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RESEARCH ARTICLE

SHV and CTX-M Extended Spectrum Beta Lactamases (ESBL) Producing Bacteria Isolated from Street Foods in and around Chennai, India

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ABSTRACT:

Street food vending in India has flourished into a prominent trade especially in the urban and semi urban regions of the country. It is also a major reservoir for the spread of food borne infections caused by microbial pathogens including multidrug resistant organisms. Management of infections caused by multidrug resistant bacteria is difficult. Extended spectrum beta lactamase (ESBL) production contribute to antimicrobial resistance that may result in therapeutic failure. The objective of this study was to analyze for the prevalence of ESBL producing food borne bacteria isolated from street foods sold in and around Chennai. A total of 78 street foods including vegetable samosa (18), chicken samosa (10), panipuri masala (11), panipuri water (31), bhelpuri (8) were tested for the presence of ESBL producing bacteria. The bacteria that were identified included *Escherichia coli* (55.1%), *Staphylococcus* sp. (25.6%), *Klebsiella* sp. (17.9%), *Pseudomonas* sp. (15.3%) and *Enterobacter* sp. (11.5%). Multidrug resistance was exhibited by the isolates and ESBL production was found in 16 out of 78 (20.5%) isolates with 9 *E. coli*, 2 *Klebsiella* sp., and 5 *Pseudomonas* sp. Polymerase Chain Reaction (PCR) revealed CTX-M-1(68.75%) and SHV genotypes (31.25%) among the isolates. The 16SrRNA analysis of F10 isolate, confirmed the presence of *Pseudomonas* sp. in street foods.

KEYWORDS: Street foods, Multidrug resistant bacteria, SHV, CTX-M ESBLs, PCR, 16SrRNA.

1. INTRODUCTION:

Street foods, also known as ready-to-eat foods, attract many people irrespective of their age and social status, thanks to their exclusive flavor and ready to eat convenience at an affordable price.[1] However, it is also notorious for the risk it poses to food safety and public health and serves as a major reservoir for the spread of food borne infections caused by microbial pathogens. These infections vary in severity from mild and self-limiting ones to debilitating and life threatening conditions.[2] The global burden of food borne diseases (FBDs) is estimated to be 600 million out of which, 420,000 people die every year.[2] In South-East Asia region, the annual burden on FBDs is greater than 150 million illnesses with 175,000 deaths.[3]

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Many reports on the prevalence of food borne pathogens in the street foods have been made and studies have identified numerous bacterial food borne pathogens including species of *Staphylococcus*, *Bacillus*, *Salmonella*, *Shigella*, *Vibrio*, *Campylobacter*, *Escherichia coli*, *Listeria* sp. etc.[4-8.] Correct diagnosis and prompt treatment is crucial in the management of FBDs. However difficulty arises if the antibiotics used for treating bacterial FBDs become ineffective. The most common cause of therapeutic failure is antimicrobial resistance and develops whenever antibiotics are used.[9]

Multidrug resistance (MDR) to antibiotics is a problem encountered throughout the world mediated by the spread of resistant bacterial species. Infections caused by these MDR pathogens are associated with difficulty and limitations of therapeutic options, increased mortality and lengthy hospital stay.[10] Production of ESBLs is an important resistance mechanism found among bacteria

and emerged as a serious issue due to their dissemination in the environment including food and water. In highly populated countries like India, the spread of these resistant strains into the environment is inevitable due to overcrowding.[11] The epidemiological factor for the emergence of antibiotic resistance is attributed to the misuse and overuse of antibiotics resulting in selective pressure on bacteria. This leads to the diversification of the resistant strains and sharing of the resistance among other species. Presence of ESBL producing bacteria in foods is an important risk for human health.[12] Hence there is a need for the analysis of ready to eat foods because of its epidemiological significance.

The objective of this study was to determine the prevalence of ESBL producing bacterial species in various chat items that are widely sold in and around Chennai streets and to identify their genotypes. ESBL positive isolates were characterized for their genotypes by Polymerase Chain Reaction (PCR) and DNA sequencing.

METHODS:

A total of 78 ready-to-eat chat items consisting of vegetable samosa (18), chicken samosa (10), panipuri masala (11), panipuri water (31), bhelpuri (8) were purchased from street vendors in KK Nagar, Ashok Nagar and Vadapalani areas of Chennai Metropolis and were included for analysis. Since the panipuri water is prepared using raw ingredients, we collected more samples (31) from different areas mentioned above. The samples were collected in sterile polythene bags and immediately transported to the microbiology laboratory of VELS University, Chennai.

