermarket	ND	ND	4.51[g]	4.61[r]
	Street vendors	ND	ND	5.51[g]	5.09[q]
Cabbage	Village field	86.5	59.6	5.24[h]	4.54[s]
	Supermarket	ND	ND	4.07[h]	4.08[s]
	Street vendors	ND	ND	5.15[h]	4.32[s]
Spinach	Village field	96	91.2	6.65[i]	4.32[t]
	Supermarket	ND	ND	6.25[i]	4.07[u]
	Street vendors	ND	ND	6.66[i]	4.44[t]
Long melon	Village field	98.3	79	5.55[j]	4.35[v]
	Supermarket	ND	ND	4.96[j]	4.02[v]
	Street vendors	ND	ND	6[j]	4.23[v]

a- level of significance at $p<0.05$

b- Desirable limit is 10/ml

ND= Not Determined (low pH of Tomato gives false positive results, true representative of water sample for vegetables washing could not be produced)

Table.3 Multiple Antibiotic Resistance (MAR index) pattern for *Salmonella spp.* Isolates

Isolates	Multiple Antibiotic Resistance Index
LM INT 25	0.6
CU INT 64	0.6
CB INT 45	0.72
CR EXT 8	0.68
TO 34	0.68
CU EXT 6	0.68
SP 44	0.68
CR INT 25	0.72
TO 57	0.72
LM EXT 5	0.64
RA INT 22	0.64
SP 31	0.72
RA INT 58	0.72
CU EXT 44	0.72
RA EXT 10	0.72
SP 6	0.8
TO 21	0.8
CB EXT 18	0.52
RA EXT 37	0.52
MTCC 773	0.36

Fig.1 Percentage occurrence of *S. enterica* in vegetables among three sources

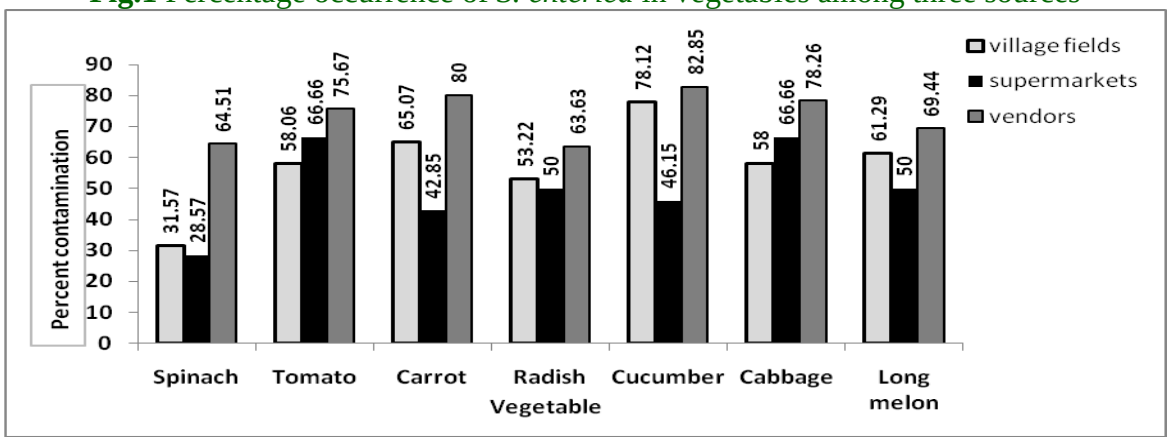


Fig.2 Percent frequency of antibiotic resistance exhibited by *Salmonella spp.* isolates

