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Development of a metagenomic DNA extraction procedure and PCR detection of human enteric bacteria in vegetable salad tissues

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Outbreaks of illness due to human enteric pathogenic bacteria via fresh vegetables warrant intensive research on changing strategies of these bacteria in alterning their hosts for survival. The systemic infection of human pathogenic bacteria in plants and the plant growth stage at which they establish endophytic relationship is poorly understood. Since cucumber and carrot are major vegetables consumed in the form of unprocessed salads in India, our study aimed at determination of infection abilities of *Salmonella enterica* sub sp. *enterica* and *Aeromonas hydrophila* in carrot and cucumber, respectively based on a metagenomic detection system. We report an optimized metagenomic DNA isolation procedure from vegetable tissues co-cultivated with bacteria under laboratory conditions. Colonization of bacteria in vegetable tissues was studied by amplification of bacterial 16S rRNA coding region from the metagenome. DNA obtained from carrot vegetable pieces inoculated with *Salmonella* resulted in expected amplification of 1.2 kb region of bacterial 16S rRNA source sequences. However, the approach failed to detect *Aeromonas* in cucumber tissues. We conclude that carrot could be a symptomless alternate host for *Salmonella* sp.

Key Words: *Aeromonas*, Carrot, Cucumber, Metagenome, *Salmonella*

Consumption of fresh vegetable produce in the form of salads has increased in recent years and a large number of minimally processed fresh-cut vegetables are available in supermarkets, food service facilities and are also prepared household. Unfortunately, the increase in the consumption of raw vegetables has resulted in the increase in frequency of outbreaks of illness (Brackett, 1999; Hodge, 1999; Thunberg *et al.*, 2002; Bhagwat, 2004). *Salmonella* spp have been isolated from several raw vegetables from many countries (Wachtel and Charkowski, 2002; Zhao *et al.*, 2001).

Microbiologists today face the challenge of providing scientific evidence for the fitness of enteric pathogens in plants and its link to the raise of food-borne incidence. An emphasis on the ecology of enteric

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pathogens in plant surfaces has placed this field of study at the cutting edge in the food safety, medical microbiology and microbial ecology, in the recent years. Despite of much information on the interactions of enteric pathogens with human and animal hosts that is known, acquisition of fundamental knowledge about their behavior in plants has just begun.

It is believed that microbial contamination in plants takes place due to pre-harvest or post-harvest agricultural practices such as use of animal feces as manures, lack of on-site sanitation facilities and handling of agricultural produce. The most common bacterial enteric pathogens associated with fruits and vegetables are *Salmonella* spp. (Thunberg *et al.,* 2002), *Escherichia coli* O157:H7 and *Aeromonas* spp. Since these species survive in water sediments, seasonal flooding of fields with overflowing stream water had added as risk factors for potential crop contamination (Himathongkham *et al.,* 1999). Work in several laboratories has shown that enteric bacteria are common inhabitants of the interior of plants. Jay *et al.* (2007) reported that the leafy green vegetables, tomatoes, cucurbits and peppers are among the vegetables linked to outbreaks of gastro intestinal illness caused by pathogenic *E. coli* and non-typhoidal *Salmonella*.

Since the past routes of human infection like non-vegetarian food are well controlled by biotechnological interventions, the food contaminating human bacteria, now have adopted themselves to colonize fruit and vegetable tissues. In the case of salads, bruised and cut surface of vegetables would exude fluids containing nutrients that may enhance the growth of not only the natural microflora but also the pathogens. However, the food-borne illness routed through vegetable salads warrants detailed investigations as understood from Beuchat (2002) who reported that manifestation of infection capabilities of enteric bacteria could be greatly influenced by intrinsic or extrinsic ecological factors naturally present in produce or imposed at one or more points during the entire system of production, processing, distribution of vegetables or preparation of salads.

