



Antibiotic Resistance Profiles, Phenotypic Analysis and Molecular Genotyping of Extended-Spectrum Beta-Lactamases-Producing Enterobacteriaceae Isolated from Red Meat Collected from Dhaka City of Bangladesh

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Abstract

Background: It is generally acknowledged that one of the most significant issues facing modern human are those with broad activity spectra that render all or most medicines in a particular treatment category ineffective. **Objective:** The goal of this study was to evaluate the microbiological quality of raw meat, specifically focusing on the frequency of Extended-Spectrum Beta-Lactamases (ESBL) production among Enterobacteriaceae, and to assess the antibiotic susceptibility of these isolates in vitro as well as to conduct a molecular characterization of ESBL to better understand the genetic mechanisms behind their production. **Methodology:** This comparative cross-sectional study was conducted in neighborhood marketplaces of Dhaka, including Bashundhara, Azimpur, and Dhanmondi Bazar, from November 2022 to August 2023. A total of 40 beef and mutton samples were collected directly from slaughterhouses and transported to the NSU laboratory in sterile Ziploc bags without freezing. For pure culture, Mac Conkey agar plates were utilized after sample collection. Muller-Hinton agar media (MHA) was used for antibiotic susceptibility testing after completion of organism detection test on Eosin-methylene blue (EMB) agar media. DNA was extracted using the hot boiling method. The purity of the DNA was assessed to ensure its quality. Gel electrophoresis was then performed to verify DNA integrity. Finally, PCR was conducted for genetic confirmation of specific sequences. **Results:** Among 40 samples, 95% of the bacteria discovered belonged to the Enterobacteriaceae family. Five (or 25%) of the beef samples showed resistance against the combination of Ceftazidime (CAZ) and Amoxicillin-Clavulanic acid (AMC). About the mutton, 20 (100%) cases were found resistant to CAZ+AMC (100%), and 12 instances were resistant to CTX (Cefotaxime) +AMC (60%). Several strains (17.5%) in our investigations demonstrated phenotypic positive for producing the ESBL enzyme. Of 40 dietary samples, 35% of the strains that create ESBL are identified in beef and mutton, respectively, with 30% and 5% of these strains producing ESBL. In this study, the focus was on two specific gene types, namely the VIM and DHA genes. The genotype known as blaDHA (Variation of the dihydrofolate reductase gene) was identified as the causative factor in 2.5% of all cases, while the genotype referred to as blaVIM (Verona -Integron encoded- Metallo-beta-lactamases) was responsible for 2.5% of the cases of total 40 strains. **Conclusion:** In conclusion antibiotic resistance is an important health issue due to the potential pathogenicity.

Keywords: ESBL, AMR, DHA, VIM

Bangladesh Journal of Medical Microbiology, January 2025;19 (1):26-34

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DOI: <https://doi.org/10.3329/bjmm.v19i1.80337>

Introduction

Antimicrobial resistance is a burgeoning global concern. Annually, 33,000 individuals succumb fatal infections caused by bacterial antibiotic resistance within the European region. According to published

article, it has been projected that the global population is going to face a significant threat to public health and economic stability in the coming years due to anti-microbial resistance¹. By the year 2050, it is estimated that approximately 10 million lives per annum and a cumulative financial output of 100 trillion USD could be jeopardized due to the emergence of drug-resistant infections. We must urgently seek proactive measures to mitigate the progression of drug resistance to safeguard public health and economic prosperity².

It is worth noting that the production of ESBLs is often associated with the emergence of multi-drug resistance³. Meat is a good substrate for many organisms to thrive because it is high in moisture, rich in nitrogenous compounds such as amino acids, peptides, and proteins and abundantly provided with mineral & auxiliary growth agents⁴. Food continue to endanger both human and animal health, despite the fact that industrialized nations have improved the hygienic of all meat production procedures. Food borne illnesses spread to humans when tainted food is consumed and toxins enter the body. This becomes one of the major global public health issues & both social and economic stability will be endangered⁵.

Generally speaking, unclean slaughter and sale facilities can harbor microorganisms resistant to antibiotics that infect raw meat. Eating meat tainted with microorganisms that are resistant to antibiotics can have serious health effect⁶ and because of cultural and religious concerns, many here consume red meat. This study was proposed to assess the microbiological quality of raw meat, the incidence of Extended spectrum-beta- lactamases (ESBL) Production in Enterobacteriaceae isolates from raw meat, and the susceptibility of these isolates to antibiotics.

