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Molecular Characterization and Antibiogram Profiling of the Isolated Food-Borne Pathogenic Bacteria on Fresh-Cut Salad Vegetables

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Abstract

Fresh cut salad vegetables (FCSV) play a significant role in our healthy and balance diet. The consumption of raw salad vegetables are vectors for the transmission of various infectious diseases, which is a public health issue in developing and developed world. Therefore, the aim of this present study was to evaluate the microbial contamination of ready-to-eat salad vegetables sold in different local vendors in Kushtia and Jhenaidah districts in Bangladesh. A total of 21different ready-to-eat FCSV samples were collected aseptically and analyzed immediately in our laboratory. A total viable bacterial count (TVBC) was enumerated up to 10^7 CFU/g in the tested samples. The highest number ($3.01\pm1.4 \times 10^7$ CFU/g) of TVBC was found in Coriander leaf (*Coriandrum sativum*) and least microbial load ($2.33\pm1.05 \times 10^5$ CFU/g) was observed in Red spinach. On the basis of morphological differentiation randomly selected bacterial isolates (*Aeromonas* sp., *Citrobacter* sp., *Enterobacter* sp., *Pectobacterium* sp., and *Staphylococcus* sp.) were identified by 16S rRNA gene sequencing. Moreover, most of the isolates were showed as multidrug-resistant bacteria via the antibiogram profiling testing. The overall antimicrobial susceptibility profile showed that the tested bacterial isolates were resistant against Erythromycin (15µg), Penicillin G (10 unites), Nalidixic acid (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), susceptible against Kanamycin (30 µg), Polymyxin B (300 units), CO-Trimoxazole (25 µg), Colistin (10 µg), Doxycycline (30 µg) and intermediate against Nalidixic acid (30 µg), CO-Trimoxazole (25 µg), and Kanamycin (30 µg). Pathogenic bacterial contamination of FCSV, hygienic practices should be maintained before consumption.

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Introduction

Fresh cut salad vegetables (FCSV) are essential elements for our sound health but they could contaminate via life threatening pathogenic microorganisms (Hamilton *et al.,* 2006). The FCSVincluding cucumber, carrots, tomatoes, green chili, cabbage and lettuce etc. were used to prepare salad recipe.

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The FCSV are sold in urban and rural market and it is usually seen the hawker sell these commodities in a public places. However, FCSV have been recognized not only source of pathogenic microbes but also different chemical contaminants (Uzeh *et al.,* 2009).They may be spoiled by a wide variety of pathogenic bacteria, fungi, viruses and parasites (Ahmed *et al.,* 2014). FCSV can also be contaminated from different environmental sources during cultivation from the soil and water. Besides they can expose via air, insects, birds, animal, equipments, and marketing etc. (Hamilton *et al.,* 2006). The most common pathogenic bacteria such as *Escherichia coli*, *Enterobacter* spp., *Salmonella* spp. *Pseudomonas* spp. *Aeromonas* spp. *Shigella* spp. were found on the surface of FCSV samples. So the environmental and food microbiologists have continued to identify and suggest control measures for hazards at all stages in the supply chain from farm to fork (Jongen, 2005). The FCSV generally consumed salad as raw and a number of food-borne outbreaks related to these raw vegetables have increased recently (Buck *et al.,* 2003; Lynch *et al.,* 2009; Olaimat and Holley, 2012).

In developing countries like Bangladesh, the different food-borne outbreaks caused by fecal contaminated vegetables which are frequent. However, foodborne disease surveillance and investigation are limited and most of outbreaks are unrecorded due to lack of the scientific study and government policy. In recent decades, antimicrobial resistance problem not only developing country but also developed world are facinga great challenge (Rabbi *et al.,* 2011). Different scientific studies havebeen reported the presence of multidrug resistant bacteria are exist on the different agricultural produces (Nipa *et al.,* 2011; Osibote *et al.,* 2014). Now-a-days, drug resistance is spreading mainly due to misuse of antibiotics, incomplete use of medications and widespread practice in livestock feeding as growth promotion (Sultana *et al.,* 2014).

Objectives

In the present study, we evaluate the total viable bacterial load, molecular identification and antibiogram profiling of selected bacterial isolates from salad vegetables samples in Kushtia, Jhenaidah city, Bangladesh.

