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Original Article

Molecular Characterization of *Enterobacter* and *Escherichia coli* Pathotypes Prevalent in the Popular Street Foods of Dhaka City and their Multidrug Resistance

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Food borne pathogenic enteric bacteria are of great concern for global public health. Among them, *Escherichia coli* and *Enterobacter* spp. are the most prevalent in the street food. In this study, 23 strains of such enteric bacteria were isolated from multiple food samples by conventional cultural technique. Isolated strains were characterized molecularly into different genotypes using RAPD, amplified ribosomal DNA restriction analysis, and partial sequencing of 16S rDNA. RAPD represents 10 different types of strains whereas ARDRA clusters them into two separate groups. 16 out of the 23 isolates were identified as *E. coli* and the rest were as *Enterobacter* spp. by biochemical tests and were further confirmed by partial 16S rDNA sequencing. Significant level of virulence traits including *stx*1, *stx*2 and *esc*V genes were identified in *E. coli* strains. Also, most of the isolates were found resistant to azithromycin and amoxicillin. This study revealed the presence of various pathogenic enteric bacteria in various street foods with multidrug resistance. Therefore, this study suggests that people consuming such street foods are at major risk of food borne illness.

Keywords: Street food, enteric bacteria, virulence genes, multidrug resistance.

Introduction

Food is an awfully effective vehicle for the conveyance of a variety of pathogens[1,2]. Lack of food safety results different food borne diseases which puts a constant threat to public health[3]. In Bangladesh, about 30 million people suffer from food borne illnesses each year of which diarrheal diseases kill approximately 2.2 million people including many children yearly[4]. Over the years, food borne diseases have been considered a major public health problem considering the socio-economic status in developing countries[5]. Many previous reports claim that consumption of non-homemade foods, especially street foods which can easily be contaminated by foodborne pathogens, is mostly responsible for food borne diseases[6]. High loads of bacterial pathogens in different street foods have been reported for several times in Bangladesh. These dangerous street foods are triggering deadly diseases, especially among children. Statistical evidence indicates children younger than five are at the greatest risk[7]. Street food vendors have little or no knowledge about food safety and hygiene in Bangladesh[8]. Hence, the chance of microbial contamination can be very high in the street foods. In recent time, bacterial contamination of the street foods has drawn most of the attention not merely due to their pathogenicity but also due to their high antibiotic resistance pattern[9]. High prevalence of multidrug resistance genes among the street food bacterial isolates have been reported in many countries. One of the major groups of these food borne pathogens comprises of enteric bacteria, mostly *Escherichia coli*[10]. Many previous reports identified high prevalence of *E. coli* into the different street foods in Bangladesh[11]. *E. coli* is highly versatile that can frequently be deadly pathogens. Considering the overall situation of occurrence of the virulence traits among the street food, *E. coli* is a growing concern in Bangladesh. This study was carried out to assess the virulence potential and to categorize the food borne *E. coli* into various pathotypes along with their antibiotic resistance pattern from different street food items in Dhaka. Various virulent traits were examined to detect different pathotypes of *E. coli* like *stx*1 and *stx*2 for shiga toxin-producing *E. coli* (STEC) and enterohemorrhagic *E. coli* (EHEC), *esc*V for enteropathogenic *E. coli* (EPEC) and *inv*E for enteroinvasive *E. coli* (EIEC).

Materials and Methods

Sample collection and preparation

Multiple vel-puri, fuchka, water and salad materials were collected in labeled sterile zip lock bag from different street food vendors around the Dhaka city in Bangladesh with a regular time interval. All the samples were brought immediately to the laboratory for processing. Samples were either directly dissolved or were homogenized in sterile saline solution before culture. Occasionally, samples were resuscitated in sterile peptone water. All the water samples were first centrifuged at 10000 rpm for 10

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minutes twice with sterile physiological saline and the pellets were collected for culture.

Isolation of bacterial strains

All samples were inoculated on MacConkey agar plates and were incubated at 37 °C for 18 hours. Pure colonies on the MacConkey agar plates were randomly selected based on their characteristics colony color and texture, and were further streaked on EMB agar plates to primarily sort out enteric bacteria. Isolates producing characteristics metallic green sheen colony on EMB agar plates were considered for further experimentation and were preserved in 20% glycerol at -80 °C.

