Bangladesh J Microbiol, Volume 34, Number 1, June 2017, pp 33-38

Original Article

Prevalence of antibiotic resistant enteric bacteria in the hands of street food vendors in Dhaka city

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Foodborne pathogens are affecting public health globally. Street foods being an important part in daily life of urban people are considered as a major source of foodborne pathogens. The major sources of street food contamination are water and the unhygienic condition of the vendors and food preparing places. This study was carried out to assess the load of antibiotic resistant enteric bacteria in the hands of street food vendors. A total of 20 street food vendors were selected from various locations in the Dhaka University campus and their hands were rinsed with 500 mL normal saline. Most of the samples contained high total viable counts, total coliforms count and total fecal coliforms count. A total of 25 selective colonies were identified as enteric bacteria including *E. coli, Klebsiella* spp. and *Acinetobacter* spp. by biochemical tests and 16S rDNA sequencing. All these isolates showed high antibacterial resistance against Amoxacilin (64%) and most of them showed sensitivity against Ciprofloxacin (76%) and Azithromycin (72%). This study revealed that the hand hygiene of street vendors is very poor and may have serious implications for public health due to possible contamination of food. This study, therefore, suggests proper training and education of street food vendors in order to improve their knowledge of personal hygiene and sanitation.

Keywords: Foodborne-pathogens, public health risks, antibiotic-resistance, street food contaminations

Introduction

Foodborne diseases are considered as a major global public health problem[1].Over the years many food-borne diseases have been reported due to contaminated non-homemade food consumption[2]. Among various food types, street foods, which are very popular, are causing serious public health threats in many countries especially in developing countries. With the growing popularity of street foods, prevalence of pathogenic bacteria in street foods and water has been reported in many countries[3]. The hands of ready-to-eat food service employees have been shown to be vectors in the spread of foodborne diseases in many countries, mainly because of poor personal hygiene[4]. It is also stated that unhealthy food handling practices contributed to approximately 97% of foodborne illnesses in food service establishments and homes[3]. Globally, statistical evidence indicates, food poisoning caused by the catering industry is 70% higher than that caused by any other sector[5].

The prevalence of enteric pathogens like *E coli, Shigella* spp. and *Salmonella enterica*, the well known etiologic agents of diarrheal diseases, has been very high in Bangladesh for decades[6,7]. Because of the climatic conditions and poor hygiene, the loads of pathogenic bacteria have been reported in household drinking water and salads as well as foods. Antimicrobial resistance among enteropathogens, including *E. coli* has been reported to be increasing in recent years[8], sometimes leading to point-break situations where no antibiotic treatment options remain[9]. These situations are of serious concern in developing countries where enteropathogens are frequently encountered and cause life-threatening infections, especially among children. Street foods are prepared in open space with visually dirty surroundings and the people handling the foods lack the knowledge of good personal hygiene. One of the major contributors of such foodborne enteric pathogens might be the person involved in preparation and serving of food. Hence, we, for the first time carried out this study to assess the loads of enteric pathogens and their antibiotic resistance patterns in the hands of street food vendors.

Materials and Methods

Sample collection and preparation

A total of 20 street food vendors were selected randomly from various parts of Dhaka University campus and nearby area. Various kinds of street food vendors were selected depending on the types of foods they prepare including, Fuchka (n=5), chotpoti (n=5), salads (n=5) and tea sellers (n=5). The persons were informed verbally about the purpose of the study and immediately after their verbal agreement, the street food vendors rinsed their hands thoroughly in 500mL sterile normal saline (0.90% w/v of NaCl), and the rinsed normal saline was then

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collected in labeled sterile plastic bags and brought in laboratory immediately for sample processing. After bringing the samples in the laboratory, 100 mL from each sample were centrifuged (Eppendorf, USA) at 11000 rpm for 10 minutes at room temperature. After centrifugation, carefully 98 mL of the supernatant was removed and the remaining 2mL of the concentrated fraction was used for various bacterial counts and isolations. For all the counting, two 10-fold serial dilutions were performed twice before spreading on different culture medium.

