Original Article

Comparison of beta-lactamase genes in clinical and food bacterial isolates in India

Mohd. Shahid, Abida Malik, Mohd. Adil, Noor Jahan, and Ritu Malik

Section of Bacteriology & Molecular Biology, Department of Medical Microbiology, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh-202002, Uttar Pradesh, India

Abstract

Background: The present study aimed to determine the occurrence of human disease-causing enteric bacteria on raw vegetables, fruits, meats, and milk products sold in Indian markets. The study further aimed to analyze antibiotic resistance rates and the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{AmpC}.

Methodology: Twenty-three food-borne and 23 clinical isolates were compared for antibiotic resistance rates and the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{AmpC}. Swabs were taken from unwashed and washed food items, as well as from some chopped food specimens, and inoculated on appropriate culture medium. Bacterial isolates were identified, antibiotic susceptibility was performed, and *bla* genes were detected by PCR.

Results: Thirty-eight bacterial isolates were obtained from the food specimens, of which 36 (94.7%) were Gram-negative and two (5.3%) were Gram-positive bacterial species. *Klebsiella pneumoniae* was the most prevalent (52.6%; 20/38) bacterial species isolated, followed by *Citrobacter koseri* (18.4%; 7/38). In food isolates, the majority of the isolates were resistant to gentamicin (33.3%) followed by amikacin (11.1%). Resistance to a third-generation cephalosporin was noticed in only 5.6% isolates. However, in clinical isolates, maximal resistance was noticed against third-generation cephalosporins followed by ofloxacin in 91.3% and 86.9% isolates, respectively, and resistance to gentamicin and amikacin was noticed in 78.3% and 52.2% isolates, respectively. The presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{ampC} in clinical isolates was noticed in 52.2%, 60.9%, 21.7%, and 43.5%, respectively. None of the isolates from food showed the presence of any of the above-cited genes.

Conclusions: Probably *bla* genes have not yet disseminated to raw-food vegetation in India.

Key Words: Food-borne bacteria, antibiotic resistance, CTX-M, TEM, SHV, AmpC, beta-lactamases

J Infect Dev Ctries 2009; 3(8):593-598.

Received 17 October 2008 - Accepted 12 August 2009

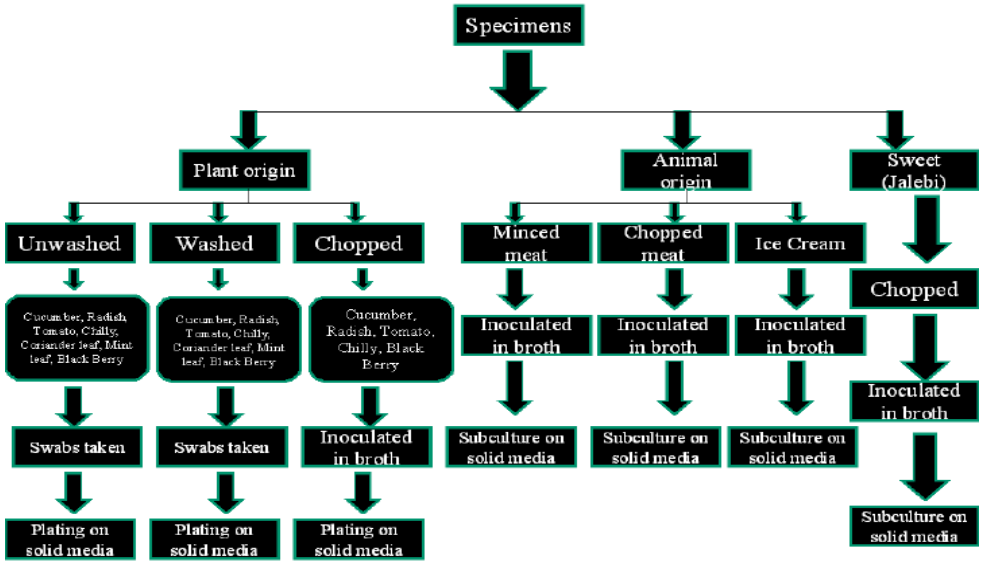
Introduction

Raw vegetables are widely consumed as salads and in other dishes in many countries, including India. In most instances these vegetables are washed with water and not treated with any antimicrobial/disinfectant solution, such as a solution of potassium permanganate. This is particularly true for food-consuming habits in India.

