PCR based detection and Biofilm formation of Salmonella from fresh coriander leaves (Coriandrum sativum)

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ABSTRACT

The aim of the study was to detect Salmonella from coriander leaves by polymerase chain reaction, determine biofilm formation and antimicrobial resistance profiling of the isolates. Four Salmonella isolates were recovered from the total of 60 samples. The isolates were confirmed by PCR. The confirmed isolates were subjected for biofilm production assay and showed that all Salmonella isolates were able to form biofilm. Biofilm forming isolates were also 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, nalidixicacid, chloramphenical, gentamicin and cephalxin. 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.

Key words: Antimicrobial resistance profiling, Biofilm, Coriander leaves, Polymerase chain reaction, Salmonella spp.

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 one of the pathogens 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.

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MATERIALS AND METHODS
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.

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 37 °C overnight for pre-enrichment.

Salmonella isolation and identification: One ml of the pre enriched sample was inoculated into 9 ml of Rappaport Vassiliadis Broth and incubated for 24 hrs 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 37 °C for 24 to 48 hrs. Characteristic colonies of Salmonella were randomly picked from each plate and inoculated into Triple Sugar Iron Agar (TSA) and Lysine decarboxylase 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).

DNA extraction and PCR amplification for detection of invA gene: After confirming the isolates as Salmonella by biochemical tests, the isolates were subcultured on Nutrient agar, a single colony of each isolate on agar plate was picked and suspended in 200 μl of distilled water. After vortexing, the suspension was boiled for 5 min, and 50 μl of the supernatant was collected after centrifuging for 10 min at 14000 rpm (Zahraei et al., 2012). All the isolates which were confirmed as Salmonella were screened by PCR using specific primers for invA gene (Salmonella Invasion Gene A) (S139: 5’ GTGAAATTATCGCCACGTT CGGGCAA 3’ and S141: 5’ TCATCGCACCGTCAAA GGAAACC 3’) as described by Rahn et al. (1992). The PCR method was conducted using 2.5 μl 10X PCR buffer, 1 μl MgCl2 (50 mM), 0.5 μl dNTP (10 mM), 0.2 μl Taq DNA polymerase (5U/μl), 3 μl DNA, 0.5 μl (10 μM stock) of each primers and 16.8 μl water. The thermal cycle included three steps as followed. Primary denaturation was performed at 94°C for two minutes as the first step. In the second step, 35cycles each included three sections as denaturation at 94°C for 60 seconds, annealing at 64°C for 30 seconds and extension at 72°C for 30 seconds were performed. Eventually, final extension was conducted at 72°C for 5 minutes as the third step. S. typhimurium (MTCC 98) was obtained from Department of Veterinary Public Health, Kerala Veterinary College used as positive control. To detect whether the specific 284 bp amplicon of invA gene exists, the PCR product was electrophoresed for 60 minutes on 1.2% agarose gel.

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.

Antimicrobial susceptibility testing: All isolates were tested for 14 antimicrobial drugs procured from Himedia, Mumbai. 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).

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. The photograph of PCR product showed in Figure 1. This incidence is less as compared to Igbinosa et al. (2015) and Lertworapreecha et al. (2013) who reported 31.0% from spinach vegetables, 51.0% from fresh cabbage in South Africa and 82.0% in 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 wastewater system when used as fertilizer could result 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 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 cephalaxin. Clindamycin and ofloxacin were showed sensitivity 20%. In Igbinosa et al. (2015) study were showed sensitivity against aminoglycosides &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 ie, tetracycline (41%:49%), ampicillin (45.5%:31.4%), erythromycin (58.1%: 52.7%), streptomycin

| Antibiotics      | S(%) | Coriander leaves (n=4) | I(%) | R(%) |
|------------------|------|------------------------|------|------|
|                  | 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     | 97.0    | 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   |
| Cefoxatime       | 90.2  | 90.0                   | 51.0  | 38.0  | 11.0    | 13.4   |
| Cephalaxin       | 10.0  | 12.0                   | 0     | 0     | 97.5    | 88.0   |

S- sensitivity, I- intermediate, R-resistant and 1,2,3&4- Positive isolates number from different markets
(54.5%:76.5%), cephalothin (35.5%:49.2%), respectively (Table. 1).

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; Parveen et al., 2007). In this study the complete resistance of ciprofloxacin, nalidixic acid and cepholaxin 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 (Gordon, 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 nowadays 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 healthcare 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.

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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