Total bacterial counts

The total viable bacterial count was carried out by spread plate technique using Plate Count Agar (PCA) (Oxoid, UK) medium. 100µL of the concentrated sample and of each dilution were finely spread onto PCA and incubated at 37°C for 18-24 hours and actual numbers of bacteria were estimated as colony forming unit (CFU)/mL of hand rinse. Similarly, total coliforms and total fecal coliforms were counted using MacConkey agar and mFC agar medium (incubated at 37ºC and 44.5ºC for 18-24 hours) respectively.

Isolation of enteric bacteria

Enteric bacteria were isolated from the MacConkey agar medium and sub-cultured to get pure cultures. Different selective and differential media like Eosine Methylene Blue (EMB) agar and MacConkey (MAC) agar were used to differentiate among different enteric bacteria. One set of all bacterial isolates were stocked in 20% glycerol and stored at -80°C.

Presumptive identification of the enteric bacteria

According to the methods described in the "Manual of Methods for General Bacteriology (American Society for Microbiology (ASM), 1981)", the isolates were presumptively identified based on their colony morphology, Gram staining and biochemical properties. Standard biochemical tests included Triple Sugar Iron (TSI), Motility Indole Urease (MIU), Methyl-Red (MR), Voges-Proskauer (VP), Citrate Utilization, Catalase test and Oxidase tests. All the experiments were performed in triplicate and the results were reproducible.

Amplified ribosomal DNA restriction analysis

ARDRA (Amplified ribosomal DNA restriction analysis) grouping of 16S rRNA gene amplicons amplified using 27F (52 -AGAGTTTGATCMATGGCTCAG-32) and 1492R (52 -GGTTACCTTGTTACGACTT-32)[10] primers were accomplished to verify the identity of the isolates[11, 12]. For PCR, boiled DNA was prepared by boiling the bacterial suspension for 10 minutes at 100°C according to the previously discussed method[13]. The conditions of thermal cycler (Eppendorf Mastercycler®, Germany) for amplification of 16S rRNA gene were: initial denaturation of 5 min at 95 °C followed by 30 cycles of denaturation of 45s at 94°C, annealing of 45s at 58 °C, extension of 2.5 min at 72°C with a final extension of 10 min at 72°C. *Alu*I (Thermo Fisher Scientific, USA) restriction enzyme was used for the digestion of 16s rRNA gene amplicons. The digestion products were resolved by agarose gel electrophoresis using 1.5% agarose (w/v) gel running for 90 minutes at 70V and the gel was observed using Alpha Imager HP Gel-documentation system (Cell bioscience, USA). Genetically related isolates were clustered together based on the restriction patterns using the tool Phoretix 1D (Totallab, UK). In this experiment, there were three experimental controls- uncut experimental DNA, digestion of commercially supplied control DNA and no-enzyme "mock" digestion. Two different size markers, 1 kb (Promega, USA) and 100 bp (Promega, USA) DNA ladders, were used to analyze different restriction fragments.

16S rDNA sequencing

16S rRNA gene amplicons of selected isolates representative of each genotype were sequenced followed by phylogenetic analysis to find out their close relatives. Amplified PCR products were purified by Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's instructions for sequencing purpose. The purified PCR products were sequenced by automated cycle sequencing from DNA sequencing lab of First BASE Laboratories SdnBhd, Malaysia. Partial sequences, obtained using forward and reverse primers, were combined to full length sequences via the SeqMan Genome Assembler[14] and aligned, checked and processed by using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0[15], an integrated tool for sequence analysis. Phylogenetic and molecular evolutionary analyses were also conducted using the MEGA version 6.0 software package. The multiple sequence alignment of the retrieved reference sequences from NCBI, EMBL or DDBJ and sequences of representative isolates was performed with the ClustalW program embedded in Mega 6. Aligned sequences were refined by sequence trimming and conserved region identification. Refined sequences were used for selecting best model and phylogenetic tree construction using the Neighbor-Joining Algorithm and selecting 1000 bootstrap replication.

Antimicrobial susceptibility tests

Susceptibility to antimicrobials was determined by an agar diffusion test using antimicrobial agents impregnated paper discs (Oxoid, USA) as described by the Clinical Laboratory Standards Institute (CLSI) guidelines[16]. The antibiotics used in this study were Ampicillin (AML 10 µg), ciprofloxacin (CIP, 5 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), gentamicin (GN, 10 µg) and Azithromycin (AZM, 5 µg). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as negative and positive controls, respectively. The diameter of the zone of inhibition were used to interpret the results according to the guidelines[16].