The solid and liquid food samples were then subjected for microbiological analysis following the procedures described in Microbiological Methods and Bacteriological Analytical Manual (2013)[13] with few modifications. Briefly, 10g of solid samples were homogenized aseptically in 100 ml of sterile Nutrient broth (HiMedia Laboratories, India). Panipuri water (10 ml) was added to 90 ml of nutrient broth. All the contents were mixed well and were then incubated at 35-37°C for 48 hours. About 10 µl of the suspension from each food sample was then inoculated in HiCrome Universal Differential Agar Medium (HiMedia Lab, Chennai) and incubated at 35- 37°C for 18-24 hours.

Antibiotic Susceptibility Testing:

The Gram negative bacterial isolates were selected for further study and were tested against a panel of antibiotics which included Ampicillin (10 µg/ml), Ceftazidime (30 µg/ml), Cefotaxime (30 µg/ml), Cefepime (30 µg/ml), Piperacillin-Tazobactam (100/10 µg/ml), Imipenem (10 µg/ml), Gentamycin (10 µg/ml), Amikacin (30 µg/ml), Tobramycin (10 µg/ml) (HiMedia Labs, India) following the Clinical Laboratory Standards Institute (CLSI) criteria. (2013). [14]

ESBL Detection:

The gram negative bacterial isolates were tested for production of ESBL enzymes following the recommendations of CLSI, 2010.[15] *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as the control strains throughout the study.

Double Disc Synergy Test (DDST):

The isolates were screened for ESBL production by testing against Amoxyclav (20 µg/10 µg) and ceftazidime (30 µg/ml) and cefotaxime (30 µg/ml) placed on Mueller-Hinton agar. Upon incubation at 35-37°C for 18-24 hours, ESBL production was detected by the formation of zone of inhibition around the cephalosporins that increases towards the amoxyclav resulting in synergy formation.

Cephalosporin/Clavulanate Combination Test (CCCT):

This test is confirmatory for ESBL production in which the isolates to be tested are swabbed on Mueller-Hinton agar and tested against ceftazidime (30 µg) and cefotaxime (30 µg) with and without clavulanic acid. A zone difference of ≥ 5 mm between the discs of cephalosporin and cephalosporin/clavulanate combination upon incubation at 35-37°C for 18-24 hours is confirmatory for ESBL production.

Molecular analysis of ESBL genes:

The isolates that were ESBL positive were analyzed for the genotypes. The genomic DNA of the isolates was extracted following the protocol of Sambrook *et al.,* 2001.[16] Multiplex PCR was carried out with the following primers that were procured from Chromous Biotech, Bangalore for amplification of the genomic DNA. For *bla* $_{TEM}$ genes, 5' AAAATTCTTGAAGACG 3' and 5' TTACCAATGCTTAATCA 3', for *bla* $_{SHV}$ genes, 5' TTAACTCCCTGTTAGCCA 3' and 5' GATTTGCTGATTTCGCCC 3' were used as forward and reverse primers based on previous report. [17] For *bla* $_{CTX-M}$ genes, CTX - M - 5'- ATGTGCAGY ACCAGTAARGT - 3' and CTX - M - 5'- TGGGTRAARTARGTSACCAGA - 3' were used as primers as reported by other study. [18] The amplification was carried out based on the protocol by other study with few modifications.[18] Briefly, amplification was performed was performed using 30 amplification cycles of 15 s at 94°C (denaturation), 30 s at 52°C (annealing) and 90 s at 72°C (chain elongation), with final elongation at 72°C for 5 min The PCR products were analyzed using 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. Since the

reports on *Pseudomonas* sp. as food borne pathogens are very few, the SHV gene found in a food isolate, *Pseudomonas* sp. F10, was sequenced and subjected to BLAST analysis and was submitted to GenBank. For further confirmation of F10 isolate as *Pseudomonas* sp. 16S rRNA of the organism was sequenced and subjected to GenBank. Phylogenetic analysis was carried out for SHV gene of F10 isolate and for the 16S rRNA gene sequence using MEGA4 software. [19]

RESULTS:

The chat food varieties showed heavy growth of bacterial isolates including *Staphylococcus* sp. (20%), *Escherichia coli* (58.9%), *Klebsiella* sp. (19.1%), *Pseudomonas* sp. (16.4%) and *Enterobacter* sp. (12.3%) (Table.1).

Table 1: Bacteria isolated from various street foods

Bacterial Isolates	Street Food Samples (n=78)					
	Vegetable Samosa (18)	Chicken Samosa (10)	Panipuri Masala (11)	Panipuri Water (31)	Bhelpuri (8)	Total (78)
E. coli	9 (50%)	6(60%)	8(72.7%)	13(41.9%)	7(87.5%)	43 (55.12%)
***Klebsiella* sp.**	2(11.1%)	2(20%)	1(9%)	8(25.8%)	1(12.5%)	14 (17.94%)
***Enterobacter* sp.**	3(16.6%)	1(10%)	2(18.18%)	3(9.67%)	-	9 (11.53%)
***Pseudomonas* sp.**	4(22.2%)	1(10%)	-	7(22.5%)	-	12 (15.3%)
***Staphylococcus* sp.**	3 (16.6%)	-	5 (45.45%)	8(25.8%)	4(50%)	20(25.64%)