Since these enteric bacteria are supposed to attach plants majorly by irrigation water before establishing endophytic behavior, a given batch of vegetables from same agricultural farm are expected to have similar type of enteric bacteria in the internal tissues. With available whole genome sequences in the database, it is now possible to use those sequences for developing genus specific molecular detection systems. Moreover, as most of the vegetables are highly perishable, usual enrichment culture technique would not demonstrate itself as reliable detection method due to lack of rapidity. A precise metagemomic approach by targeting the bacterial genome in the plant tissues would help detect the internalized bacteria in real time. With these given challenges, we have designed and validated a strategy to characterize the colonization by *Salmonella* and *Aeromonas* of vegetable tissues like carrot and cucumber by a metagenomic-PCR approach.

Materials and Methods

Bacterial cultures

Type cultures of *Salmonella enterica* subsp. *enterica* (MTCC 3229) and *Aeromonas hydrophila* subsp. *hydrophila* (MTCC 1739) were obtained from Institute of Microbial

Technology, Chandigarh, India. The cultures were retrieved and the single colony isolate was maintained on nutrient agar slants.

Collection of vegetable samples and processing

Fresh vegetables (carrot and cucumber) were collected from retail market at Vellore, India. The vegetables were washed thoroughly with sterile water for several times and small pieces of approximately 1X1 cm dimension were cut out with sterile scalpel under aseptic conditions. The vegetable pieces were inoculated in test tubes containing 3 mL of sterile water. The tubes were incubated in a rotary shaker at 120 rpm for 24 h at room temperature. The contents of the tubes were serially diluted and plated on to nutrient agar plates by spreading with sterile L rod. The plates were incubated at 37°C for 48 h and observed for growth of any native *Salmonella* sp and *Aeromonas* sp from the vegetable tissues.

Co-cultivation

Bacteria were plated by streaking on to nutrient agar plates. Single colony from the overnight culture was inoculated in 100 mL of nutrient broth and incubated in a rotary shaker at 120 rpm overnight. When the OD value at 600 nm reached 1.0, fresh cut vegetable tissues were added aseptically into the culture flasks. Co-cultivation was performed with carrot – *S. enterica* and cucumber – *A. hydrophila* combination. Bacterial cultures without vegetable pieces and sterile nutrient broth served as positive and negative control respectively. The co-cultivation flasks were incubated for 48 h in rotary shaker at 120 rpm.

Washing-off of surface bacteria

The vegetable pieces were removed from bacterial culture flasks by filtering under aseptic conditions in Whatman No.1 filter paper and washed in repeated changes of sterile water. Washing of the vegetable pieces to remove surface bacteria was performed by adding the pieces to sterile beaker and adding 50 mL of sterile water followed by vigorous shaking. Fifteen rounds of washing were performed and about 0.5 mL of 10^{th} to 15^{th} washings were plated by spreading on to nutrient agar plates.

Bacterial DNA isolation

Genomic DNA from both bacterial cultures was isolated by the method of Esteban *et al.* (1993) with several modifications. Briefly, the bacterial cells were pelleted down by centrifuging at 8000 rpm for 5 min. The pellet was resuspended in 567 µL of TE buffer, pH 7.5 by repeated pipetting. About 30 µL of 10% SDS and 3 µL of proteinase K stock (10mg/mL) were added. The suspension was mixed by inverting and incubated at 37°C for an hour. About 100 µL of 5M NaCl was added and mixed by inverting. This was followed by addition of 80 µL of CTAB/NaCl solution (10% CTAB, 0.7 M NaCl). Tubes were incubated at 65°C for 10 min and the contents were extracted with equal volume of chloroform: isoamyl alcohol. After centrifugation at 14,000 rpm for 5 min, the aqueous layer was transferred to fresh tube and 0.6 volume of ice-cold isopropanol was added. DNA was precipitated by centrifuging at 12,000 rpm for 5 min and the pellet washed with 70% ethanol, air dried and suspended in 50 µL of TE buffer.