Results

Total bacterial counts

For each sample, total viable counts, total coliform counts and total fecal coliform counts were performed. All the samples showed very high counts after 18-24 hours incubation as shown in Table 1.

Table 1. *Total bacterial counts in the hand rinse samples*

Vendor type	Samples	Total viable counts (cfu/mL)	Total coliform (cfu/mL)	Total fecal coliform (cfu/mL)
Tea	1	2.3×10^6	2.5×10^4	2.0×10^4
	2	5.7×10^5	3.6×10^4	2.6×10^4
	3	3.6×10^5	2.4×10^4	7.0×10^4
	4	2.6×10^6	6.1×10^4	2.8×10^4
	5	2.5×10^6	9.6×10^4	1.7×10^4
Fuchka	1	4.4×10^6	3.6×10^4	3.7×10^4
	2	2.9×10^5	1.1×10^4	1.9×10^3
	3	3.7×10^6	6.1×10^5	6.1×10^4
	4	3.5×10^5	1.0×10^4	5.3×10^3
	5	8.5×10^5	4.1×10^4	2.7×10^3
Chotpoti	1	6.1×10^6	1.2×10^5	3.4×10^4
	2	1.3×10^5	6.1×10^4	6.6×10^4
	3	1.8×10^6	2.4×10^4	7.4×10^3
	4	3.5×10^5	4.2×10^4	1.7×10^4
	5	7.8×10^5	5.6×10^4	1.7×10^4
Salad	1	1.3×10^5	9.5×10^4	4.4×10^3
	2	2.1×10^6	9.6×10^4	6.0×10^3
	3	2.9×10^5	4.1×10^3	4.5×10^3
	4	3.4×10^5	3.0×10^4	1.7×10^4
	5	3.9×10^5	5.1×10^4	1.9×10^4

Bacterial isolates

A total of 25 enteric bacteria were isolated from the samples based on the cultural characteristics on MacConkey agar medium and mFC medium (Table 2). All the 25 isolates were stored at -20°C before further analysis.

Table 2. *Classification of the isolates in different morphgroups based on their culture*

Source	Number of Isolates	Growth Characteristics on Different Differential and Selective Media						Microscopic Characteristics			
		Media	Appearance	Form	Elevation	Margin	Consistency	Gram-staining	Size	Shape	Arrangement
Hand rinse	16	MAC	Dark pink	C	F	E	D	Gram-negative	S	SR	Single
		EMB	Blue black with GMS	C	F	E	D				
	8	MAC	Light pink	C	R	E	G	Gram-negative	M	R	Single
		EMB	Brown, dark centered	C	R	E	G				
	1	MAC	Purple	C	R	E	G	Gram-negative	S	SR	Single
		EMB	Blue	C	R	E	G				

Legends for Growth and Microscopic Characteristics: C=Circular, F=Flat, R=Raised, E=Entire, D=Dry, G=Gummy, S=Short, M=Medium, SR=Short Rod, Rd=Rod, GMS=Green Metallic Sheen

Presumptive identification of the isolates

Based on the colony characteristics on different selective and differential media, 3 morphological groups were found. The isolates were all Gram-negative and further analyzed by different biochemical tests such as Kligler's Iron Agar (KIA) test, Motility-Indole-Urease (MIU), IMViC (Indole, Methyl-red (MR), Voges-Proskauer (VP), Citrate, Oxidase test and catalase tests. The outcome of the tests was used to presumptively identify the isolates. It was presumed that 16 of the isolates belonged to the Genus *Escherichia,* 8 of the isolates belonged to the genus *Klebsiella* and 1 isolate belonged to the genus *Acinetobacter* (Table 3).

Genotyping by Amplified Ribosomal DNA Restriction Analysis

25 isolates belonging to 3 different phenotypic groups (based on morphological and biochemical characteristics) were clustered into individual genotypes by Amplified Ribosomal DNA Restriction Analysis (ARDRA). There was a good correlation between the phenotypic groups and ARDRA genotypes where 3 genotypic patterns were revealed by completely digesting 16S rRNA gene amplicons using *Alu*I (Figure 1 & 2).