Table 2. Antibiogram profile of gram negative bacterial isolates obtained from street foods

Bacterial Isolates Showing Resistance	Antibiotics								
	Ampicillin	Ceftazidime	Cefotaxime	Cefepime	Piperacillin-Tazobactam	Imipenem	Gentamycin	Amikacin	Tobramycin
***E. coli* (n=43)**	37 (86%)	19(44.1%)	27(62.7%)	3(6.9%)	23(53.4%)	0	13(30.2%)	16(37.2%)	22(51.1%)
***Klebsiella* sp.(n=14)**	12(85.7%)	2(14.2%)	4(28.57%)	2(14.2%)	9(64.2%)	0	9(64.2%)	7(50%)	3(21.4%)
***Enterobacter* sp.(n=9)**	5(55.5%)	3(33.3%)	4(44.4%)	0	2(22.2%)	0	3(33.3%)	2(22.2%)	3(33.3%)
***Pseudomonas* sp.(n=12)**	12(100%)	7(58.3%)	5(41.6%)	4(33.3%)	8(66.6%)	0	9(75%)	4(33.3%)	7(58.3%)
Total (n=78)	54(69.2%)	31(39.7%)	38(48.7%)	9(11.5%)	42(53.8%)	0	34(43.5%)	29(37.1%)	35(44.8%)

Antibiotic Susceptibility Testing:

The isolates showed multidrug resistance to the antibiotics tested. Maximum resistance was exhibited against ampicillin (69.2%). All the isolates were completely inhibited by imipenem. (Table.2).

ESBL Detection:

The DDST and CCCT tests revealed ESBL production in 16 out of 78 (20.51%) isolates including 9 *E. coli* (56.2%), 2 *Klebsiella* sp. (12.5%), and 5 *Pseudomonas* sp (31.25%) (Fig.1).

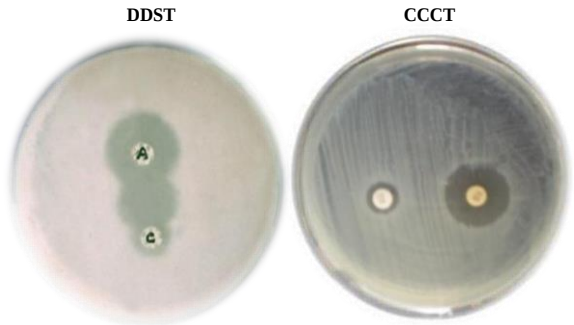

Fig.1: ESBL Detection

Molecular Analysis of ESBL Gene:

PCR analysis of genomic DNA of the ESBL positive isolates showed the presence of *bla* $_{CTX-M}$ and *bla* $_{SHV}$ genotypes while none of the isolates harbored *bla* $_{TEM}$ genes. The prevalence of CTX-M and SHV ESBLs among the food isolates was found to be 68.75% and 31.25% respectively. Sequence analysis of SHV genes revealed 447 base pair sequence of *bla* $_{SHV}$ gene with GenBank Accession Number KY271368. BLAST analysis and phylogeny confirmed the gene as SHV and shared 99% identity with SHV-12 producing *E. coli* (GenBank Accession No. LT621755) (Fig.2). Based on the microbiological characters biochemical and physiological results and the isolated bacterial nucleotides, phylogeny relationships with NCBI nucleotide database has clearly revealed that, the given sample belongs to the taxonomy of *Pseudomonas aeruginosa.* The 16Sr RNA sequencing and phylogenetic relationships with NCBI nucleotide database, the *Pseudomonas* sp. F10 isolate was confirmed as *Pseudomonas aeruginosa* having 734 bp with GenBank Accession Number KY271369 (Fig.3).

