Characterization of Multi-Drug Resistant Gram-Negative Bacteria Present in Fresh Leafy & Salad Vegetables in Dhaka, Bangladesh

Shahrin Akter Aurin, Sohana Parvin Chowdhury, Maruf Abony, Jarin Rifa, Avijit Banik, Aneeka Nawar Fatema, Oshin Ghurnee, and Zakaria Ahmed

Abstract — This study was conducted to determine the microbiological quality of fresh raw and unwashed leafy and salad vegetables in Dhaka, Bangladesh. Six different types of samples (red amaranth, spinach, carrot, radish, tomato, and cucumber) were collected in pre-sterilized zip-lock bags from various local markets in Dhaka. All samples were transported to the Centre of Excellence for Global Health Research Laboratory of Primeasia University at earliest convenience. Samples were enriched in Nutrient Broth media and were then cultured on selective media for isolation purpose. Serial dilution was performed for the total viable count. Biochemical and carbohydrate profiling was conducted for the presumptive identification of the isolates. Commercial antibiotic discs were used for antibiogram by Kirby-Bauer disc diffusion method on Mueller-Hinton agar medium. The total microbial load ranged from 8x10⁶ to 4.70x10⁸. Total 36 isolates were identified, having 7 different organisms. The most predominant organism was Vibrio sp. (23%) followed by Klebsiella sp.(20%), Acinetobacter sp. (19%), Pseudomonas sp. (19%), Salmonella sp. (8%), Moraxella sp.(8%) and Escherichia coli (3%). 11% of the Vibrio sp. isolates were V. cholerae, found from 4 samples. No presence of V. cholerae was observed in the tomatosamples. E. coli was observed only in Carrot sample. Antibiotics from 7 different groups were tested against the organisms among which Imipenem showed the highest sensitivity (86%). Following Ceftriaxone (100%), Nitrofurantoin (94%), Erythromycin (89%) and Amoxicillin (83%) had the highest resistance against the isolated organisms. Moreover, most of the isolates showed a multi-drug resistance pattern where they were resistant to at least four drugs. Prevalence of pathogenic bacteria in raw unwashed vegetables can cause potential adverse health effects and therefore the consumers need to be conscious about the matter.

Index Terms — Fresh raw vegetables, Food-borne illness, Gram-negative bacteria, Multi-drug resistance.

I. INTRODUCTION

Different types of vegetables are consumed raw worldwide for its highly nutritional value. Usually salad vegetables are often unwashed before consumption. Vegetables are rich in carbohydrates, anti-oxidants, minerals, vitamins and fibers and often consumed uncooked [1]. In recent years many countries have undertaken various initiatives to encourage consumers to eat more vegetables as they are an essential ingredient of a healthy diet [2]. In the daily diet, vegetables have been strongly associated with improvement of gastrointestinal health, good vision, and reduced risk of heart disease, stroke, chronic diseases such as diabetes, and some forms of cancer. Some phytochemicals of vegetables are strong antioxidants and are thought to reduce the risk of chronic disease by protecting against free radical damage, by modifying metabolic activation and detoxification of carcinogens, or even by influencing processes that alter the course of tumor cells [3].

In Bangladesh Total vegetable consumption reached 4,049 kt in 2013 in Bangladesh, according to Faostat. This is 0.226 % more than in the previous year [4]. Hence consumption of raw vegetables for diet as salad has become more common among health-conscious people in recent years.

Recently vegetables have been identified and confirmed as a significant source of pathogens due to the phytounitrients present in vegetables that act as effective media for the transmission of pathogens [2]. Consumption of raw vegetables contaminated with harmful microorganisms may result in food poisoning due to the fact that there is no killing step such as heating during preparation that would inactivate the harmful microorganisms [5].

Microorganisms capable of causing human disease may be found in raw produce. Sometimes they are part of the fruit or vegetable microflora as incidental contaminants from the soil and surroundings. In other instances, they are introduced into or on food by poor handling practices in agricultural production or post-harvest processes [6].

The primary sources of microbial contamination of fresh vegetables include human and animal fecal matter, contaminated water, soil, dust, surroundings and handling equipment and poor sanitary practices throughout the production chain [6]. Contaminations may also occur at post-harvest stage through dirty wash water, cross-contamination, and consumption of raw or uncooked vegetables.
Based previous investigations, pathogenic (disease-causing) strains of (STEC), *Salmonella*, Norovirus and *Listeria monocytogenes* are responsible for causing foodborne illness via fruit [7]. Multiple investigations involved *E. coli* O157:H7 illnesses linked to leafy greens [8]. Bangladeshi people are more prone to microbial outbreak due to the relatively dense population with unsanitary condition. It is estimated 30 million people in Bangladesh suffer from foodborne illnesses annually [9]. Children are more susceptible to unsafe food in comparison to adults which contributes to child mortality [10].