Phylogenetic analysis

16S rDNA sequencing was carried out and phylogenetic and molecular evolutionary analysis revealed that all three matched with the biochemical identification results as *Klebsiella* spp., *Acinetobacter* spp. and *E. coli* respectively as shown in Figure 3.

Antibacterial resistance

Antibacterial resistance analysis of all the isolated strains revealed that most of the isolates are resistant to amoxicillin (64%), while most of them are sensitive to ciprofloxacin (76%), azithromycin (72%), sulfamethoxazol (84%) and gentamycin (56%) respectively (Figure 4).

Table 3. *Biochemical test results of the 3 ARDRA groups*

KIA				Motility	Indole	Urease activity	MR	VP	Citrate	Oxidase test	Catalase test	Presumptive organism
Slant	Butt	Gas	H_2S									
A	A	-	-	-	-	+	-	-	-	+	-	*Klebsiella* spp.
A	A	+	-	-	-	+	-	+	+	+	-	*Acinetobacter* spp.
K	A	+	-	+	+	-	+	-	+	-	-	*Escherichia* spp.

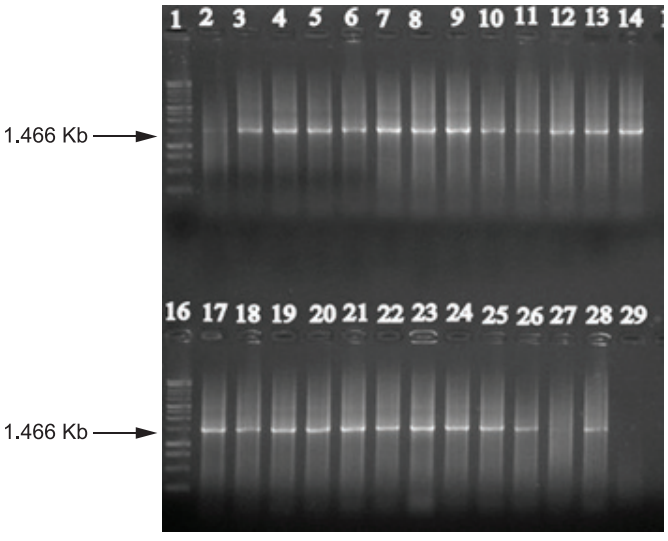

Figure 1. *Amplicons of 16S rRNA genes [Lane 1, 16-Molecular Marker (1Kb), Lane 2-14, 17-28-contain approximately 1.5 Kb band, Lane 29- Negative control].The 1kp ladder was originated from Promega, USA*