There are reports from many parts of the world indicating bacterial contamination of raw vegetables [1]. Furthermore, there are also reports that implicate food as a source of resistant bacteria, raising concerns of disease transmission when these food items are consumed raw [2,3]. However, reports looking for the occurrence of *bla* genes in food-associated enteric bacteria are elementary, particularly in India.

This preliminary study was performed to look for the occurrence of human disease-causing enteric bacteria on raw vegetables and fruits sold in Indian markets by taking swabs from unwashed food items to analyze the bacterial occurrence and load before and after washing them with sterile water (mimicking a condition of consumption of salads). Moreover, we also attempted to look for the occurrence of bacterial contamination in chopped food items. The Gram-negative bacterial isolates obtained from food items were further studied for antibiotic resistance rates and the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{AmpC}. In addition to food items of plant origin, other foods, including minced meat, chopped meat and ice cream (as a source of animal-origin food) and sweets (Jalebi), which are sold openly in unhygienic condition in Indian markets, were also analyzed in a

Figure 1.



Algorithm showing various food-originated specimens used in this study as well as their processing steps.

similar manner. A small subset of pathogenic bacteria obtained from clinical specimens was also included in the study to compare the results with bacterial isolates of food origin.

Materials and Methods

The present prospective study was conducted in the department of Microbiology of Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, UP, India, during August 2008 to October 2008.

Specimens

Food-borne specimens

The detailed algorithm showing various food-borne specimens used in this study as well as their processing steps is shown in Figure 1. All the food items were collected in sterile polythene bags and transported to the laboratory immediately for further processing.

Specimen processing

Swabs were taken from unwashed and washed food items by rubbing and rolling a sterile swab over a rectangular section of 5.0 cm x 2.5 cm from various surfaces of food items. In the case of coriander and mint leaves, the swabs were collected from a surface of 2.5 cm x 2.5 cm from both the surfaces. These swabs were inoculated over blood agar (BA) and McConkey agar (McA) for bacterial culture and the plates were incubated overnight at 37^{o}C in aerobic conditions. The detailed descriptions of food items processed by taking the swabs are shown in Figure 1. After taking the swabs, the washed food items were chopped in sterile petriplates and 1 gm of chopped material was inoculated in 10 ml of sterile nutrient broth and incubated for six hours at 37^{o}C. A loopful of broth was then subcultured over BA and McA for bacterial identification. Colonies grown on the plates from unwashed and washed specimens were counted and analyzed for the elimination or reduction of bacterial load in washed specimens.

Minced meat, chopped meat, ice cream, and Jalebi were first inoculated in sterile broth and processed in the same manner described above.

Clinical Specimens

Clinical samples of pus and urine submitted to the Department of Microbiology of Jawaharlal Nehru Medical College and Hospital of Aligarh Muslim University, during the specified period of this study, were also included for comparative analyses of the occurrence of *bla* genes. Clinical specimens were subjected to direct microscopy and culture on Blood Agar and McConkey Agar, and culture plates were incubated aerobically for 18 hours at 37^{o}C. A small subset of bacteria (*E. coli* = 17, *K. pneumoniae* = 6)

Table 1. Primers used and the expected amplicon size for dectection of bla_{CTX-m}, bla_{TEM}, bla_{SHV}, and bla_{AmpC}