Biochemical characterization of the isolates

Isolates were characterized by their response to different biochemical tests that includes Kligler Iron agar (KIA), Motility Indole Urease (MIU), Methyl-Red (MR), Voges-Proskauer (VP), Triple Sugar Iron (TSI), citrate utilization, catalase and oxidase tests[12].

Molecular characterization of the isolates

Randomly amplified polymorphic DNA (RAPD) method, amplified rDNA restriction analysis (ARDRA) method, and partial sequencing of 16S rDNA gene of the isolates were performed for further characterization. For RAPD, PCR reactions were carried out in 25-µL volumes containing: 12.5 µl of 2X mastermix, 1 µl of 10 pmol primer (sequence: 52 -GCGATCCCCA-32)[13], 3 µl template DNA and nuclease-free water up to the volume. Amplification was performed using PCR thermal cycler with condition: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 40 °C, and 2 min at 72 °C with a final extension of 10 min at 72 °C. After amplification, PCR product was subjected to agarose gel electrophoresis and visualized under UV-transilluminator. 16s rDNA amplicons were generated by PCR was accomplished using forward primer: 52 -AGAGTTTGATCMATGGCTCAG-32 and reverse primer: 52 -GGTTACCTTGTTACGACTT-32 primers[14]. Reaction conditions of thermal cycler were: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C with a final extension of 10 min at 72 °C. For ARDRA, a single restriction enzyme *Alu*I (Thermo Fisher Scientific, USA) was used for the digestion of 16s rDNA amplicons overnight at 37 °C. After digestion, the products were resolved in 1.5% agarose gel. For partial sequencing of 16S rDNA gene, the 16s rDNA amplicons were purified with the Wizard PCR SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manual instruction. The purified PCR products were sequenced by automated cycle sequencing from DNA sequencing lab of First BASE Laboratories, Malaysia.

Bioinformatic analysis

Sequences were assembled in SeqMan Genome Assembler and the complete sequences were blasted at NCBI to identify individual strains. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 7.0 software package. Trees were generated using neighbor-joining algorithm with 1000 bootstrap replication. A *Mycobacterium* spp. sequences was used as an out-group.

Detection of different virulent genes

Four different virulent genes (*stx*1*, stx*2, *esc*V*,*and *inv*E) was detected by PCR using the primers listed in Table 1. The reaction conditions were optimized by slightly modifying a protocol described previously[15]. The target genes were amplified individually.

Antimicrobial susceptibility tests

Antimicrobial susceptibility of the isolated strains was determined by standard Kirby-Bauer disk diffusion method[16]. The antibiotics tested were ciprofloxacin, amoxicillin, nitrofurantoin, trimethoprim, azithromycin and gentamicin (Oxoid, UK). Briefly, a colony of individual strain was transferred into 5 ml of nutrient broth and was allowed to grow at 37 °C for 8 h with shaking to obtain young culture adjusted to McFarland 0.5 standard (2×10^8 cfu/ml). A sterile swab dipped into this standardized suspension was streaked evenly in two directions over the entire surface of a Muller-Hinton agar (Oxoid, UK) plate to obtain uniform lawn of bacterial culture. The antibiotic disks were then applied to the inoculated plates with sterile forceps and were incubated at 37 °C for 24h. Then, the diameters of the zones of complete inhibition were measured using a scale. The results were interpreted as mentioned by Clinical and Laboratory Standards Institute (CLSI) standard guidelines[17].

Table 1. *Primers used for PCR amplification of different virulence genes.*

Target	Primer	Primer sequence (52 -32)	T_m (°C)	Amplicon (bp)
*stx*1	stx1 F	AGTTAATGTGGTGGCGAAGG	60	347
	stx1 R	CACCAGACAATGTAACCGC		
*stx*2	stx2 F	TTCGGTATCCTATTCCCGG	58	589
	stx2 R	CGTCATCGTATACACAGGAG		
*inv*E	invE F	GCAGGAGCAGATCTTGAAG	58	208
	invE R	GAAAGGCACGAGTGACTTTC		
*esc*V	escV F	GGCTCTCTTCTTCTTTATGGCTG	62	534
	escV R	CCTTTTACAAACTTCATCGCC		

Results

Basic biochemical characteristics of the isolated strains

23 individual Gram negative bacterial strains producing metallic green sheen on EMB agar were isolated from the studied street food samples. Colonies of these strains were circular, raised or flat and dry or gummy. They produced gas and acidic reaction in both butt and slant of KIA. Also, they were motile and positive for urease, VP, citrate and catalase tests, but negative for H_2S, indole, MR and oxidase tests. These data indicated that these strains were enteric bacterial strains, from which 16 isolates were *Escherichia coli* and the remaining 7 isolates were *Enterobacter* spp.