Materials and Methods

Sample collection and processing

A total (n = 21) consisting of seven category commonly consumed fresh vegetable samples green Chili (*Capsicum annuum*), Coriander leaf (*Coriandrum sativum*), Carrot (*Daucus carota subsp. sativus*), Red spinach (*Amaranthus dubius*), Cucumber (*Cucumis sativus*), Pudina leaf (*Mentha*), and Tomato (*Solanum lycopersicum*) were collected for analysis. The microbiological tests were carried out in the microbiology laboratory, Dept. of Biotechnology and genetic Engineering, Islamic University, Kushtia. Samples were collected in the sterile polythene zipper bags to avoid any handling contamination and transported to laboratory for microbial analysis. The twenty gram (20g) of each sample was aseptically mixed (1:10) ratio with 180 mL distill water (Muhammad *et al.,* 2017)

Microbiological analysis of fresh cut salad vegetables

The FCSV samples were suspended in distill water and serially diluted up to 10^{-6} (Muhammad *et al.* 2017) and a volume (0.1 mL) of each diluted sample was spread onto the viable cell counting media i.e. nutrient agar (NA) for counting the total viable bacterial count (TVBC). The plates were incubated at 37 ºC for 24-48 h (Muhammad *et al.,* 2017). The distinct morphological colonies on Nutrient agar were preserved for further analysis. On the basis of their morphological difference the distinguished bacterial colonies were isolated for further studies. Pure cultures were stored by glycerol stock at -70 ºC for further studies (Mamun *et al.,* 2016).

Molecular identification by polymerase chain reaction (PCR) using 16S rRNA gene sequencing

The template DNA was prepared by a slight modified boiling method described by Rawool *et al.* (2007). In order to molecular identification, bacterial isolates were sent to Invent Technologies Limited (Dhaka, Bangladesh). PCR protocol was slightly modified according to Rahman *et al.* (2017) where initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 47°C for 30 s and extension at 72 °C for 1 min and 30 s and the final extension was conducted at 72 °C for 10 min.

Antimicrobial susceptibility test

Antimicrobial susceptibility testing (AST) is used to assess the clinically used antibiotic resistance profiles of bacterial isolates. A standardized procedure was develop for antimicrobial susceptibility testing using disc diffusion method (Bauer *et al.,* 2012). AST process such as minimum inhibitory concentration (MIC) determination is a quantitative method which determinethe MIC using a conventional broth dilution method. The resulting MIC value can be compared to the standardized CLSI procedure. According to disc diffusion method, zone of inhibition was observed on Mueller Hinton Agar after incubation at 35-37 °C for 18-24 h for standard cultures (Bauer *et al.,* 2012). In this study, ten (10) different antibiotic disc i.e. Erythromycine (15 µg), Kanamycin (30 µg), Penicillin G (10 unites), Nalidixic acid (30 µg), Polymyxin B (300 unites), Ceftriaxone (30 µg), Ceftazidine (30 µg), CO-Trimoxazole (25 µ g), Colistin (10 µg), and Doxycycline (30 µg) were used for antibiotic susceptibility testing.

Results and discussion

Isolation, morphological identification and total viable bacterial count

In order to assess the TVBC count (N = 21) on samples consisting of seven types of vegetable samples were tested. The TVBC ranges on FCSV were different from sample types and collection places. The highest number ($3.01\pm1.4 \times 10^7$ CFU/g) of TVBC was found in Coriander leaf (*C. sativum*) and least microbial load ($2.33\pm1.05 \times 10^5$CFU/g) was observed in Red spinach (*A. dubius*). From NA agar media, the distinct morphological colonies were preserved for further molecular analysis.An outbreak of human disease was observed related to the consumption of FCSV not only developing countries but also hasseen frequent in developed countries over the few decades. On the basis of Hazard Analysis and Critical Control Point-Total Quality Management (HACCP-TQM) technical guidelines, the raw produc eranges are containing aerobic plate count of $<10^4$ CFU/g as “Good”, 10^4-5×10^6 CFU/g as “Average”, 5×10^6-5×10^7 CFU /g as “Poor” and $>5 \times 10^7$ CFU/g as “Spoilt” (Mamun *et al.* 2016). Moreover, the reference

value of per gram vegetable is 10^3 to 10^5 CFU/g. Presence of *E. coli, Salmonella spp.* and *Shigella spp.* are also indicate the connection of poor sanitary practices and they might be create a potential risk of food borne illness to human health (Mamun *et al.,* 2016).