Metagenomic DNA isolation

Several methods of plant DNA isolation was used to isolate metagenome as the major component of the metagenome is plant. A modified method using extraction with step wise increasing concentrations of

CTAB followed by CTAB precipitation solution, was optimized and the procedure is as follows: About 0.5 g of vegetable tissue sample was extracted with CTAB extraction solution (2% (w/v) CTAB; 100 mM Tris.Cl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl) containing 2% 2-mercaptoethanol and 1% PVP. The samples were heated to 65°C for one hour and extracted with equal volume of chloroform: isoamyl alcohol and centrifuged at 10,000 rpm for 5 min at 4°C. The acqueous phase was mixed with CTAB/NaCl solution (10% CTAB; 0.7 M NaCl) and extracted again with chloroform: isoamyl alcohol. After centrifugation, acqeous phase was mixed with equal volume of CTAB precipitation solution (1% (w/v) CTAB; 50 mM Tris.Cl, pH 8.0; 10 mM EDTA, pH 8.0). The samples were centrifuged at low speed (3000 rpm) for 5 min at 4°C. For samples which had no visible pellet, more of CTAB precipitation solution was added, incubated at 37°C for 1 h and centrifuged again. The pellets were dissolved in 0.5 mL of high-salt TE buffer (10 mM Tris.Cl, pH 8.0; 0.1 mM EDTA, pH 8.0; 1 M NaCl). One sixth volume of isopropanol was added to the tubes and centrifuged at 10,000 rpm for 15 min at 4°C. After washing the pellet with 70% ethanol, the DNA pellet was dissolved in TE buffer.

Both bacterial DNA and metagenomic DNA were quantified spectrophotometrically and electrophoresed in 0.8% agarose gel to check the integrity.

PCR target sequences and primers

The whole genome sequence of *A. hydrophila* subsp. *hydrophila* (ATCC7966) and *S. enterica* subsp. *enterica* serovar Newport Str. SL254 were accessed from the NCBI database. From the structural RNA gene bank database of the whole genome, the first rRNA coding region of both bacteria was selected to obtain the 16S rRNA gene sequences. Primer3 software was used to design primers for the amplification of 16S rRNA coding region. The oligonucleotides were synthesized at Genei, Bangalore, India. The primer sequences used in the study are presented in Table 1.

Table 1. Primers used in the study for the amplification of 16S rRNA coding region

Primer ID	Bacteria	Sequence (5′ to 3′)
SBSal1F	*Salmonella*	TCATGGCTCAGA TTGAACGC
SBSal1R	*Salmonella*	CATTGTAGCACG TGTGTAGC
SBAer1F	*Aeromonas*	CAGAAGAAGCA CCGGCTAACTC
SBAer1R	*Aeromonas*	TTACCTTGTTAC GACTTCACC

PCR analysis

PCR amplification of the 16S rRNA coding region of *Salmonella* and *Aeromonas* was carried out with both bacterial DNA and metagenomic DNA as templates. Metagenomic DNA from un-inoculated vegetable tissues served as negative control and bacterial genomic DNA served as positive control. The PCR reaction mixture contained 1 µg of template DNA, 0.5 µM each of the primer, 10 µl of PCR master mix (Genei, Bangalore, India) and nuclease free water in a 20 µL reaction mixture. Amplification was carried out in a thermal cycler (Applied Biosystems, USA) with PCR steps of initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 7 min. The amplified products were separated in 0.8% agarose gel with molecular weight marker.

Results

Co-cultivation of bacterial pathogens with vegetables

Cut pieces of carrot and cucumber were co-cultivated with *S. enterica* and *A. hydrophila* culture under aseptic conditions for *in vitro* infection. The co-cultivation of carrot tissues with bacterial suspension in conical flasks is presented in Fig. 1. A control nutrient broth flask with pieces of each vegetable was maintained separately to check for any contaminating bacteria from fresh vegetables. Absence of turbidity in control flasks indicated that the growth of the bacteria in test flasks is due to inoculated bacteria.