Targets	Primer sequence (5' to 3')	Amplicon size (bp)
bla_{CTX-M}	F-ATGTGCAGYACCAGTAARGT R-TGGGTRAARTARGTSACCAGA	593
bla_{TEM}	F- KACAATAACCCTGRTAAATGC R-AGTATATATGAGTAAACTTGG	936
bla_{SHV}	F-TTTATCGGCCYTCACTCAAGG R-GCTGCGGGCCGGATAACG	930
bla_{AmpC}	F-CCCCGCTTATAGAGCAACAA R-TCAATGGTCGACTTCACACC	634

Y Wobble (C + T); R Wobble (A + G); S Wobble (C + G); K Wobble (G + T)

obtained from urine and pus cultures were included for further study.

Bacterial identification

All the bacterial isolates (both Gram-negative and Gram-positive) were identified to the species level based on catalase test, oxidase test, motility, coagulase test, carbohydrate fermentation, and other specific biochemical tests according to the standard protocol [4].

Antibiotic susceptibility

Antimicrobial susceptibility testing was performed on Mueller Hinton agar (HiMedia, India) by the standard disk diffusion method per the Clinical Laboratory Standard Institute (CLSI; formerly NCCLS) guidelines [5]. The following antibiotics and their concentrations (μg) were used: cefotaxime (30), ceftazidime (30), cefoperazone (75), cefpirome (30), gentamicin (10), amikacin (30), ofloxacin (5), Imipenem (10), piperacillin/tazobactam (100/10), cefoperazone/sulbactam (75/10). The antibiotic disks used were procured from HiMedia Lab. Ltd., India.

Phenotypic detection of extended-spectrum beta-lactamases (ESBLs)

ESBL production was inferred phenotypically by combination disk method using cefoperazone (Cs) and cefoperazone/sulbactam (Cfs) disks. An increase in zone diameter of $\geq$ 5 mm in Cfs in comparison to Cs alone was taken as potential ESBL production.

Preparation of DNA template

Template DNA from clinical and food-borne bacteria was prepared from freshly cultured bacterial isolates by suspending 3-5 colonies in 50 μl of molecular grade water, and then heating at 95°C for 5 minutes and immediately chilling at 4°C. Positive controls harboring bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{AmpC} and negative control (*E. coli* ATCC 25922) were processed in the same way for DNA extraction.

Detection of bla genes by PCR

Molecular detection of bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{AmpC} was performed in all the food and clinical isolates by using polymerase chain reactions (PCRs) according to the methods described previously with

Figure 2.

