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ORIGINAL ARTICLE

Biofilm Formation of *Salmonella* Species Isolated from Fresh Coriander Leaves Sold in Puducherry, India

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Abstract

The aim of the study was to isolate *Salmonella* from coriander leaves, determine biofim formation and antimicrobial resistance profiling of the isolates. Four *Salmonella* isolates were recovered from the total of 60 samples. The isolates were subjected for biofilm production assay and showed that all *Salmonella* isolates were able to form biofilm. Biofilm forming isolates were subjected for antimicrobial resistance profiling. Three isolates were showed sensitivity against norfloxacin (92.5%). One was showing sensitivity against cefotaxime (90%). All the four isolates were showing complete resistant to ciprofloxacin, nalidixic acid, chloramphenical, gentamicin and cephalexin. Clindamycin and ofloxacin were showed sensitivity 20%. The finding of this study shows that coriander leaves are potential host for the transmission of *Salmonella* to human and animals. The ability of the isolates to form biofilm reveals the potential of the isolates to persist on the green leafy vegetables and the pathogenic status of the isolates as well as ability to resist antimicrobial chemotherapy.

Keywords: Biofilm, *Salmonella* spp, Antimicrobial resistance profiling, Coriander leaves.

1. Introduction

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. This has led to significant rise in the demand of fresh produce, changes in life styles and major shifts in consumption trends (Abadias *et al*., 2008; Tang *et al*., 2012). Vegetables can become contaminated with microorganisms capable of causing human diseases while still on the fields (Mukherjee *et al*., 2006). Bacteria such as *Clostridium botulinum*, *Bacillus cereus* and *Listeria monocytogenes* capable of causing diseases are normal inhabitants of many soils, 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 faeces, sewage, untreated irrigation water or surface water (Cliver, 1997). Fresh farm produce can be a vehicle for the transmission of bacterial, parasitic and viral pathogens capable of causing human illness. *Salmonella* is able to colonize in animal intestinal tracts, mainly swine and chicken, and shed in the faeces. Therefore, *Salmonella* contamination in pork or chicken is unavoidable in the human food supply chain. Additionally, probably due to the use of manure fertilizer in their cultivation, *Salmonella* contamination can also be found in various types of vegetables. The incidence and frequency of foodborne outbreaks caused by contaminated fresh vegetables is on the increase (Wegener *et al*., 2003; Raufu *et al*., 2014). *Salmonella* is one of the pathogen most frequently linked to consumption of fruit and vegetables (Sivapalasingam *et al*., 2004). The factors influencing the increase in *Salmonellosis* due to vegetables are changes in agricultural practices, eating habits and increases in the worldwide commerce of fresh produce (Raufu *et al*., 2014). An increasing number of antimicrobial resistant *Salmonella* has been reported in both developed and developing countries. In present times, bacterial biofilms have been more linked to food safety issues globally. Biofilm is formed when bacterial cells attach to one another and stick on to a contact surface. Biofilms are hazardous as they can become a persistence source of contamination (Houdt and Michiels, 2010). The existence of pathogenic organisms in biofilms has been linked to foodborne illness outbreaks in cantaloupe melons, apples, and

leafy greens (Annous *et al*., 2009). They are capable of adhering to plant surfaces and forcefully infect the plants interior (Schikora *et al*., 2012). Once biofilm forms on fresh produce surface, they not only can cause cross contamination to other food produce or processing equipment surfaces in industry, they also result in a potent health hazard to consumers (Tang *et al*., 2012). So, this work aimed at investigating *Salmonella* contamination in coriander leaves sold in Puducherry, to determine their antibiotic resistance pattern as well as the adherence and pathogenic status of *Salmonella*.

2. Materials and Methods

2.1 Sample Collection

Sixty samples of fresh coriander leaves were collected from vegetable markets (January to March, 2016) located in Puducherry town. Samples were kept in separate sterile plastic bags, and placed in a cooler with frozen gel packs and transport to the laboratory. All the chemicals used in this study were procured from Himedia, Mumbai.

2.2 Sample Preparation

The method of Selleh *et al*. (2003) was used for the preparation of samples with modification. Briefly, the coriander leaves were placed on a working bench in aseptic environment and carefully processed. The coriander leaves were washed with sterile distilled water and leaves were made into small pieces and mixed. From that preparation 5 ml was transferred into 225 ml of buffered peptone water (BPW) and incubated at 37ºC overnight for pre enrichment.

2.4 Salmonella Isolation and Identification

One ml of the pre enriched sample was inoculated into 9ml of Rappaport Vassiliadis Broth and incubated for 24hrs at 37°C. A loopful of the enriched broths was streaked onto Mac Conkey Agar, Xylose Lysine Deoxycholate Agar (XLD) and *Salmonella-Shigella* Agar, plates were incubated at 37ºC for 24 to 48hrs.

Characteristic colonies of *Salmonella* were randomly picked from each plate and inoculated into Triple Sugar Iron Agar (TSA) and Lysine decorboxylase Agar (LDA). Each culture showing presumptive positive TSI and LDA results were maintained in glycerol broth.