Molecular identification

Morphologically distinct identified bacterial isolates were picked from salad vegetables. In order to molecular identification, bacterial isolates were sequenced with 16S rRNA primer and the collected gene sequences were compared for similarity with those of bacteria deposited in GenBank, using the NCBI BLAST.

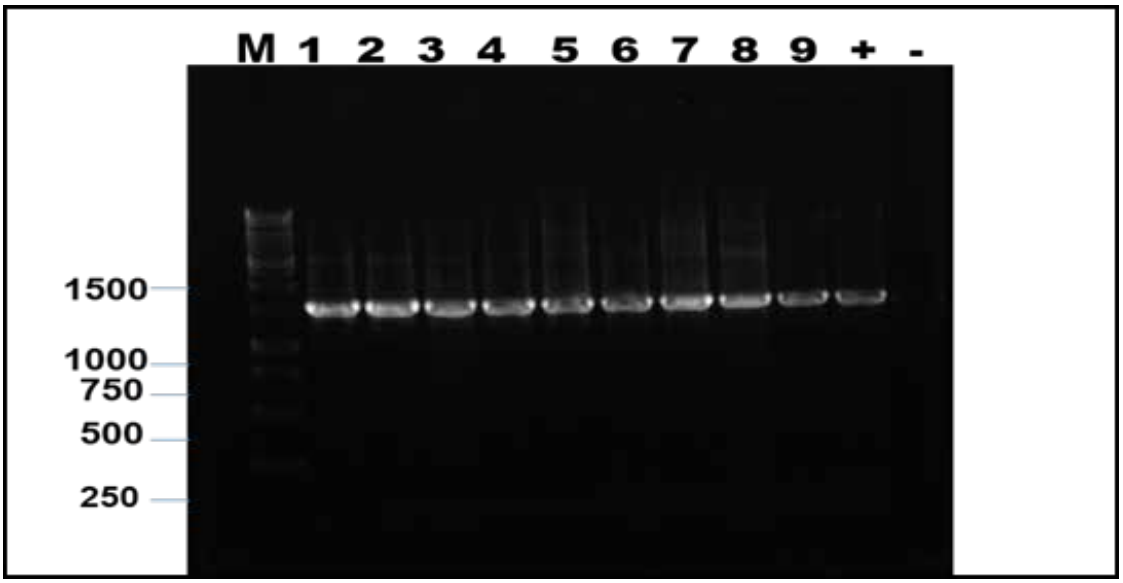

Fig. 1. PCR amplification of 16S rRNA (27F and 1492F) primer sets with nine bacterial isolates. PCR band 'M' indicate 1Kb DNA ladder (marker). The gel lane numbers are as follows: lane No. 1-9 asthe tested bacterial isolates and "+" indicates positive control as E. coli O157:H7 (ATCC-95150)and "-" means negative control only water.

Antibiogram profiling of the isolated and identified pathogenson fresh cut salad vegetables

A total of nine (n = 9) isolates including six (n = 6) categories bacteria was subjected to antimicrobial susceptibility testing (Fig. 2). Most of the isolates were showed as multidrug-resistant bacteria via the antibiogram profiling testing. The overall antimicrobial susceptibility profile showed that the tested bacterial isolates were resistant against Erythromycin (15 µg), Penicillin G (10 unites), Nalidixic acid (30 µg), Ceftriaxone (30 µg), Ceftazidime (30 µg), susceptible against Kanamycin (30 µg), Polymyxin B (300 units), CO-Trimoxazole (25 µg), Colistin (10 µg), Doxycycline (30 µg) and intermediate against Nalidixic acid (30 µg), CO-Trimoxazole (25 µg), and Kanamycin (30 µg). The isolates (5/10) were resistant to multiple drugs and few (2/10) of them were intermediate (Fig. 3).