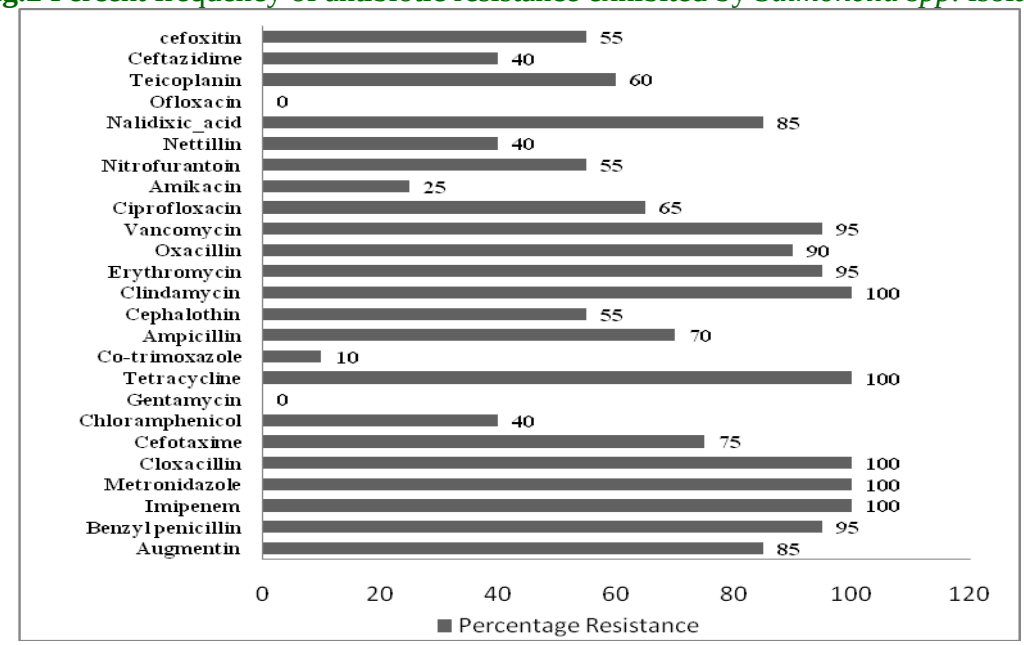


Fig.3 Agarose gel electrophoresis of PCR products from representative *Salmonella enterica* isolates. M lane stands for the molecular weight standard: 1kbp ladder (Invitrogen). Lanes 1-5 contains products of *S. enterica* isolates showing the 16S rDNA (312bp fragment) along with positive control of *S. enterica* MTCC 733

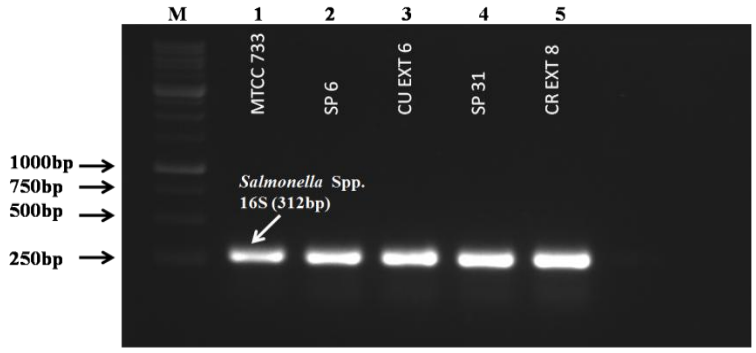


Fig.4 Agarose gel electrophoresis of PCR products from representative *Salmonella enterica* isolates. M lane stands for the molecular weight standard: 1kbp ladder (Invitrogen). Lanes 1-14 contains products of *S. enterica* isolates showing the *sipB* gene (875bp fragment) along with positive control of *S. enterica* MTCC 733

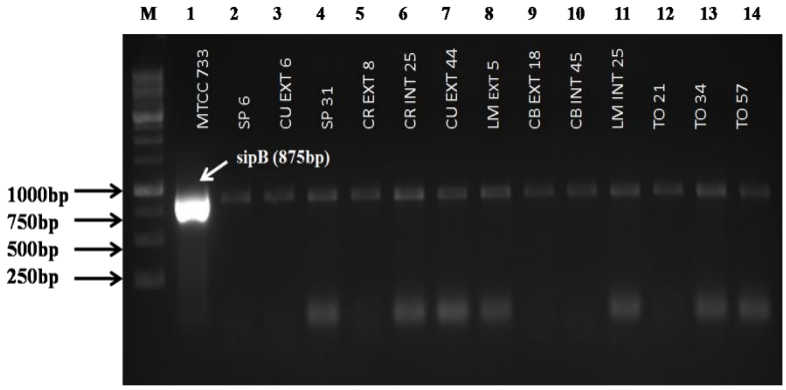


Fig.5 Agarose gel electrophoresis of PCR products from representative *Salmonella enterica* isolates. M lane stands for the molecular weight standard: 100bp ladder (Invitrogen). Lanes 1-16 contains products of *S. enterica* isolates showing the *sopB* gene (220bp fragment) along with positive control of *S. enterica* MTCC 733