Often the vegetables are not properly washed before consumption due to the unawareness of the people living in Bangladesh about the hazards associated with it. To prevent the occurrence of foodborne disease and its spread from raw vegetables it is necessary to minimize the contamination with microbes. This study was undertaken to determine the prevalence of Gram-negative pathogens in fresh raw leafy and salad vegetables, without wash from different local markets in the Dhaka city and thereby analyze the quality of the products. By analyzing the quality of these products, an estimated condition of the vegetables sold at random local markets in Dhaka city can be comprehended. Previously many researchers had also conducted similar studies in both developed and developing countries as well as in Bangladesh [11]-[13]. Many of them evaluated the presence of microbes after washing with tap water and/or other disinfectants to assess the proper cleansing method of vegetables. Where our study focused on the presence of Gram-negative bacteria particularly, on the fresh and unwashed vegetables to increase public awareness of the importance of good domestic hygiene practice.

**II. METHODOLOGY**

**A. Sample Collection and Enrichment**

A total number of 12 samples of 6 different kinds were collected: red amaranth, spinach, tomato, cucumber, carrot and radish, were collected from different local markets in Dhaka city. The sampling area included Banani, Mirpur, Mohammadpur and Abdullahpur. The samples were transported to the Centre for Excellence Laboratory of Department of Microbiology, Primeasia University [14-15]. Each sample was enriched to increase the small microbial growth to a detectable level. 10g of each sample was weighed and aseptically added to 90ml of sterile Nutrient Agar and incubated at 120rpm and 37°C in a reciprocal shaker for overnight [16].

**B. Determination of Bacterial Count**

Bacterial count was determined using pour plate technique. The plates were then incubated at 37°C and the counts were taken after a day [17].

**C. Isolation of Gram-Negative Pathogens**

Different Gram-negative bacteria were isolated by cultivating on different selective (e.g. Cetrimide agar, SS agar etc.) and differential media (e.g. MacConkey Agar etc.) and identified initially by the morphological characteristics of the colonies.[16] The presumptive organisms were further tested for biochemical, carbohydrate profiling and antibiotic susceptibility test in order to obtain a more precise identification [14-15].

**D. Identification of the Isolated Strains**

Identification of the Isolated Strains was done by conducted microscopy and biochemical characterization. The biochemical characteristics of a bacterium provide many traits that are useful for classification and identification. Triple Sugar Iron (TSI), Motility, Indole Test, MR-VP Test, Citrate Utilization, Nitrate Reduction, Catalase, Oxidase, Urease Test, Gelatin Hydrolysis, Starch Hydrolysis [15] was conducted along with Carbohydrate Fermentation of mannitol, sorbitol, inositol, rhamnose, raffinose [18].

**E. Antibiotic Susceptibility Test**

For antibiotic susceptibility test, the disc diffusion method of Kirby-Bauer was used. Bacterial suspension of test isolates were uniformly spread using a sterile cotton swab on sterile Mueller- Hinton agar (MHA) petri dish [19]. The antibiotic disks were placed using sterile forceps and plates were incubated for 18–24 h at 35-37°C. Following incubation, the diameter zone of inhibition (ZOI) formed was measured in millimeter (mm).

**III. RESULTS**

All of the samples used in the present study were found to be contaminated with more or less organisms (Table 1). The microbial load ranged from 8x10^7 to 1.70x10^8 CFU/ml with the lowest in carrot and the highest in cucumber (Fig. 1).

**Table 1: Number of each Organism Isolated per Sample**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vibrio cholerae</th>
<th>V.parahaemolyticus</th>
<th>V.parahaemolyticus</th>
<th>Acinetobacter sp.</th>
<th>Klebsiella sp.</th>
<th>Pseudomonas sp.</th>
<th>Pseudomonas sp.</th>
<th>Moraxella sp.</th>
<th>Salmonella sp.</th>
<th>Escherichia coli</th>
<th>Total number of organisms per sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red Amaranth</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tomato</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cucumber</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carrot</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radish</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of the Organisms Found in The Sample</td>
<td>11</td>
<td>6</td>
<td>6</td>
<td>19</td>
<td>20</td>
<td>19</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Salmonella sp. was detected from Red Amaranth, Cucumber and Carrot. Presence of Vibrio cholerae, Salmonella sp. and E. coli found from the present study are concerning for public health.