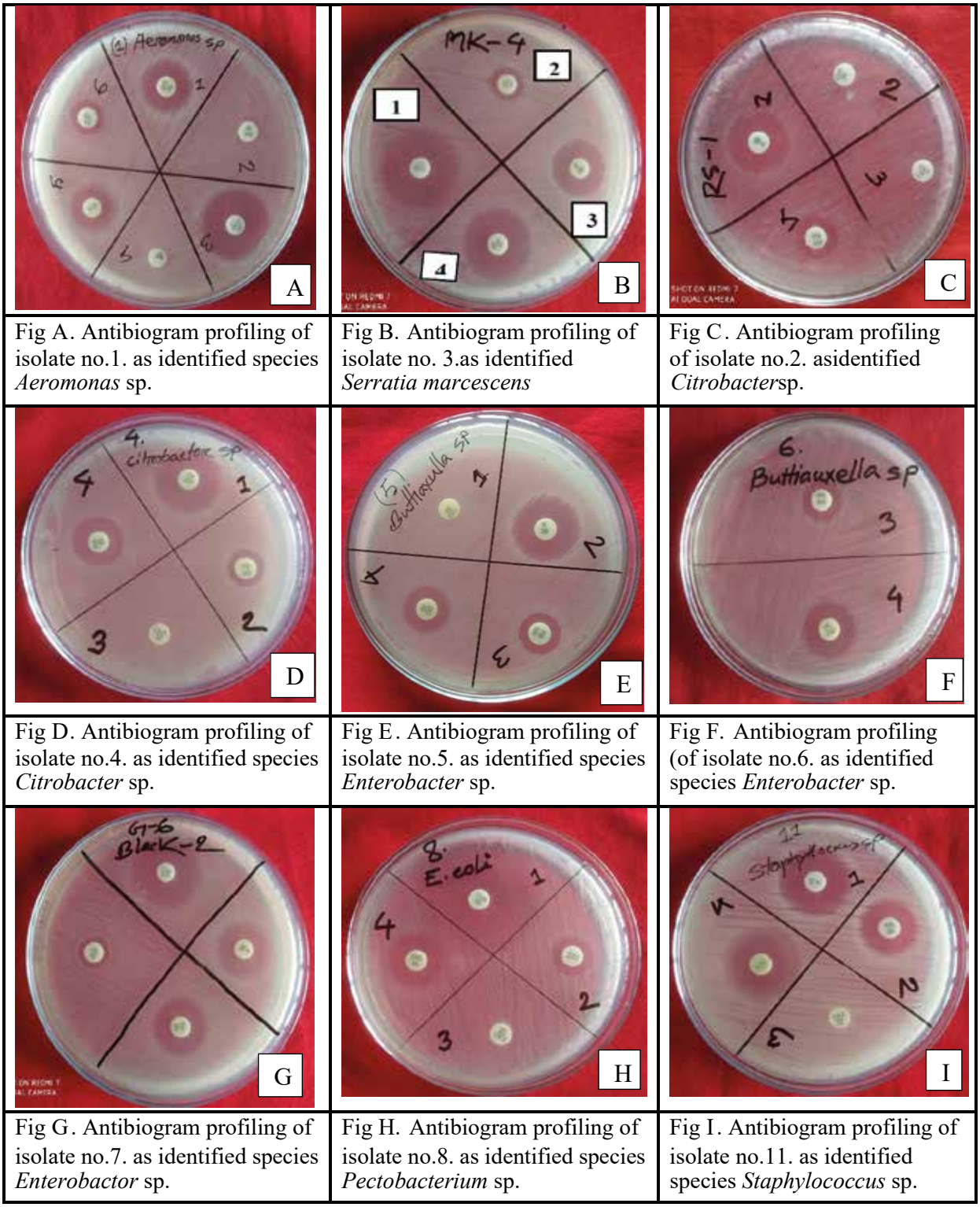

Fig A. Antibiogram profiling of isolate no.1. as identified species *Aeromonas* sp.

Fig B. Antibiogram profiling of isolate no. 3.as identified *Serratia marcescens*

Fig C. Antibiogram profiling of isolate no.2. asidentified *Citrobacter*sp.

Fig D. Antibiogram profiling of isolate no.4. as identified species *Citrobacter* sp.

Fig E. Antibiogram profiling of isolate no.5. as identified species *Enterobacter* sp.

Fig F. Antibiogram profiling (of isolate no.6. as identified species *Enterobacter* sp.

Fig G. Antibiogram profiling of isolate no.7. as identified species *Enterobactor* sp.

Fig H. Antibiogram profiling of isolate no.8. as identified species *Pectobacterium* sp.

Fig I. Antibiogram profiling of isolate no.11. as identified species *Staphylococcus* sp.

Fig. 2. (A-I). Antibiogram profile of different bacteria in ready-to-eat salad vegetables was observe on nutrient agar media.