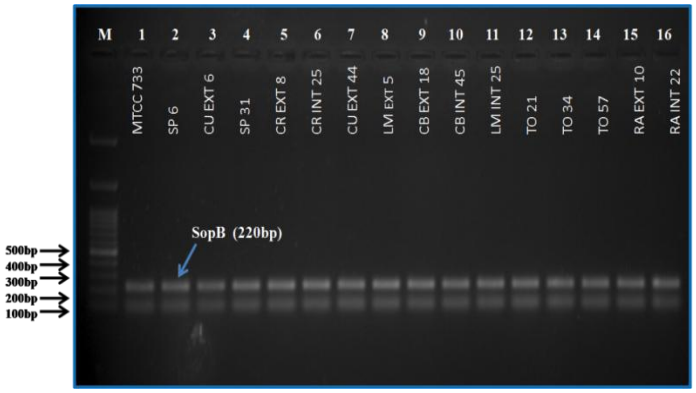


Fig.6 Agarose gel electrophoresis of PCR products from representative *Salmonella enterica* isolates. M lane stands for the molecular weight standard: 1kbp ladder (Invitrogen). Lanes 1-14 contains products of *S. enterica* isolates showing the *spiA* gene (550bp fragment) along with positive control of *S. enterica* MTCC 733

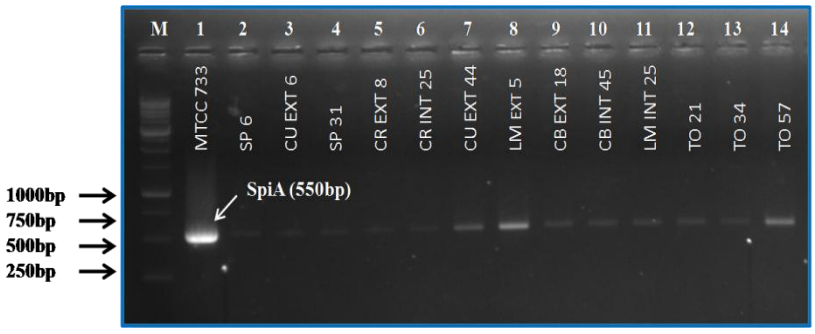
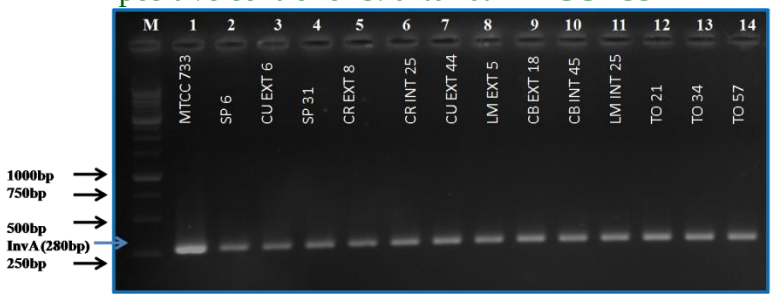


Fig.7 Agarose gel electrophoresis of PCR products from representative *Salmonella enterica* isolates. M lane stands for the molecular weight standard: 1kbp ladder (Invitrogen). Lanes 1-14 contains products of *S. enterica* isolates showing the *invA* gene (280bp fragment) along with positive control of *S. enterica* MTCC 733



Phenotypic characteristics of *S. enterica* isolates

Strains of *Salmonella* spp. isolated from vegetables were biochemically characterised and found that all the isolates were motile, majority non-haemolytic, H_2S producers, glucose fermenters but could not ferment lactose, sorbitol, xylose, rhamnose, raffinose and adonitol (data not shown).

Clindamycin (2mcg), Tetracycline (30mcg), Metronidazole (5mcg), Imepenem (10mcg) and Cloxacillin (5mcg) had shown 100% resistance to vegetable isolates of *Salmonella* spp. These isolates have high level of resistance to penicillin (95%), Augmentin (30 mcg) (85%), Vancomycin (30 mcg) (95%), Oxacillin (1 mcg) (90%), Erythromycin (15 mcg) (95%) and Nalidixic Acid (30 mcg) (85%) (Fig. 2).