Molecular characteristics of the isolated strains

10 different RAPD patterns were identified in these isolated strains. Figure 1 represents a typical photograph showing six different RAPD patterns. Such observation indicates that these enteric bacterial strains are possibly different from each other. To further validate this and to genotype these strains, ARDRA was performed. Figure 2 represents a typical ARDRA pattern of the isolated strains. ARDRA showed that these strains can be grouped in two types: one group of 7 isolates showing three bands of about 100 bp, 250 bp and 550 bp, and the other group of 16 isolates showing two bands of about 100 bp and 250 bp. 16S rDNA sequencing of these strains confirms that the isolates showing three bands in ARDRA were actually *Enterobacter* spp. (7 isolates) and the remaining isolates were *E. coli* (16 isolates). Figure 3 represents a typical phylogenetic tree showing clustering of *Enterobacter* spp. *E. coli*.

Presence of different virulence genes in the E. coli isolates

Among the 16 *E. coli* isolates, 5 were *stx*1 positive, 4 were *stx*2 positive and the remaining 7 were *esc*V positive (Figure 4). All of the *E. coli* isolates were *inv*E negative (data not shown). So among the different pathotypes of *E. coli*, this study identified 9 shiga toxin-producing *E. coli* and enterohemorrhagic *E. coli* (*stx*1 and *stx*2 positive), and seven enteropathogenic *E. coli* (*esc*V positive) in the street food samples.

Antibacterial resistance

Antibacterial resistance analysis of all the isolated bacteria revealed that most of the isolates were resistant to azithromycin (72%) and amoxicillin (64%). However most of them are sensitive to ciprofloxacin (76%), nitrofurantoin (84%), trimethoprim (72%) and gentamycin (80%) respectively (Figure 5).

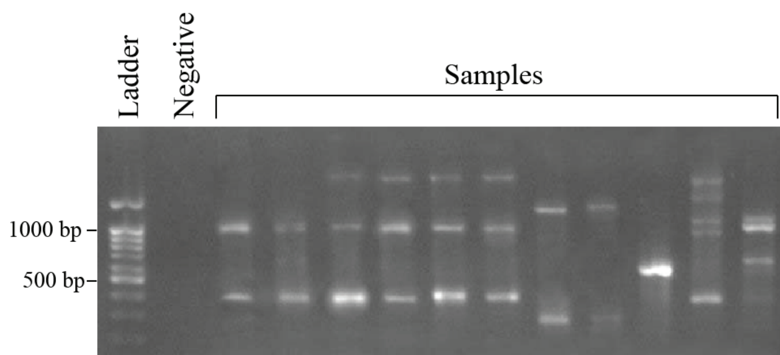

Figure 1. *RAPD analysis of the isolated strains. Six different RAPD patterns are shown here.*

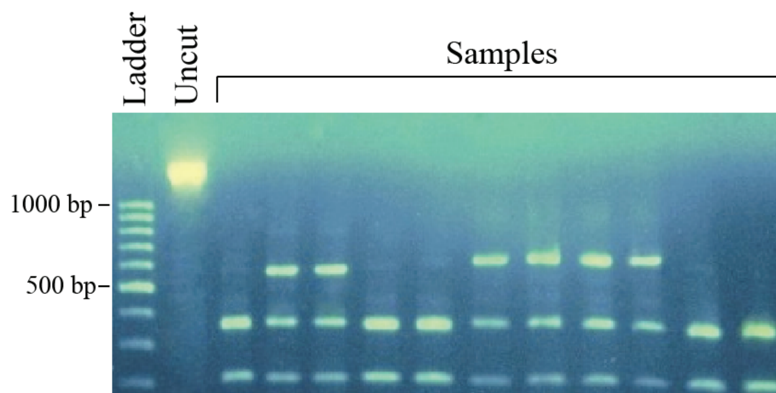

Figure 2. *ARDRA of the isolated strains. The isolated strains can be grouped in two types based on ARDRA pattern.*