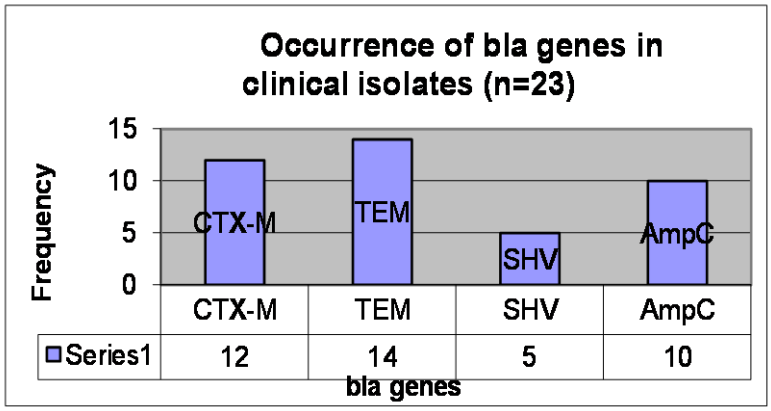


Table 2. Showing bacterial isolates obtained from various food specimens

SPECIMENS	BACTERIAL ISOLATES
Plant-origin food specimens	
Unwashed	
Cucumber	*Klebsiella pneumoniae* *Klebsiella pneumoniae*
Radish	*Citrobacter freundii*
Tomato	*Citrobacter koseri*
Chilli	*Klebsiella pneumoniae* *Klebsiella pneumoniae*
Coriander leaves	*Klebsiella pneumoniae* *Citrobacter koseri*
Mint leaves	*Escherichia coli*
Black Berry (Jamun)	*Citrobacter koseri* *Klebsiella pneumoniae*
Washed	
Cucumber	*Klebsiella pneumoniae* *Klebsiella pneumoniae*
Radish	*Citrobacter freundii*
Tomato	*Citrobacter koseri*
Chilli	*Klebsiella pneumoniae* *Klebsiella pneumoniae*
Coriander leaves	*Klebsiella pneumoniae* *Citrobacter koseri*
Mint leaves	*Escherichia coli*
Black Berry (Jamun)	*Klebsiella pneumoniae* *Citrobacter koseri*
Chopped	
Cucumber	*Klebsiella pneumoniae* *Klebsiella pneumoniae*
Radish	*Citrobacter freundii*
Tomato	*Klebsiella pneumoniae* *Citrobacter koseri*
Chilli	*Klebsiella pneumoniae* *Pseudomonas aeruginosa*
Black Berry (Jamun)	*Klebsiella pneumoniae* *Acinetobacter* spp
Animal-origin Food specimens	
Minced meat	*Klebsiella pneumoniae*
Chopped meat	*Klebsiella pneumoniae* *Staphylococcus aureus*
Ice Cream	*Klebsiella pneumoniae* *Escherichia coli* *Acinetobacter* spp
Sweet	
Jalebi	*Enterococcus* spp.

minor modifications [6,7]. The primers used for respective detection of these genes are shown in Table 1. The cycling conditions for detection of bla_{AmpC}, bla_{TEM}, bla_{SHV} were as follows: initial denaturation at 95°C for 15 minutes; 35 cycles of 94°C for one minute; 58°C for 2 minutes; 72°C for 3 minutes; and a final elongation at 72°C for 10 minutes. However, the cycling conditions for bla_{CTX-M} detection were as follows: initial denaturation at 94°C for 7 minutes; 35 cycles of 94°C for 50 seconds; 50°C for 40 seconds; 72°C for one minute; and a final elongation at 72°C for 5 minutes. PCR products were analyzed by gel electrophoresis after running 5 µl products in 2% agarose gels. Controls harbouring bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{AmpC} were used as molecular weight markers.

Results

Bacterial isolates obtained from food specimens

A total of 38 bacterial isolates were obtained from 23 food-borne specimens. Out of the 38 bacterial isolates, 36 (94.7%) were Gram-negative and 2 (5.3%) were Gram-positive species. *Klebsiella pneumoniae* was the most prevalent (52.6%; 20/38) bacterial species noticed, followed by *Citrobacter koseri* (18.4%; 7/38). *S. aureus* was isolated from chopped meat and *Enterococcus faecalis* from the sweet specimen. The details of the bacterial isolates obtained from various food specimens are shown in Table 2.

Antibiotics resistance

The details of the antibiotic resistance rates are shown in Table 3. Among the food-borne bacterial isolates, maximum resistance was noticed against gentamicin (33.3%), followed by amikacin (11.1%). Maximal resistance to a third-generation cephalosporin was noticed in only 5.6% isolates and all the isolates were found susceptible to piperacillin/tazobactam and imipenem. Among the clinical isolates, maximum resistance was noticed against third-generation cephalosporin followed by ofloxacin in 91.3% and 86.9% isolates respectively. Resistance to gentamicin and amikacin was noticed in 78.3% and 52.2% isolates respectively.

Phenotypic ESBL detection in food and clinical isolates

Based on combination disk tests using Cs and Cfs, ESBL could be inferred in 5.6% (2/36) and 91.3% (21/23) food-borne and clinical isolates, respectively.

Occurrence of bla genes

The frequency of the occurrence of various *bla* genes in clinical isolates is shown in Figure 2. TEM was found to be the most prevalent ESBL noticed in 60.9% (14/23) followed by CTX-M in 52.2% (12/23) isolates. AmpC and SHV beta-lactamases were noticed in 43.5% and 21.7% isolates, respectively. It

was noteworthy to observe that none of the ESBL genes could be detected from food-borne bacterial isolates.

