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A Small-Scale Survey of Antibiotic Resistant Enteric Bacteria in Raw Vegetables

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A B S T R A C T

Fresh fruits and vegetables are essential components of a healthy diet. During agricultural production and harvesting, fruits and vegetables can become contaminated with antibiotic-resistant pathogens or commensals from animal and human sources. Plant foods, like fresh salad vegetables, when eaten raw, have been reported to act as vehicle of antibiotic resistant population. World Health organization has launched a Global Antimicrobial Resistance Surveillance System or GLASS in 2015 to support global surveillance and research in order to strengthen the evidence base on antimicrobial resistance (AMR).' Though their initial emphasis was on bacterial pathogens they also emphasized the need of surveillance system for foodborne AMR with time. In this study, we have surveyed the presence of antimicrobial-resistant strains of enteric bacteria in raw vegetables used as salad items like, carrot and cucumber, from two different markets in and around Kolkata. Many multidrug resistant (MDR) strains were detected in these fresh raw vegetable samples. Resistance was detected against streptomycin, ampicillin, tetracycline, chloramphenicol, amikacin, gentamycin and erythromycin. The MDR strains were further characterized to understand the extent of multidrug resistance pattern and the mechanisms by which these strains are exhibiting resistance to multiple drugs.

***Key words*:** Antibiotic resistance, MDR strains, Raw vegetables, Enteric bacteria

Fresh produce like fruits, leafy and non-leafy vegetables are a part of a healthy diet as they are rich source of essential nutrients, such as vitamins, fibers, minerals, and have many health benefits. WHO and FAO have promoted fruits and vegetables consumption since 2003 due to their high nutrient density and low energy density [1-2]. Since many leafy and non-leafy vegetables, root vegetables, sprouts and fruits are eaten raw they may act has a source of foodborne illness as well as antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) [3-4]. There are various potential pathways of contamination of fresh produce with antibiotic resistant bacteria or antibiotic resistant genes [4]. The pre-harvesting pathways include use of untreated or contaminated water for irrigation, use of soil contaminated with animal waste whereas post-harvesting pathways may be washing with contaminated water [5], poor hygiene and sanitation practices of the food handler [6], or cross-contamination from animal foods [7].

Several studies have been conducted worldwide that assessed the presence of ARB and ARG in fresh produce. Twenty different bacterial genera mainly *Escherichia coli*, *Salmonella. enterica*, *Listeria spp*., *Pseudomonads*, *Enterococci* and *Bacillus cereus* were identified [3]. ESBL-producing pathogens were detected on fresh vegetables in Japan [8]. Various pathogenic strains of *E. coli* like diarrheagenic *E. coli*, Shiga toxin-producing *E coli*, enteropathogenic *E coli*, enterotoxicogenic *E. coli* were detected in two studies one from Pakistan [9] and another from Mexico [10]. Multidrug resistant *E coli*, *Pseudomonas* and *Salmonella spp* on fresh vegetables were detected in various other countries like Germany, Czech Republic, USA and Canada, that have the potential of causing disease outbreak [11-14]. A recent literature review showed that the most commonly observed ARB was *E. coli* followed by *Klebsiella spp.* and *Salmonella spp.* and were mainly detected on leafy vegetables [4]. They also reported a significant variation in the prevalence of contamination among developed and developing countries.

In India also there are quite a few reports about ARB prevalence in raw fruits and vegetables. 97.3% of fruit and vegetables sampled from wholesale markets and retail shops in Delhi-NCR region were contaminated with pathogens and even ESBL producing *E. coli* could be detected in 5.7% of the isolates [15]. Multidrug resistant *Salmonella* was detected in leafy and root vegetables collected from two north Indian cities, Kanpur and Bareilly [16]. Antimicrobial resistant Shiga-toxin producing *E. coli* and *Salmonella* were detected in vegetables and fruits collected from local markets and agricultural fields of Rajasthan [17]. However, there is not much report from eastern part of India. In this study the presence of enteric bacterial population was surveyed in fresh vegetables like carrot and cucumber that are largely eaten raw or with minimal processing,

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collected from in and around Kolkata. Further these isolates were characterized to understand the extent of multidrug resistance pattern and the mechanisms by which these strains are exhibiting resistance to multiple drugs.

MATERIALS AND METHODS

Cucumber and carrot samples were collected from local markets of Uttarpara and Baguihati area of Kolkata. Samples were collected in sterile zipped bags and transferred to the laboratory on ice.

Enumeration of total viable count of enteric bacterial population

Samples were washed thoroughly with sterile water to remove the surface contaminants. Then 10 gram of each sample was taken and homogenized with ice cold sterile 0.9% NaCl solution. The homogenate was centrifuged at 7000 rpm for 5 minutes and supernatant was collected in a fresh tube which was treated as the stock solution for each sample. The stock was serially diluted up to 10^{-3} dilutions. 0.1 ml of stock and all dilutions were plated on MacConkey agar medium to determine the total viable count of enteric bacteria. The plates were incubated at 37 °C for 18 hrs. All platings were done in duplicates.