Gram reaction and oxidase test were carried out on the presumptive isolates. Suspected *Salmonella* colonies were confirmed by biochemical reactions as per Standard procedure (Veterinary Microbiology and Microbial Diseases, 2007).

2.5 Biofilm Formation Assay (Slime Production Assay)

Qualitatively, biofilm formation among *Salmonella* isolates was assessed using Slime Production Assay method as described by Dhanalakshmi *et al*. (2015). Briefly, Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved. The isolates were inoculated and incubated aerobically for 24 to 48 hours. The ability of the isolates to produce bio-films was indicated by black colonies with a dry crystalline consistency.

2.6 Antimicrobial Susceptibility Testing

All isolates were tested for 14 antimicrobial drugs. List of the antibiotics were used in this study showed in Table 1. Isolates were sub cultured on nutrient agar plates incubated for 24 hrs at 37°C. Colonies were picked from the agar plates, and suspended in normal saline (0.85% w/v), and the density of the suspension was adjusted to 0.5 McFarland standard. The bacterial suspension was spread on the Mueller Hinton agar plates using a sterile swab stick, allowed to dry, and impregnated with antibiotic disk (Igbinosa *et al*., 2013). Plates were incubated at 37°C for 24 hrs. Diameters of the zones of inhibition were measured and interpreted, as susceptible, intermediate or resistant according to the Clinical Laboratory Standard Institute guidelines (CLSI, 2006).

3. Results and Discussion

In this study, coriander leaves were found to harbour *Salmonella*. The incidence and predominance of *Salmonella* in green leafy vegetables including lettuce and cabbage has been documented (Nillian *et al*., 2011). This is also in agreement with the findings of Chia *et al*. (2007). Where leafy vegetables might permit more surface attachment that contributes to the high rate of *Salmonella* survival. These vegetables are top soil creeper hence soil may be a potential source of contamination especially if animal waste have been used as fertilizer (Nillian *et al*., 2011).

Four (6.8%) isolates were recovered from a total of 60 samples. This incidence is less compared to Igbinosa *et al*. (2015) and Lertworapreecha *et al.* (2013). Who reported 31.0% from 100 samples (spinach vegetables), 51.0% from 100 fresh cabbage in South Africa and 82.0% in a total of 41 vegetable samples in Thailand. Whereas in India it is higher than the Nair *et al.* (2015) reported from Uttar Pradesh. Who reported 4.0% incidence in a total of 50 fresh vegetable samples. Animal waste such as fresh faeces or human faeces from incompletely discomposed sludge from waste water system when used as fertilizer could result-

Table 1: List of antibiotics used in the study

Antibiotics code	Antibiotics	Disk content	Antibiotics group
TET	Tetracycline	10µg	Tetracyclines
CHL	Chloramphenicol	30µg	Phenicols
AMP	Ampicillin	25µg	Penicillins
CIP	Ciprofloxacin	5µg	Fluoroquinolones
GEN	Gentamicin	10µg	Aminoglycosides
NAL	Nalidixic acid	30µg	Quinolones
CEP	Cephalothin	30µg	Cephalosporins
			(First Generation)
STR	Streptomycin	10µg	Aminoglycosides
TXM	Trimethoprim- Sulfamethoxazole	25µg	Sulfonamides
OFL	Ofloxacin	5µg	Fluoroquinolones
ERY	Erythromycin	15µg	Macrolides
CTX	Cefotaxime	30µg	Cephalosporins
			(Third Generation)
NX	Norfloxacin	10µg	-
CP	Cephalexin	30µg	-
CD	Clindamycin	2 µg	-

to a primary source of contamination of the farm vegetables. Use of untreated waste water for irrigation or irrigation water from a contaminated source is a major contributing factor to contamination. During the cultivation stage, pathogenic organisms can establish themselves on growing crops. The risk can be enlarged after harvest either by further direct contamination or by proliferation of existing pathogen populations during processing and post harvest handling activities (Berger *et al*., 2010).