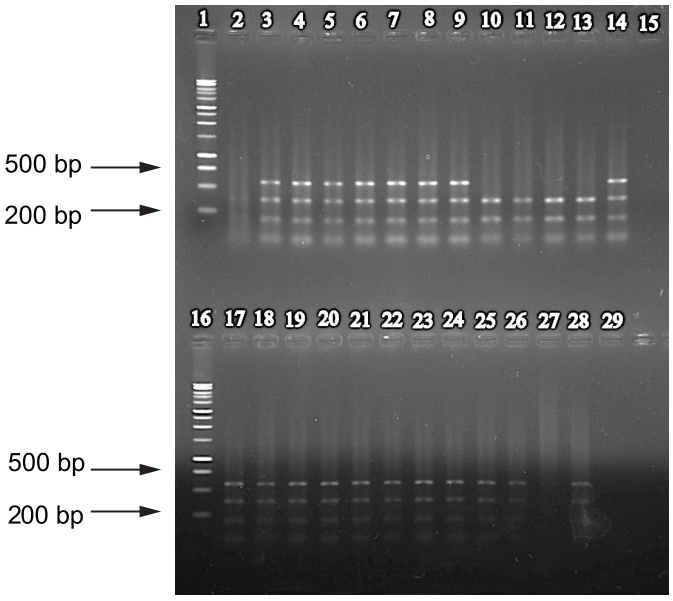

Figure 2. *ARDRA pattern of 25 isolates using Alu1 restriction enzyme*

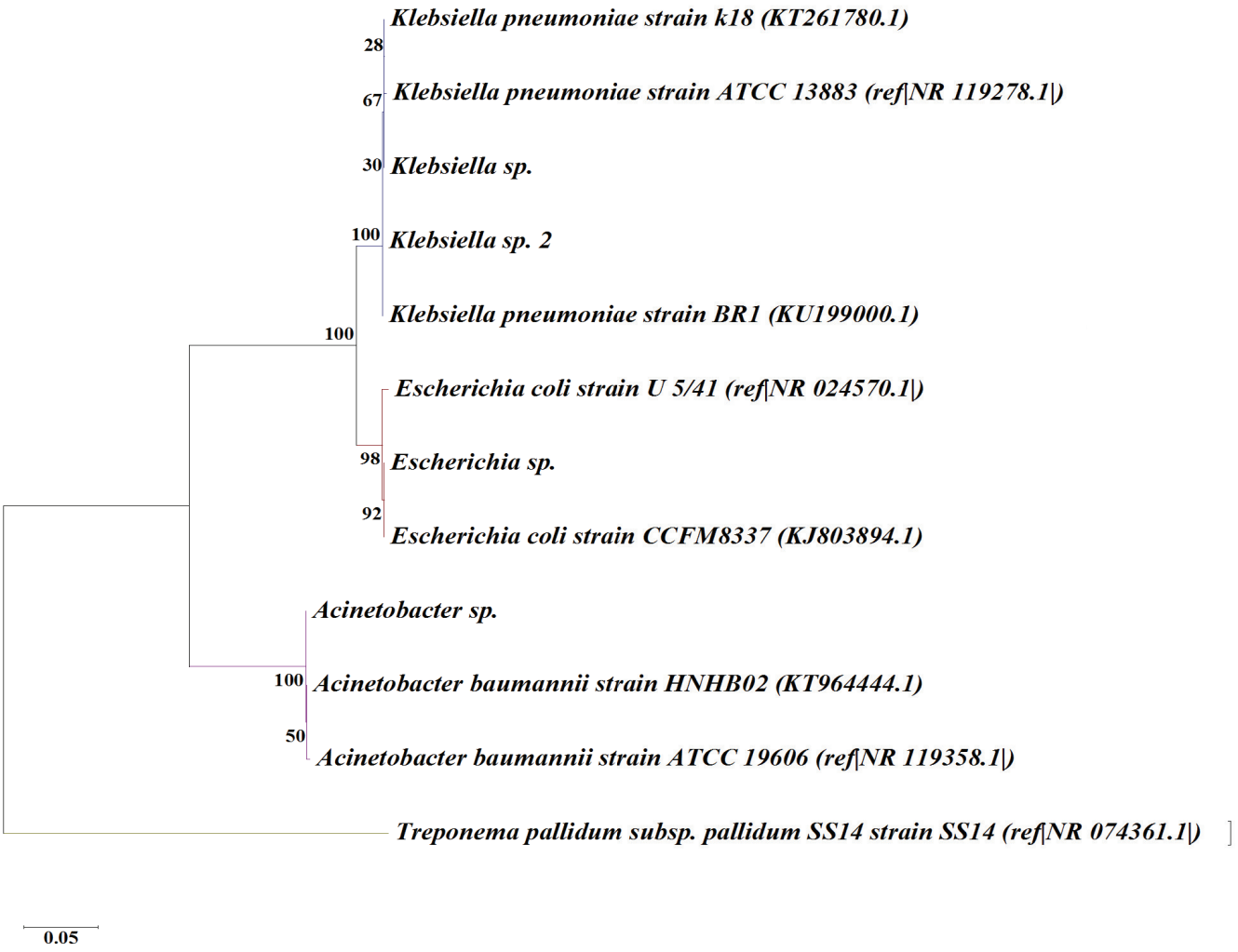

Figure 3. *Phylogenetic tree constructed with MEGA 6 based on ARDRA. The optimal tree with the sum of branch length = 0.62275713 is shown*