Replica plating and selection of antibiotic resistant bacteria

The MacConkey plates having colonies in the countable range were selected and the colonies were replica plated on two sets of MacConkey agar supplemented with antibiotics tetracycline (30ug/ml) and streptomycin (100 ug/ml) respectively using a sterile velvet pad. The experiments were performed in duplicate sets and incubated at 37 °C for 18 hrs. Total eight distinct streptomycin resistant colonies, three from Uttarpara samples and five from Baguihati samples, were selected randomly and streaked and restreaked several times on streptomycin containing media till pure culture was obtained. The resistance pattern of these isolates towards multiple antibiotics was tested by disc diffusion method.

Antibiotic susceptibility assay

The streptomycin resistant isolates were individually grown in Luria-Bertani (LB) broth at 37 °C for 18 hrs. and next day 100 ul of these cultures were plated on LB-agar plates and discs of different antibiotics chloramphenicol (10ug/disc), erythromycin (15ug/disc), ampicillin (10ug/disc), amikacin (10 ug/disc), gentamicin (50 ug/disc), tetracycline (30ug/disc) were placed aseptically. The plates were incubated at 37 °C for 18 hrs. and diameters of zone of inhibition were measured. The susceptibility or resistance of the organisms to each drug tested was determined according to the CLSI guidelines [18]. Two isolates resistant to six of the seven antibiotics tested were selected and further characterized by gram staining. All antibiotic discs used in this study were purchased from Himedia, Mumbai, India.

Biofilm assay

The selected multidrug resistant isolates were tested for their ability to form biofilm by microtiter plate method. In two microtitre wells of volume 300 ul, 200 ul of LB broth was taken and to it 50 ul of culture of the two isolates were added and incubated at 37 °C for 18 hrs. The content of the wells were removed completely and the empty wells were washed with sterile water twice. The wells were blotted dry by inverting on filter paper and to them 200 ul of LB broth was added and incubated at 37 °C for 18 hrs. The culture was collected and gram stained. The morphology and gram characters were matched with that of the mother culture.

RESULTS AND DISCUSSION

The fresh vegetables used were carrots and cucumbers that are essential components of salads and are often eaten raw. The root vegetable carrot and the fruit cucumber procured from local market of Uttarpara have much higher load of enteric bacteria than those obtained from Baguihati market (Table 1). Initially two different groups of antibiotics tetracycline and an aminoglycoside streptomycin was used to assess the resistance pattern of the enteric bacteria by replica plating on MacConkey media supplemented with antibiotics. The antibiotic resistant bacterial load was highest in the root vegetable sample of Uttarpara market. Almost 80% of the enteric bacterial population was resistant to streptomycin and more than 60% was resistant to tetracycline (Fig 1). The cucumber samples from Baguihati market had almost 50% of its bacterial load resistant to tetracycline. For all other samples the ARB population was greater than 20% (Fig 1).

Table 1 Comparative chart of antibiotic resistant enteric bacterial population

Samples	Total viable count (cfu/ml)	Antibiotic resistant population (cfu/ml)	
		Tetracycline	Streptomycin
	Uttarpara		
Carrot	8.3×10^4	5.2×10^4	6.6×10^4
Cucumber	2.73×10^5	6.0×10^4	5.5×10^4
	Baguihati		
Carrot	9.1×10^3	2.3×10^3	2.1×10^3
Cucumber	6.1×10^3	2.9×10^3	1.9×10^3

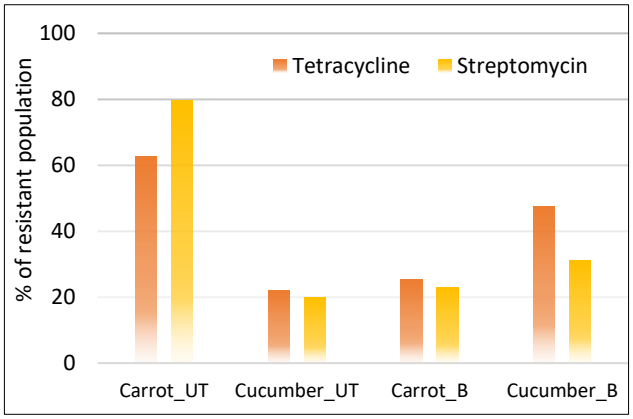

Fig 1 Plot showing proportion of total bacteria population resistant to antibiotics. Uttarpara samples are denoted by UT and Baguihati samples with B

Streptomycin resistant colonies were selected - three from Uttarpara samples and five from Baguihati samples and streaked on fresh antibiotic containing media. To obtain the pure culture repeated sub-culturing was performed. There were five isolates from carrot samples that were denoted as R1-R5 and the three isolates from cucumber samples were denoted as L1-L3. The purity of all the isolates were confirmed by staining and antibiotic susceptibility of these strains were checked against six different antibiotics by disc diffusion assay. Five different groups of antibiotics - ampicillin, a macrolide erythromycin, a fluoroquinolone ciprofloxacin, tetracycline and two aminoglycosides gentamicin and amikacin were tested. Antibiotic susceptibility assay revealed that many of the isolated strains were multidrug resistant (Table 2).