A number of studies have shown that *Salmonella* spp are capable of adhering and forming biofilms on diverse surfaces including metal, glass and rubber surfaces (Hood and Zottola, 1997; Joseph *et al*., 2001; Stepanovic´ *et al.,* 2004). The assessment of biofilm formation by *Salmonella* on Brain heart infusion agar plate showed that all *Salmonella* isolates were able to form biofilms. This is also in agreement with findings of Igbinosa *et al*. (2015) in South Africa. The bacteria under study were able to form biofilm on slime production assay potentiating its ability to form biofilm on different surfaces. The study reveals that *Salmonella* isolated from vegetable is able to form biofilms. A correlation between the capacity to produce biofilms and the attachment to leaves, with *Salmonella* showing the efficient adhesion to lettuce leaves has been documented (Patel and Sharma, 2010; Schikora *et al*., 2012). Hence, the biofilm forming ability demonstrated by these *Salmonella* isolates reveals the pathogenic status of the isolates. Bacteria can use multiple hosts as channel to human or other animals. Generally, *Salmonella* infection is self-limiting, however when symptoms persist, antimicrobial therapy is used. Hence the antimicrobial susceptibility of the isolates was carried out. The isolates showed diverse susceptibility profiles against the antibiotics under studied. Multiple antibiotic resistances were found against different classes of antibiotics. Three isolates were showed sensitivity against norfloxacin (92.5%). One was showing sensitivity against cefotaxime (90%). All the four isolates were showing complete resistant to ciprofloxacin, nalidixic acid, chloramphenical, gentamicin and cephalexin. Clindamycin and ofloxacin were showed sensitivity 20%. In Igbinosa *et al*. (2015) study were showed sensitivity against aminoglycosides and quinolones and 82% sensitivity ofloxacin, but in this study all isolates are showing 100% resistance for aminoglycosides and quinolones. Variable resistance patterns was observed between first three and last isolates *i.e.* tetracycline (41%:49%), ampicillin (45.5%:31.4%), erythromycin (58.1%:52.7%), streptomycin (54.5%:76.5%), cephalothin (35.5%:49.2%), respectively (Table 2).

Several studies have documented high resistance of *Salmonella* to the tetracyclines (Yoke-Kqueen *et al*., 2008; Learn-Han *et al*., 2009), which is in agreement with the result obtained in this study. The high resistance phenotypes rate of tetracycline observed in the study could be as a result of the use of tetracycline in food animal production which has led to worldwide spread of tetracycline resistance observed in *Salmonella* isolates (White *et al*., 2001; Logue *et al*., 2003; Parveen *et al*., 2007).

In this study the complete resistance of ciprofloxacin, nalidixic acid and cepholexin also reported because these antibiotics are commonly used in veterinary and human medicine. *Salmonella* resistance to the fluoroquinolones (*ciprofloxacin*) is of great concern to public health as invasive forms of *Salmonellosis* are treated with these compounds (Gord-

Table 2: Antimicrobial pattern of *Salmonella* isolates from coriander leaves

Coriander leaves (n=4)						
	S (%)		I (%)		R (%)	
Antibiotics	1, 2, 3	4	1, 2, 3	4	1, 2, 3	4
Tetracycline	16.1	25.8	20.2	30.3	41.0	49.0
Ampicillin	36.2	33.5	54.0	48.4	45.5	31.4
Nalidixic acid	0	0	0	0	100	99.3
Norfloxacin	92.2	92.0	17.0	15.3	13.4	12.5
Cephalothin	41.9	47.0	19.0	21.0	35.5	49.2
Gentamicin	0	0	0	0	97	98.2
Streptomycin	64.5	66.1	20.0	28.0	54.5	76.5
Trimethoprim-Sulfamethoxazole	32.0	16.0	51.5	35.6	13.5	16.0
Ciprofloxacin	0	0	0	0	98.6	100
Ofloxacin	20.0	20.2	19.0	17.5	37.4	51.0
Erythromycin	29.0	12.9	58.0	19.6	58.0	52.4
Clindamycin	20.5	20.0	0	0	51.0	45.5
Cefotaxime	90.2	90.0	51.0	38.0	11.0	13.4
Cephalexin	10.0	12.0	0	0	97.5	88.0

S-sensitivity, I-intermediate, R-resistant and 1, 2, 3, 4 - Sample number

on, 2000; White *et al*., 2001; Learn-Han *et al*., 2009). The absence of resistance to the fluoroquinolones by *Salmonella* serovars from vegetable in Nigeria has been documented (Raufu *et al*., 2014). Even though the sensitivity was noticed with cefotaxime it is quite costlier than other commonly available antibiotics. *Chloramphenicol* is not frequently used now-a-days due to many side effects but in this study complete resistance of *chloramphenicol* is noticed with three isolates and last isolates showed sensitivity around 26%. This result agreed with Igbinosa *et al*. (2015). Who reported 24% of *chloramphenicol* resistant *Salmonella* isolates from Cabbage in South Africa.

The occurrence of multi-drug resistance *Salmonella* from fresh vegetable is of global health concern as this could led to major health care challenge since multi-drug resistance hinder the possibility of therapeutic treatments. The health benefits of consumption of vegetables has led to significant rise of eating of vegetables among the pregnant, young, old, and ill challenged individuals thereby leading to higher risk of infection among these group of consumers. Hence this is vital in the risk assessment and management of the consumption of vegetables.

4. Conclusion

In this study, coriander leaves are found to harbour *Salmonella*. The biofilm forming ability demonstrated by these *Salmonella* isolates reveals the pathogenic status of the isolates. Bacteria can use multiple hosts as channel to human or other animals and also multiple antibiotic resistances are found against different classes of antibiotics. So, the awareness about the hazard present in the coriander leaves and hygienic measures to prevent the pathogens transmission should be educated among the consumers.

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