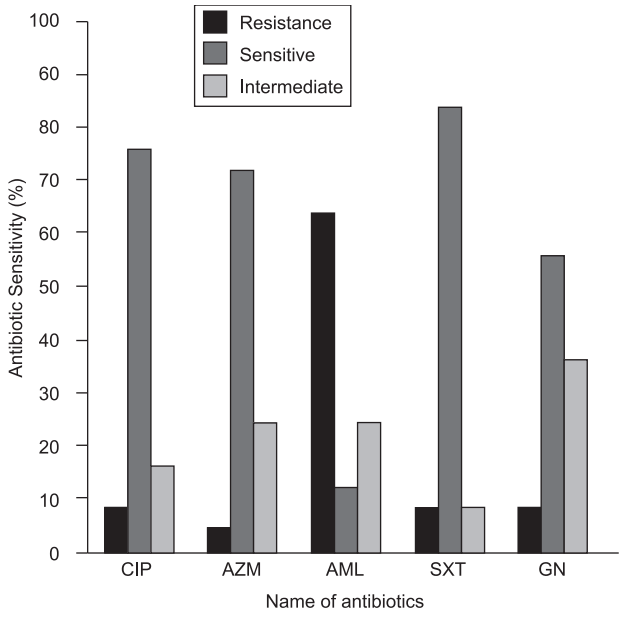

Figure 4. *Antibiotic sensitivity of the 25 isolates against common antibiotics used for enteric bacterial infections*

Discussion

Due to the climatic and the socio economic condition of Bangladesh, the pathogenic bacteria are very prevalent in the environment and in food chains. Because of the high density of population, street foods are very abundant in Dhaka city as well as many big cities and urban areas in Bangladesh. Previous reports have already identified a number of pathogenic bacteria in the food chain of Bangladesh. Considering this prevalence of pathogenic bacteria and the poor hygienic condition of the personnel involved in food preparation and vending, this study was carried out to see the loads of enteric bacteria in the hands of the food handlers in Dhaka city.

As part of this pilot project, we found a very high load of total viable bacteria as well as very high count of total coliforms and fecal coliforms. This reflects the poor personal hygiene among these food handlers. This is very consistent with the results of some of the similar experiments carried out in other parts of the world[17]. The loads of fecal contamination suggest the prevalence of enteric bacteria among the people. We successfully isolated 25 different bacterial colonies showing characteristic colonies of different enteric bacteria. For analysis of such bacterial colonies,

molecular techniques like ARDRA is used now a days to reduce the huge workloads like conventional biochemical tests for each strains and probably expensive serological tests. By performing ARDRA using *Alu*1 restriction enzyme, we observed 3 different patterns from all the isolates. Following this, conventional biochemical tests confirmed the 3 different groups as *Klebsiella* spp., *Acinetobacter* spp. and *E. coli*. The 16S rDNA sequencing and phylogenetic analysis further confirmed the identification of the bacteria. Antibacterial resistance assay was performed to analyze the susceptibility of the isolated enteric bacteria to the commonly used antibiotics as antibiotic resistance is found very often from the isolates in Bangladesh and neighboring countries [6,7,18,19]. Very high resistance was observed with amoxicillin and interestingly, there was high susceptibility to other antibiotics. This might be related to the usage and price of the antibiotics. Among the low-income people, the most commonly used antibiotic is amoxicillin. However, further studies with different economic status might explain these resistance patterns better.

This study successfully isolated and identified 3 types of pathogenic enteric bacteria from hand rinse of street food vendors. The overall result of this study is putting a huge threat to the public health of Bangladesh as people are becoming more interested in street foods. The personal hygiene is too poor of these vendors and they clearly lack the knowledge of food safety. The future studies can be carried out to regularly monitor the prevalence of the enteric pathogens from street foods as well as foods from different restaurants and hospitals. All these enteric bacteria can further be well characterized to study their toxigenicity and other pathogenic roles to aware people about such situations. Strict guidelines and proper authorization is required to overcome such situation. Government authorities like food safety authority and related organizations can regularly monitor and take measures to train such food handlers for the protection of public health.

Acknowledgement:

We thank Professor Dr. Sabita Rezwana Rahman, Professor Dr. Chowdhury Rafiqul Ahsan and Professor Dr. Anwar Hossain for their kind support with the lab space and instruments. We also thank Mr. Shamsur Rahman and Mr. Rafiqul Islam for their technical assistance throughout the project. There is no conflict of interests among the authors.

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