Table 3. Percent antibiotic* resistance rates in Gram-negative isolates obtained from food and clinical specimens

Antibiotics tested	% Resistance (n)	
	Food isolates (n = 36)	Clinical isolates (n = 23)
Gentamicin	33.3 (12)	78.3 (18)
Amikacin	11.1 (4)	52.2 (12)
Ofloxacin	2.8 (1)	86.9 (20)
Ceftazidime	2.8 (1)	86.9 (20)
Cefotaxime	5.6 (2)	91.3 (21)
Cephoperazone	5.6 (2)	91.3 (21)
Cefpirome	2.8 (1)	91.3 (21)
Piperacillin/Tazobactam	0 (0)	52.2 (12)
Imipenem	0 (0)	4.3 (1)

* *P. aeruginosa* was also tested against piperacillin and found resistant to piperacillin

Discussion

bla$_{CTX-M}$ is the among the most prevalent and widely disseminated genes in the clinical bacterial population in India [6,8]. In the present study, we found *bla*$_{TEM}$ as the most prevalent ESBL gene followed by *bla*$_{CTX-M}$. The high prevalence of these resistant genes is attributed to the irrational use of antibiotics in India since antibiotics are freely available over the counter in this country [9,10]. These resistance genes may freely disseminate to the vegetation-associated bacterial population during irrigation using sewage water, which is a common irrigation practice in India. Keeping these facts in mind, we planned this study to determine the occurrence of enteric pathogens on vegetation and to screen for the presence of *bla* genes.

We also included samples of animal-origin food and sweets that are openly sold in Indian markets, as well as a small subset of clinical bacteria to compare their resistance patterns and occurrence of resistance genes.

The most prevalent organism isolated in the present study was *Klebsiella pneumoniae* followed by *Citrobacter koseri*. The majority of the specimens provided mixed bacterial growth and there was a predominance of Gram-negative over Gram-positive bacteria. The strain of *S. aureus* isolated from chopped meat was identified as a methicillin-resistant strain based on the detection of mecA and femB genes (data not shown) using a protocol described earlier [11]. It was alarming to note that, after washing the specimens, the bacteria could not be eliminated completely; however, the bacterial load was reduced markedly (data not shown). This observation emphasizes that the common practice of eating salad vegetables after only washing with water is not sufficient, and that these vegetables should be disinfected by using some antiseptic solutions such as potassium permanganate.

A marked difference was noticed in the susceptibility patterns of food-borne and clinical isolates. Food-borne isolates showed maximum resistance to gentamicin (33.3%) followed by amikacin (11.1%). Maximal resistance to any of the third-generation cephalosporins was noticed only in 5.6% isolates (see Table 3). On the other hand, the clinical isolates showed maximal resistance to cephalosporins (91.3%) followed by fluroquinolones, then ofloxacin (86.9%). Resistance to gentamicin, amikacin and piperacillin/tazobactam was noticed in 78.3%, 52.2%, and 52.2% isolates respectively. One *K. pneumoniae* isolate also demonstrated resistance to imipenem (Table 3). Similarly, a marked difference was noticed in the phenotypic demonstration of ESBL production in clinical versus food isolates.

It was interesting to observe that none of the food-borne isolates demonstrated the presence of any of the *bla* genes; however, *bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{ampC}$ were detected in 52.2%, 60.9%, 21.7%, and 43.5% respectively in clinical isolates.

Although these resistance genes have not yet disseminated to food vegetation, they remain a global threat. The findings from this study suggest that we must check and introduce strict irrigational and antibiotic policies to prevent the wide dissemination of these genes. Moreover, strict policies should be

instituted and over-the-counter availability of antibiotics should immediately be stopped in India. To the best of our knowledge, this is the first report from India looking for these resistance genes in food-borne bacterial populations and also among the premier reports simultaneously looking for *bla* genes, including CTX-M, in Indian clinical isolates.