Salmonella enteritidis and *Salmonella typhimurium* isolates obtained from the raw vegetables (*ulam*) were found to exhibit high resistance against ampicillin (100%), erythromycin (100%), amoxicillin/clavunic acid (81.3%), cephalothin (75%), streptomycin (50%) and ciprofloxacin (50%) (Najwa *et al.*, 2015). Other studies have found a certain level of tolerance in *Salmonella* strains against nalidixic acid, ciprofloxacin, chromaphenicol, co-trimoxazole, ampicillin, gentamycin, augmentin and amoxicillin (Chaudhary *et al.*, 2013; Adeshina *et al.*, 2012). The resistance pattern quite similar in our study has proved the indiscriminate use of antibiotics whose further practise may lead to complete resistance to tested antibiotics.

Scallan *et al.*, (2011) reported that more than 90% of the foodborne infections worldwide are due to *Salmonella* infections. These isolates may potentially transmit their virulence factors and cause human infections. The virulence and MDR of these isolates directly reflects the substantial contamination water of Buddah Nallah used for irrigation purpose or else its percolation to the soil that is used for cropping. The high incidence of *Salmonella* in vegetables and control of this spread of resistance needs attention and effection empirical therapy should be instituted.

The high MAR index range of 0.36-0.8 for *Salmonella* spp. isolated from fresh vegetables has been observed and could pose a serious health risk to the consumers when infectivity reaches a certain threshold (Table 3).

High MAR index recorded in the study has found in complete agreement with those discussed by Adeshina *et al.*, (2012) and Najwa *et al.*, (2015) in which *Salmonella* strains from raw salad vegetables had found with MAR index range of 0.27 to 0.82.

The detection of *Salmonella* is an indication of contamination of sewage of human origin, which was most detected in the study was also observed to be most resistant and hence implies human use/misuse of antibiotics. Although, it is possible that isolates may have acquired the genes for resistance to multiple antibiotics from other enteric bacteria.

Molecular detection of *Salmonella enterica* and its virulence gene distribution

All the 20 *Salmonella* strains (isolated and MTCC 733) were identified with 16S rDNA PCR assay and subsequent electrophoresis generated 312bp gene fragment (Fig. 3).

The 16S rDNA and 16S–23S rDNA internal transcribed spacer (ITS) region have been the most popular target for bacterial taxonomy. Furthermore, the ITS sequences of *Salmonella* serovars might be useful for analysis of the phylogenetic tree for *Salmonella* serovars.

Present study had also illustrated that all the *Salmonella* isolates were positive for *sipB* of 875bp (Fig. 4), *sopB* of 220bp (Fig. 5), *spiA* of 550bp (Fig. 6) and *invA* of 280bp gene fragment (Fig. 7) whereas none of the strain was positive for *hilA* (169bp).

These genes were also screened by other researchers in meat, poultry and food samples (El Allaoui *et al.*, 2013; Karmi, 2013; Rowlands *et al.*, 2014) which are in substantial agreement with our findings.

International Standard procedure for detection of *Salmonella* targets invasion gene *invA*, whose presence in our isolates has, confirmed its potential virulence (Amini *et al.*, 2010). The *inv*A gene which is responsible for invasion of cells, have the capacity to invade and survive in macrophages (Gole *et al.*, 2013). Presence of virulence genes such as *spiA*, *sopB* and *sipB* required for effective colonization and survival in host cell has been observed which corroborates it as potentially threatening entity. Although, *Salmonella* strains isolated from vegetables in the study vary in their biochemical and antibiotic susceptibility pattern as indicated by the clusters, the presence of virulence genes supports the potentiality of these isolates to cause human infections.

In conclusion, the present study provides considerable insight to level of contamination of fresh raw vegetables with *Salmonella enterica* which are routinely being consumed in daily households as well as sold to the restaurants. The high risk of illness associated with its raw consumption has been assessed and correlated with the virulence factors present in the isolates of *Salmonella enterica*. The high plate count of *Salmonella enterica* in seven vegetables can be marked for its well establishment in local environment. Presence of the target genes in indigenous isolates from this study is the evident of well-established salmonella pathogenicity islands (SPI). The prevalence of virulence genes and their expression in-vivo may contribute to emerging risks of illness. By understanding and determining these critical control points, contamination intervention strategy can be implemented stringently.

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