The isolate No.1 (*Aeromonoas* sp) and isolate No.8 (*Pectobacterium* sp.) which confer resistant (40%) to tested different antibiotics, similarly isolate No.4 showed resistant (33%) to tested antibiotics (Figure 3). However, antibiotic resistance caused mainly by using of widely and misuse antimicrobials in humans and other domestic animals, and transmission of acquired resistant strains between humansand animals. Moreover, growing antibiotic resistance has also been related to dumping of incomplete treatment of the pharmaceutical effluents where bulk drugs are manufactured (Gullberg *et al.* 2011). Antimicrobial resistance is increasing last few decades in the whole world due to greater access to antibiotic drugs. Estimates are that a number of people such as 700,000 to several million deaths result per year (Nordea 2016).

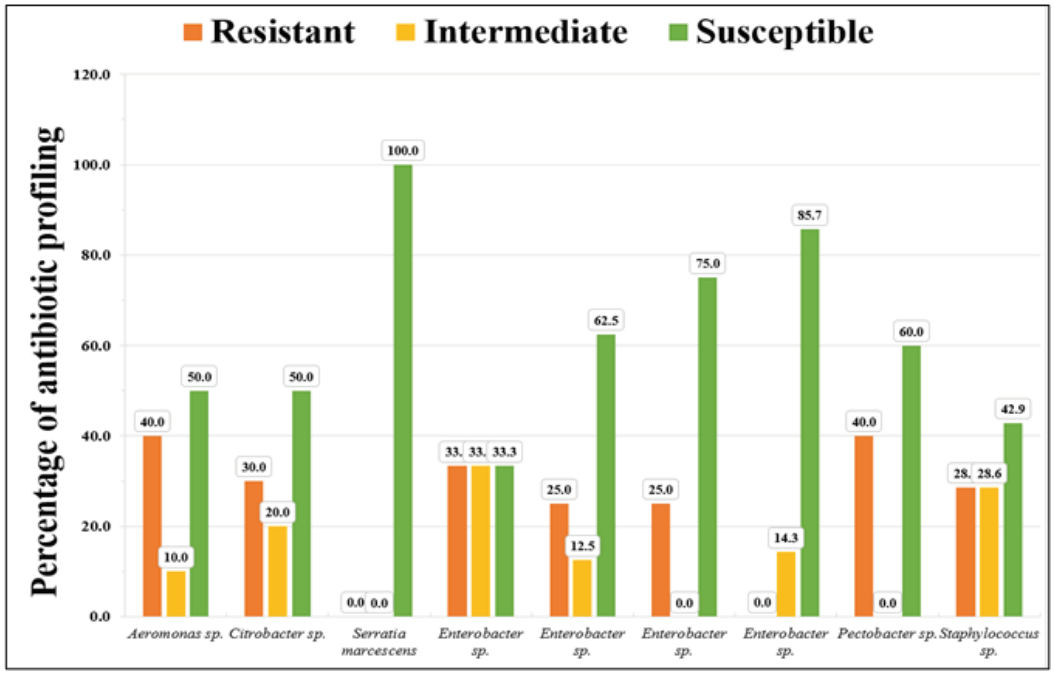

Fig. 3. Antibiotic resistance profiling (%) of selected bacterial isolates using different antibiotics

Conclusion

This study indicate that the fresh cut salad vegetables contain higher viable bacterial counts which would be highly risk and threaten to public health. We observed the tested FCSV samples were contaminated with various pathogenic bacteria (*Aeromonas* sp., *Citrobacter* sp., *Serratia* sp., *Pectobacterium* sp., *Enterobacter* sp., and *Staphylococcus* sp.) which can cause serious public health hazards. The pathogenic bacterial isolates from the salad vegetables has been linked to a major risk to public health are not only developing country like Bangladesh but also developed country. However, the pathogenic bacterial isolates present in the commonly consumed salad vegetables showed resistance against the regular antibiotics (Penicillin G-10, CO-Trimoxazole-25) which is threatened to public health in globally. As an extensive research of identification, antibiotic profiling and molecular characterization of pathogenic

bacteria, we should analyze more samples for accurate evaluation of this research. Nevertheless, our study contributea significance information of some pathogenic bacterial strains and their antibiotic resistance profile of the fresh cut salad vegetables which would be useful for the all stakeholders.

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