Fig. 1. Co-cultivation of *Salmonella enterica* and carrot

Post co-cultivation processing

The vegetable pieces after artificial inoculation were processed with several rounds of washing as described earlier. Plating of an aliquot of the washing every time was performed and the number of colonies of bacteria were counted. The number of colonies of inoculated bacteria from surface washings reduced with increase in number of washings. After eleventh washing, there were no bacterial colonies. However, fifteen washings were done to ensure complete elimination of surface bacteria from vegetable tissues.

Bacterial and metagenomic DNA

Genomic DNA of bacteria and the metagenome (plant DNA+inoculated bacterial DNA+ DNA from any symptomless endophytic microorganisms) were checked spectrophotometrically for their purity and yield. Few of the procedures did not result in intact metagenome and showed shearing of DNA as represented by smear in the agarose gel. The described protocol was successfully used to isolate intact metagenome (Fig. 2). Results of the determination of quantity and purity of DNA are presented in Table 2.

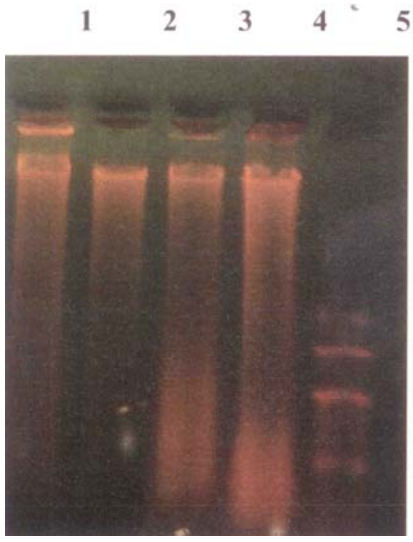

Fig. 2. Metagenomic DNA Lanes 1- uninfected carrot; 2 – carrot infected with *Salmonella*; 3 – uninfected cucumber; 4 – cucumber infected with *Aeromonas;* 5 – 1 kb DNA ladder

PCR analysis

Metagenomic DNA obtained from cucumber infected with *A. hydrophila* failed to result in amplification of the bacterial 16S rRNA coding sequence. However, carrot infected with *S. enterica* resulted in 1.2 kb amplified product (Fig. 3) which is of the expected size.

Table 2. Yield and purity of bacterial and metagenomic DNA

DNA Sample	Concentration* (µg / mL)	Purity * (A260/A280)
Salmonella	45.0	1.63
Aeromonas	75.0	1.82
Carrot infected with *Salmonella*	57.5	2.55
Cucumber infected with *Aeromonas*	102.5	1.41

*Mean of three replicated extractions.

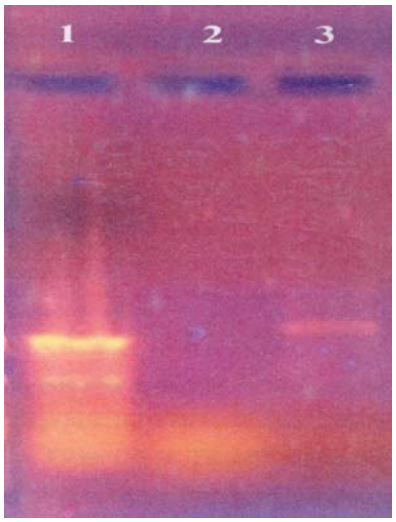

Fig. 3. PCR detection of *Salmonella* in carrot metagenome Lanes 1 - 1 kb DNA ladder; 2 – uninfected carrot; 3 – carrot infected with *Salmonella*