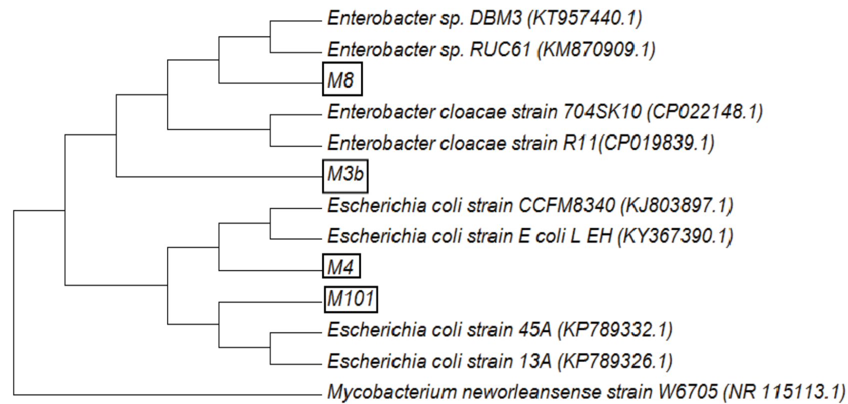

Figure 3. *Phylogenetic tree constructed from partial16S rDNA sequences of the isolated strains. Two strains, namely M8 and M3b clusters with Enterobacter spp., and M4 and M101 clusters with E. coli. Mycobacterium neworleansense was used as an out-group organism.*

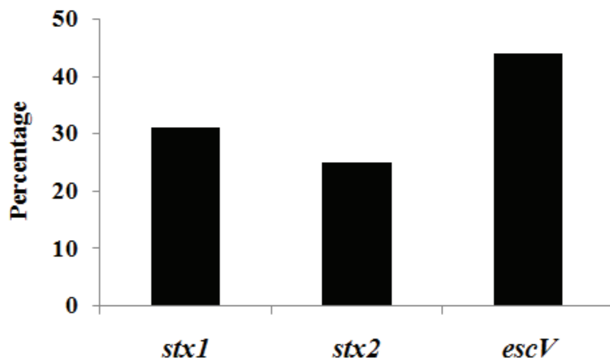

Figure 4. *Percentage of different virulence genes found in the isolates. Most of the isolates carry escV gene.*

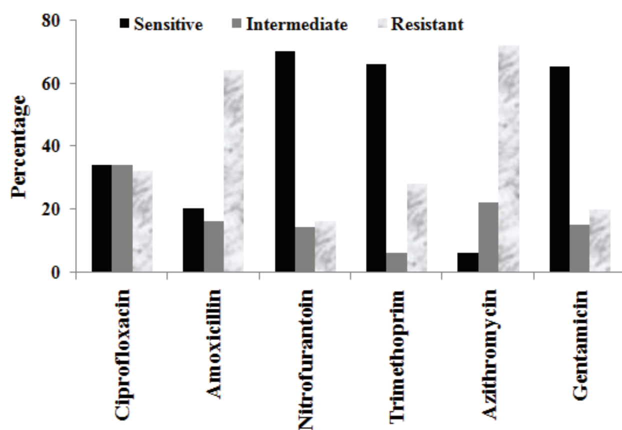

Figure 5. *Antibiotic sensitivity of the 23 isolates against common antibiotics used for enteric bacterial infections.*

Discussion

Disease outbreaks associated with food borne diarrheagenic *E. coli* are frequently reported in the developed countries due to the existence of a comprehensive surveillance system[18]. Although the socioeconomic condition of Bangladesh indulges vary high prevalence of pathogenic bacteria in the food chain, the current surveillance system is poorly defined[8]. Street food vending is very common and popular in busiest city Dhaka in Bangladesh[19]. Most people like these street foods both for its cheap price as well as for its mouth-watering taste. But a good number of investigations reported that street foods are dangerous, especially for enteric bacteria[8,20]. Hence, this study was carried out to comprehend the virulence potential as well as multidrug resistance status of the enteric bacteria in the street food items in Dhaka city.