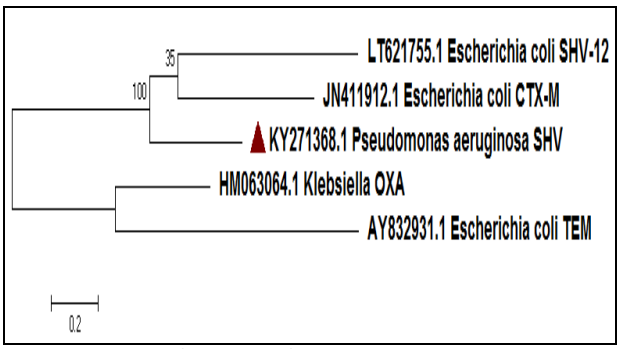

Fig. 2: Phylogeny of SHV gene from bacterial isolate F10

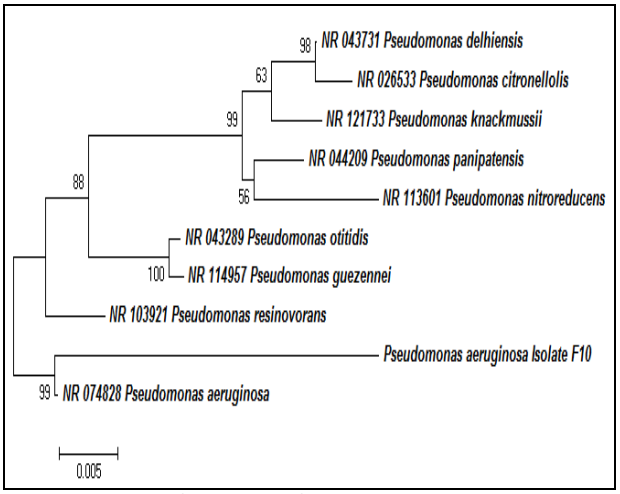

Fig. 3: Phylogeny of 16s rRNA of *Pseudomonas* sp. F10

DISCUSSION:

Street vended are preferred by many people, especially, the urban population, as they are cheaper and time saving.[7] Ready-to-eat foods like panipuri, samosa, bhelpuri etc. are very popular street foods that are available in every nook and corner of India. They have gained popularity due to their cheaper cost, convenience and exclusive taste. However particular attention has to be given in terms of their safety and the threat posed in serious food poisoning outbreaks. Microbiological contamination of street foods could be due to improper and unhygienic handling practices, use of contaminated water, raw materials of poor quality, incongruous surroundings etc. In this study, *E. coli* (55.12%) was the most prevalent bacteria followed by *Staphylococcus* sp. (25.64%) (Table.1). This is in accordance with other reports.[1, 20, 21] However, only limited reports have been published on the prevalence of *Pseudomonas* sp. from street foods.[5, 22] Nevertheless, *Pseudomonas* sp. were reported to be involved in food spoilage including fish, [23] other raw materials (vegetables, fruits, salads, meat and meat products etc.) and food products.[24-26] Presence of coliforms in foods could be due to fecal contamination and unhygienic food handling procedures.[27] Most of the organisms of Enterobacteriaceae are reported to cause serious infections and to become increasingly resistant to presently existing antibiotics.[28] In this study, all the isolates were susceptible to imipenem while 4% resistance by the isolates obtained from street foods from Nigeria was reported.[29] The isolates showed maximum resistance to ampicillin (69.2%) followed by piperacillin-Tazobactam (53.8%). Against the Aminoglycosides tested, the isolates exhibited 43.5% and 44.8% resistance to gentamycin and tobramycin respectively. These results are lower than other studies [29] and higher than the studies done in Turkey [28] and in Switzerland. [30]

Presence of ESBLs in street foods is a matter of health issue as they confer resistance to many antibiotics and co-resistance to other antibiotics as well. Reports on ESBL producing enterobacteriaceae which included 80% *E. coli*, 9.1% *Enterobacter cloacae* and 3.6% *K. pneumoniae* were made from food samples of animal origin [12] while this study reported ESBL producing *E. coli* (11%) and *Pseudomonas* sp. (40%) isolated from chicken samosa. Also, this study reported an overall ESBL production of 20.5% which is more similar to a study from Switzerland reporting 18.3% ESBL prevalence from vegetable samples imported from India. [30] This study detected SHV and CTX-M type ESBLs among the food isolates which is in concordance with other reports. [31, 32] Phylogenetic analysis of SHV gene of *Pseudomonas* sp. isolate F10 showed 99% similarity to SHV-12 producing *E. coli* (Gen Bank Accession No. LT621755). The detection of CTX-M and SHV ESBLs in the food isolates is of epidemiological significance suggestive of the spread of the resistant genes from pathogenic strains to the environment.

CONCLUSION:

Street food mediated food poisoning outbreaks can be curbed by taking adequate measures to minimize microbiological contamination. This study substantiates that street foods are a source of ESBL producing bacteria and the detection of ESBL genes in foods is of significant concern due to its potential of outbreaks. Hence appropriate measures for controlling the dissemination of resistant genes need to be taken immediately. They may include, implementation of regulations and recommendations to specify codes of practices suitable for street foods by local authorities following the guidelines of Joint FAO/WHO Codex Alimentarius Commission. Proper sanitation, quality of potable water, utensils and containers for foods, hygienic practices by the vendors and food handlers, conducting awareness programs for food handlers and vendors, educating consumers, especially children, on hygienic street foods will contribute to restriction of street food mediated infections and outbreaks. Likewise, prudent and careful use of antibiotics decreases the selective

pressure in bacteria and could control not only the spread of antibiotic resistance among bacteria but also will hinder the evolution of new resistance genes.

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CONFLICT OF INTEREST:

None declared.

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