Discussion

There are a number of reports indicating that raw vegetables may harbour potential foodborne pathogens (Nguyen-the and Carlin, 1994). Initially, *Listeria monocytogenes* (Schlech *et al.*, 1983), *Salmonella* (Doyle, 1990), and *E. coli* (Nguyen-the and Carlin, 1994) were reported from raw vegetables. In recent years, as the traditional routes of infection are better controlled, large outbreaks of nontyphoidal *Salmonella* infection have been attributed to fruits, vegetables, and processed foods (Andrews-Polymenis *et al.*, 2009). It is not yet certain how these bacteria enter plant tissues and spread within them (Teplitski *et al.*, 2009). *Salmonella* and enterovirulent *E. coli* are capable of spending at least a part of their life cycle as plant-associated endo- or epiphytes. However, several important questions about the genetics and physiology of these interactions still need to be answered before plants are designated as true alternate hosts for these enteric pathogens. The ability of *S. enteritidis* to grow on melon, watermelon and papaya pulp stored at different times and temperatures was investigated by Penteado and Leitao (2004). It has been demonstrated that *S. typhimurium* overcomes the innate immune response of *Arabidopsis thaliana* and shows an endopathogenic lifestyle (Schikora *et al.*, 2008). Guo *et al.* (2001) reports that tomato stems and flowers are possible sites at which *Salmonella* may attach and remain viable during fruit development, thus serving as routes or reservoirs for contaminating ripened fruit. Itoh *et al.* (1998) demonstrated the presence of viable enterohemorrhagic E. coli O157:H7 in the inner tissues and stomata of cotyledons of radish sprouts. Many of the interactions that occur between foodborne pathogens and the fruits and vegetables they contaminate are just beginning to be elucidated (Critzer and Doyle, 2010). A general lack of efficacy of sanitizers in removing or killing pathogens on raw fruits and vegetables has been attributed, in part, to their inaccessibility to locations within structures and tissues that may harbor pathogens. Understanding the ecology of pathogens and naturally occurring

microorganisms is essential before interventions for elimination or control of growth can be devised.

Metagenomics has been defined as function-based or sequence-based cultivation-independent analysis of the collective microbial genomes present in a given habitat (Riesenfeld *et al.,* 2004). The developed metagenomic technologies are used to complement or replace culture-based approaches and bypass some of their inherent limitations. Although metagenomics represent the complete analysis and high-throughput second generation sequencing of all the microbes in a habitat, it also includes function-based analysis as referred by Riesenfeld *et al.* (2004).

In the present study, we were successful in isolating about 57.5 and 102.5 µg / mL of DNA in a pure and intact form, from the vegetable tissue metagenome. The procedure optimized in the study deserves adoption for any plant based metagenome involving molecular studies of microbes including beneficial microbes. The primers based on *Salmonella* 16S rRNA coding region proved successful in amplifying the bacterial DNA in the carrot metagenome. This also confirms that carrot can serve as symptomless alternate host for human pathogenic *Salmonella* sp. Experiment with *A. hydrophila* 16S rRNA region in artificially infected cucumber showed no amplification which leads us to two possible conclusions i) the bacteria might not have had the abilities to colonize cucumber or ii) the number of bacterial cells and in turn the genome could have been less contributing to the metagenome and remained in an undetectable level. The results obtained in our study represents preliminary observations which warrant further investigations involving metagenomic PCR analysis at different time points after co-cultivation and altering amplification conditions, before *A. hydrophila* could be declared as a non-colonizer of cucumber.

Our approach of detection of bacteria in an unusual habitat which ultimately aims to characterize the interactions of the human enteric bacteria in alternate ecology bypasses culturing steps in the culturable bacteria. Hence the approach represents a sub branch of the metagenomics, utilizing some of the components of this cutting-edge science for a faster and easy bacterial detection. Supporting declaration is from Simon and Daniel (2009) who reported that metagenomics could allow the assessment and exploitation of the taxonomic and metabolic diversity of microbial communities on an ecosystem level.

Application of the standard metagenomic technologies have resulted in the generation of large sequence and metabolic datasets derived from various environments, mainly soil and water. Metagenomic analysis in plant tissues, as optimized in the present study, with plant genome and genome of many symptomless endophytic microorganisms as components of the metagenome, stands novel. Our approach, other than for the rapid detection of bacteria, can be adopted for determination of functions and interactions of microbial communities in plant micro environment.

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