From different street foods, 23 enteric bacteria like isolates on selective agar were analyzed using molecular techniques like ARDRA. This molecular analysis made it convenient to conduct the conventional biochemical tests as well as reduced the load of 16S rDNA sequence analysis for all isolates. ARDRA grouping revealed 2 different patterns from all the isolates (Figure 2) which later presumptively confirmed as *E. coli* and *Enterobacter* spp. by conventional biochemical tests. In this study, RAPD typing was also accomplished which further differentiated two ARDRA group isolates into 10 different patterns (Figure 1). Isolates were selected for sequencing based on 16S rDNA gene from 2 different ARDRA groups with different RAPD types to interpret the close relative species. The 16S rDNA sequencing and phylogenetic analysis further confirmed the identification of the bacteria from two different ARDRA groups as *E. coli* and *Enterobacter* spp (Figure 3). Isolates from the same ARDRA group with different RAPD type showed the same genus of bacteria.

To characterize the virulence traits of the identified *E. coli* strain, a PCR based assay was employed for *in vitro* determination of the *stx*1and *stx*2 genes encoding shiga-like toxin (Stx), *esc*V gene encoding EscV protein of Type III secretion systems (T3SSs) and *inv*E gene encoding InvE protein for invasion which is atypical for enteroinvasive *Escherichia coli*. Determination of the *stx*1 and*stx*2 confirmed the presence of diarrhoeagenic *E. coli* (especially STEC and EHEC) strains in the street food

samples. Detection of *esc*V gene confirmed the presence of enteropathogenic *E.coli* (EPEC) in the food samples (Figure 4). No band for *inv*E confirmed the absence of EIEC strains in the food samples. Therefore, the pathogenic *E. coli* strains found in this study belong to three different pathotypes: STEC, EHEC and EPEC. EPEC (43%) represents the most frequent pathotype in the street food samples followed by STEC (17%). The prevalence of *stx*1 gene was 17% among the strains tested whereas *stx*2 gene was 55%. *stx*2 is more associated than *stx*1 with the EHEC strains that cause hemolytic-uremic syndrome. Results of the prevalence of potential pathogenic *E. coli* strains in the street food samples could be explained by the fact that these foods might be contaminated by fecal coliform. Another reasonable fact could be that most of the street food items were sampled near hospital area where the foods might get contaminated with clinical sewage. In addition, most of the homeless people living in Dhaka city without sanitary latrines, usually defecate in the roadside through which the vending items might get contaminated.

Irrational use of antibiotics has already made the situation worse in Bangladesh. As anticipated, high resistance against commonly used antibiotics was found. Many of the isolates studied here were found multi-drug resistant. Most people in Bangladesh use antibiotic indiscriminately even in case of viral infection. As a result multidrug resistant bacteria thrived in the environment. Highest resistance was observed with the amoxicillin and azithromycin (Figure 5) and moderately resistance to other antibiotics. So it appears that none of the drugs could be prescribed exclusively that would be effective against all the tested pathogens.

Conclusion

This study successfully identified pathogenic *Escherichia coli* with some dangerous virulence traits from different street food samples and also found them resistant to commonly used antibiotics. Hence, this is really an alarming situation putting a huge threat to the public health of Bangladesh. One of our previous studies also reported the high load of enteric bacteria in the hands of street food vendors[10]. Therefore, both the street foods and the personnel involving street food vending could be an exclusive reason to cause enteric diseases to the consumers[7-9]. To overcome such situation, proper regulation and monitoring in street food vending activities are warranted. Food handlers and street food vendors need to train about personal hygiene and microbiological quality environment for food preparation.

Conflict of interest

The authors declare no conflict of interest.