One of the isolated Salmonella sp. was resistant to 6 of the drugs (86%). Six of Klebsiella sp., two of Pseudomonas sp. and Acinetobacter sp. and one of Moraxella sp. was resistant to 5 of the drugs. All of the Vibrio sp. isolates was resistant upto 4 drugs (Fig. 3-5).

All of the isolates were also tested for their susceptibility to a number of different antibiotics. In the present study, all of the isolates were resistant to Amoxicillin, except Pseudomonas sp. Imipenem showed the highest sensitivity (86%) among the tested antibiotics against all the organisms, followed by Ciprofloxacin (65%) and Chloramphenicol (42%) (Fig. 4).

IV. DISCUSSION

The increasing consciousness in people regarding the nutrition has resulted in the increased consumption of raw fresh unprocessed vegetables and fruits. These foods carry indigenous microflora besides pathogenic microorganisms. A number of diseases outbreaks due to consumption of these produce have been reported previously [11], [20]. The present study was focused on the detection of Gram-negative pathogenic bacteria from unwashed raw vegetables and thus determining the quality of those products. The total bacterial count showed higher bacterial load in Cucumber ($1.70 \times 10^8$) than other samples which is similar to the findings of study conducted in Dhanbad, India [21].
Among 35 isolated organisms Acinetobacter sp., and Klebsiella sp. were present in all of the samples. However, Pseudomonas sp. was absent only in Tomato samples. According to current study, V. cholerae was isolated from 4 samples out of 12 (33.3%), including red amaranth, spinach, cucumber and radish. Consumption of vegetables and fruits from fields where raw sewage was used for irrigation showed association with a cholera outbreak in Peru [22].

In current study Salmonella sp. was isolated from 3 vegetables out of 12 (8%), which is higher than the findings of Mrittunjay et. al. (21) but close to findings of Kumar. (7.8%) [23]. It also reported that pond water used for irrigation, cleansing and sprinkling of vegetables by vegetable farmers and vendors might be the primary source of contamination of by Salmonella sp.

The presence of E. coli was observed only (6%) in the Carrot sample in current study, while it was the predominant (38.3%) bacterial pathogen from 50 different salad vegetables sold in Amravati City, India. [24] Another study reported 8.1% presence of E. coli in carrots, lettuce, green onions, and spinach samples [25].

According to our study, most of the isolates were resistant to at least four drugs. Ceftriaxone showed complete resistance to all of the isolates, which is a matter of great concern. Consumption of these multi-drug resistant (MDR) pathogen contaminated vegetables can result in serious illness to the consumers. Following Ceftriaxone (100%), Nitrofurantoin (94%), Erythromycin (89%) and Amoxicillin (83%) had the highest resistance against the isolated organisms (Fig. 5). Over the last 50 years, various studies have shown that the excessive use of antimicrobial drugs to treat human and animal infections has resulted in selective pressure, leading to antibiotic resistance. Recent studies have demonstrated that reservoirs of genes coding for antibiotic resistance are present in humans, as well as in animals, plants, and the environment, and that these genes can be transferred to human pathogens by direct contact or indirectly via ingestion of contaminated foods [26].

V. CONCLUSION

Outbreaks of human infection associated with the consumption of raw fruit and vegetables often occur in developing countries and have become more frequent in developed countries over the past decade. Results obtained from this study showed the presence of various pathogenic bacteria on the surface of unwashed fresh raw vegetables from different local markets in Dhaka city. Moreover, most of the pathogens were multi-drug resistance. It indicates the necessity of proper hygiene practice during and post-harvesting stage of the vegetables. For raw consumption further processing of these products is compulsory to ensure their quality and safety of the user. Proper washing with water and/or pretreatment with different antimicrobial agents can reduce the chance of such contaminations. The presence of MDR bacteria in the raw leafy and salad vegetables indicates the poor sanitation during and post-harvesting stage, even during transportation from the fields to the market and then to the consumers. This study showed that consumption of raw vegetables without wash can cause serious illness. To reduce the risk of getting infected by these MDR bacteria, the raw vegetables must be washed properly before the consumption.

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VI. REFERENCES

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