Table 2 Antibiotic susceptibility profile of the eight isolates

Antibiotics	R_1	R_2	R_3	R_4	R_5	L_1	L_2	L_3
Ampicillin	R	R	R	R	R	R	S	R
Chloramphenicol	R	S	S	S	S	R	S	R
Erythromycin	R	R	R	R	R	R	R	R
Tetracycline	R	R	R	R	R	R	R	R
Streptomycin	R	R	R	R	R	R	R	R
Gentamycin	S	-	-	S	S	R	-	S
Amikacin	R	-	-	-	S	S	-	S

R: Resistant, S: Susceptible, (-): Inconclusive

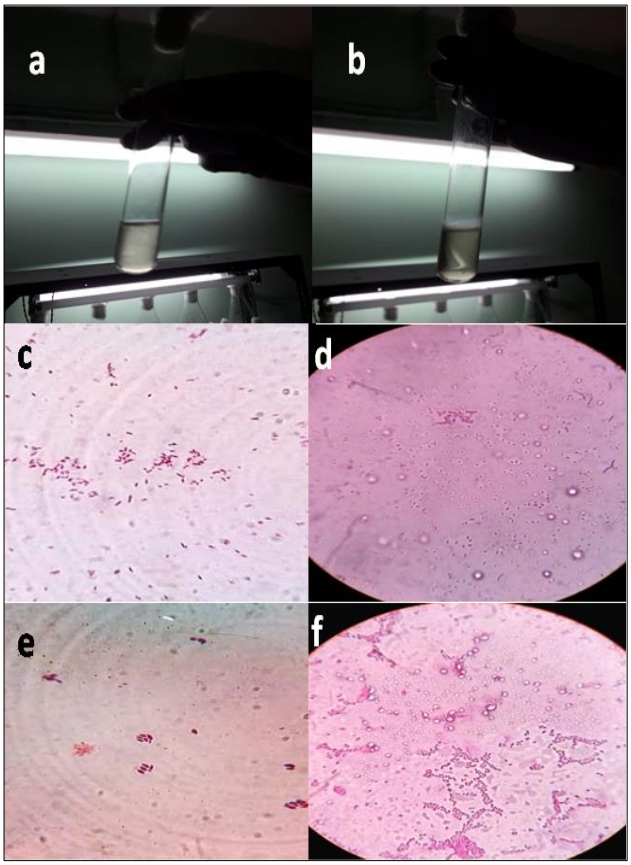

Fig 2 Culture tubes indicates the formation of biofilm at the surface for both L_1(a) and R_1(b) isolates. Gram-stained cells of L_1(c) and R_1(e) isolates as viewed under microscope. Gram-stained cells after biofilm assay of L_1 (d) and R_1 (f) as viewed under microscope

All the isolates were resistant to at least three of the seven antibiotics tested. 50% of the isolates were found to be resistant to four of the seven, 25% were resistant to six antibiotics and 12.5% were resistant to five of them. Thus, high prevalence of MDR strains of enteric bacteria that have the ability to colonize the human gastrointestinal tract could be detected in fresh vegetables, which are often consumed raw and thus pose a threat to human health.

Two of the isolates, L_1 and R_1, which were resistant to six out of seven antibiotics were further characterized. They were confirmed to be gram negative by staining (Fig 2c & 2e).

The probable mechanism by which they acquire resistance to multiple drugs was also investigated. Multidrug resistance is often caused by reduced accumulation of antibiotics inside the cell due to increased activity of efflux pump. The activity of the efflux pump was measured by ethidium bromide cartwheel assay [19]. However active efflux pump could not be detected in any of the isolates. Another major reason of bacterial resistance to multiple antibiotics is biofilm formation [20-23]. Both L_1 and R_1 strains were found to produce biofilm as detected by the microtiter plate method (Fig 2).

CONCLUSION

The organisms that are able to colonize human gastrointestinal tract such as the enteric bacteria are considered as useful bacterial indicators of antimicrobial resistance and are recommended by European Food Safety Authority for harmonized monitoring of antimicrobial resistance. This small-scale survey could detect the presence of antibiotic resistant enteric bacteria in fresh produce from in and around Kolkata that may be detrimental for public health. As high as nearly 80% of the bacterial population was found to be resistant to tetracycline. All the isolates were resistant to three or more drugs. Such high prevalence of MDR strains of enteric bacteria is really alarming. They can act as vehicle to transfer antimicrobial resistance genes to pathogens in the intestine, which can lead to disease outbreak. Moreover, two of the most resistant isolates were capable of biofilm formation. Biofilm is a assembly of cells enclosed in a self-produced matrix that can adhere to various surfaces. Biofilm-producing bacteria exhibit greater resistance to antibiotics due to limited diffusion of the drug through the matrix. Thus, multidrug resistance of the isolates could be attributed to their biofilm forming ability.

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