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References

1. Rabbani GH and Greenough WB. 1999. Food as a vehicle of transmission of cholera. *J. Diarrhoeal Dis. Res*. **17**: 1-9.
2. Abdullah WZW, Mackey BM and Karatzas KAG. 2017. High Phenotypic Variability among Representative Strains of Common *Salmonella enterica* Serovars with Possible Implications for Food Safety. *J. Food Prot*. **78**: 93-104.
3. Borchers A, Teuber SS, Keen CL and Gershwin ME. 2010. Food safety. *Clin. Rev. Allergy Immunol*. **39**: 95-141.
4. Baker KK, O'Reilly CE, Levine MM, Kotloff KL, Nataro JP, Ayers TL, Farag TH, Nasrin D, Blackwelder WC, Wu Y, Alonso PL, Omore R, Faruque AS, Das SK, Ahmed S, Saha D, Sow SO, Sur D, Zaidi AK, Quadri F and Mintz ED. 2016. Sanitation and Hygiene-Specific Risk Factors for Moderate-to-Severe Diarrhea in Young Children in the Global Enteric Multicenter Study, 2007-2011: Case-Control Study. *PLoS Med*. **13**: e1002010.
5. Ma L, Chen H, Yan H, Yang L and Wu L. 2017. Multiple attribute decision making model and application to food safety risk evaluation. *PLoS One*. **12**: e0189835.
6. Greksa LP, Islam A, Okamoto R and Omori K. 2017. Dietary Patterns and Dietary Adequacy of Street Children in Dhaka, Bangladesh. *Eco.l Food Nutr*. **56**: 479-492.
7. Islam S, Nasrin N, Rizwan F, Nahar L, Bhowmik A, Esha SA, Talukder KA, Akter M, Roy A and Ahmed M. 2015. Microbial Contamination of Street Vended Foods from a University Campus in Bangladesh. *S. Asian J. Trop. Med. Public Health*. **46**: 480-5.
8. Al-Mamun M, Rahman SM and Turin TC. 2013. Knowledge and awareness of children's food safety among school-based street food vendors in Dhaka, Bangladesh. *Foodborne Pathog. Dis*. **10**: 323-30.
9. Al-Mamun M, Rahman SM and Turin TC. 2013. Microbiological quality of selected street food items vended by school-based street food vendors in Dhaka, Bangladesh. *Int. J. Food Microbiol*. **166**: 413-8.
10. Yaici L, Haenni M, Metayer V, Saras E, Mesbah Zekar F, Ayad M, Touati A and Madec JY. 2017. Spread of ESBL/AmpC-producing *Escherichia coli* and *Klebsiella pneumoniae* in the community through ready-to-eat sandwiches in Algeria. *Int. J. Food Microbiol*. **245**: 66-72.
11. Islam MA, Mondol AS, Azmi IJ, de Boer E, Beumer RR, Zwietering MH, Heuvelink AE and Talukder KA. 2010. Occurrence and characterization of Shiga toxin-producing *Escherichia coli* in raw meat, raw milk, and street vended juices in Bangladesh. *Foodborne Pathog. Dis*. **7**: 1381-5.
12. Farmer JJ, Davis BR, Hickman-Brenner FW, McWhorter A, Huntley-Carter GP, Asbury MA, Riddle C, *et al*. 1985. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. *J. Clin. Microbiol*. **21**: 46-76.
13. Marialouis XA and Santhanam A. 2016. Antibiotic Resistance, RAPD-PCR Typing of Multiple Drug Resistant Strains of *Escherichia coli* From Urinary Tract Infection (UTI). *J. Clin. Diagn. Res*. **10**: 5-9.
14. Vaneechoutte M, Rossau R, Vos DP, Gillis M, Janssens D, Paepe N, De Rouck A, Fiers T, Claeys G and Kersters K. 1992. Rapid identification of bacteria of the Comamonadaceae with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiol. Lett*. **72**: 227-33.
15. Muller D, Hagedorn P, Brast S, Heusipp G, Bielaszewska M, Friedrich AW, Karch H and Schmidt MA. 2006. Rapid identification and

differentiation of clinical isolates of enteropathogenic *Escherichia coli* (EPEC), atypical EPEC, and Shiga toxin-producing *Escherichia coli* by a one-step multiplex PCR method. *J. Clin. Microbiol*. **44**: 2626-9.

16. Bauer AW, Kirby WM, Sherris JC and Turck M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*. **45**: 493-6.

17. Hindler JF and Stelling J. 2007. Analysis and presentation of cumulative antibiograms: a new consensus guideline from the Clinical and Laboratory Standards Institute. *Clin. Infect. Dis*. **44**: 867-73.

18. Ao TT, Rahman M, Haque F, Chakraborty A, Hossain MJ, Haider S, Alamgir AS, Sobel J, Luby SP and Gurley ES. 2016. Low-Cost National Media-Based Surveillance System for Public Health Events, Bangladesh. *Emerg. Infect. Dis*. **22**: 720-2.

19. Khairuzzaman M, Chowdhury FM, Zaman S, Al Mamun A and Bari ML. 2014. Food Safety Challenges towards Safe, Healthy, and Nutritious Street Foods in Bangladesh. *Int. J. Food Sci*. **2014**: 483519.

20. Hassan MM, Chakrabarty RP, Siddique MA and Rahaman MM. 2017. Prevalence of antibiotic resistant enteric bacteria in the hands of street food vendors in Dhaka city. *Bangladesh J. Microbiol.* **34**: 25-29.