Acknowledgements

M. Shahid is grateful to Department of Science and Technology, Ministry of Science and Technology, Government of India, for receiving the "Young Scientist Project Award" (FT/SR-L-111/2006). The authors are also thankful to the Indian Council of Medical research (ICMR) for providing a Short Term Student (STS) Fellowship to M. Adil, under supervision of Dr. M. Shahid. The authors also wish to thank Prof. Daniel Jonas, Department of Environmental Health Sciences, Breisacher Straße 115 B,D-79106 Freiburg, Germany, for kindly providing the control strains harboring $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{ampC}.

References

1. Khan MR, Saha ML, Kibria AHMG (1992) A bacteriological profile of salad vegetables in Bangladesh with special reference to coliforms. Lett Appl Microbiol 14: 88-90.
2. Ashenafi M (1989) Microbial load, incidence and antibiotic resistance of some disease-causing microorganisms on raw food items consumed in Ethiopia. MIRCEN J Applied Microbiol Biotech 5: 313-319.
3. Geldreich EE, Bordner RH (1971) Fecal contamination of fruits and vegetables during cultivation and processing for markets. J Milk Food Tech 34: 184-195.
4. Collee JG, Fraser AG, Marmion BP, Simmons A (1996) Mackie and McCartney Practical Microbiology, 14th edition. London: Churchill Livingstone.
5. National Committee for Clinical Laboratory Standards (2003) Performance standards for antimicrobial disk susceptibility tests, 8th ed. Approved standard. NCCLS document M2-A8. National Committee for Clinical Laboratory Standards, Wayne, Pa.
6. Ensor VM, Shahid M, Evans JT, Hawkey PM (2006) Occurrence, Prevalence and Genetic Environment of CTX-M Beta-lactamase in Enterobacteriaceae from Indian Hospitals. J Antimicrob Chemother 58: 1260-1263.
7. Shahid M, Ensor VM, Hawkey PM (2009) Emergence and dissemination of Enterobacteriaceae with plasmid-mediated CMY-6 and CTX-M-15 beta-lactamases in a community in North-India. World J Microbiol Biotech 25: 1439-1446.
8. Walsh TR, Toleman MA, Jones RN (2007) Comment on: Occurrence, prevalence and genetic environment of CTX-M ß-lactamases in Enterobacteriaceae from Indian hospitals. J Antimicrob Chemother 60: 187-188.
9. Akram M, Shahid M, Khan AU (2007) Etiology and antibiotic resistance patterns of community acquired urinary tract infections in the JN Medical hospital, Aligarh, India. Annals Clin Microbiol Antimicrob Agents 6: 4.
10. Shahid M, Malik A, Akram M, Agrawal LM, Khan AU, Agrawal M (2008) Prevalent Phenotypes and Antibiotics Resistance in *Escherichia coli* and *Klebsiella pneumoniae* in an Indian Tertiary Care Hospital: Plasmid-Mediated Cefoxitin resistance. Int J Infect Dis 12: 256-264.
11. Khan AU, Sultan A, Tyagi A, Zahoor S, Akram M, Kaur S, Shahid M, Vaishnavi CV (2007) Amplification of *mecA* gene in multi-drug resistant *Staphylococcus aureus* strains from hospital personnel. J Infect Developing Countries 1: 289-295.

Corresponding author

Dr. M. Shahid
Department of Microbiology
JN Medical College & Hospital
Aligarh Muslim University
Aligarh-202 002, UP, India
Tel.: +91-571-2720382
Mobile: 009411802536
Fax: +91-571- 2721776
+91-571- 2720382
Email: shahidsahar@yahoo.co.in
drmohdshahid123@yahoo.com

Conflict of interest: No conflict of interest is declared.