Methodology

Study Settings and Population: This was a comparative cross-sectional study carried out in various neighborhood markets of Dhaka, including Bashundhara, Azimpur, and Dhanmondi Bazar, from November 2022 to August 2023. The study focused on beef and mutton samples collected directly from slaughterhouses, with a total of 40 tissue samples from cows and goats. These samples were transported to the NSU laboratory in sterile Ziploc bags without being frozen. Samples of beef and mutton were utilized in this cross-sectional investigation.

Laboratory Procedure: To properly isolate the bacteria, beef and mutton samples were obtained

straight from the slaughterhouse, placed in a sterile Ziploc bag, and then delivered to the lab immediately without freezing. There were 40 tissue samples collected from various portions of cows and goats. After that, samples were immediately swabbed directly with a sterile cotton swab over MacConkey agar using the direct cotton swab technique. After that, overnight incubation was performed at 37 degrees Celsius aerobically & maintaining the same procedures subculture was done. For stock preparation, Luria-Bertani broth, a nutrient-rich medium frequently used for the growth of bacteria and 500 microliters of 50% glycerol were used. At last, -20 and -80 degrees Celsius were utilized to store two copies of the stock for each of these copies. Organism detection tests were done on Eosin-methylene blue media and Kirby-Bauer, a double-disk diffusion test was done over Mueller-Hinton agar media using two different antibiotics along with beta-lactamase inhibitor⁷. Bauer used the disc diffusion technique to assess the susceptibility of ESBL-producing *E. Coli* by Clinical Laboratory Standards Institute recommendations (CLSI 2018).

Using the boiling technique, DNA was extracted from bacterial samples. For this technique, an ESCO Class II Bio Safety Cabinet was used⁸. Using the reverse and forward primer pairs, β -lactamase genes (*blaVIM*, *blaDHA*) were discovered by PCR. The DNA template was created using a boiled suspension of bacterial cells and the cycle conditions were only slightly altered⁹. After PCR, gel electrophoresis and visualization of DNA was done.

Statistical Analysis: Statistical analysis was performed by Windows based software named as Statistical Package for Social Science (SPSS), versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Categorical data were summarized in terms of frequency counts and percentages. Continuous data were expressed as mean, standard deviation, minimum and maximum.

Ethical Clearance: All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration 2013) and also with the ethical guidelines of the Institutional research ethics. Participants in the study were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during the data collection periods. All data were collected anonymously and were analyzed using the coding system.

Results

Among 40 samples, 95% of the pieces exhibited strain positivity and 5% no growth over MacConkey agar plates (Figure I). Eosin methylene blue (EMB) medium organism detection test findings show that all 20 samples of beef are *Klebsiella* species whereas 10 and 8 samples of mutton are *E. coli* and *Klebsiella* species (Table 1).

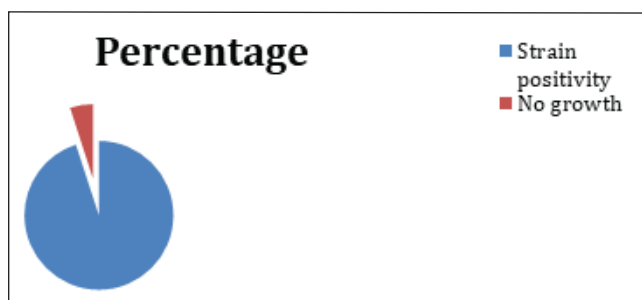


Figure I: A graphic illustrates the appearance of Enterobacteriaceae in total samples on MacConkey agar plates by direct swab approach

species). Regarding the mutton, there were 20 that were resistant to CAZ+AMC (100%), and 12 were resistant to CTX+AMC (60%) (Figure II).

The distribution of ESBL-producing bacteria among various food types. The strains that produce ESBL account for 35%, of which 30% and 5% are found in beef and mutton, respectively among 40 food samples (Figure III).

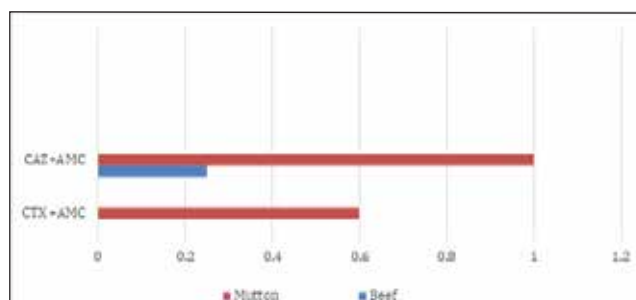


Figure II: This column chart represents AMC resistance results in overall samples.

Table 1: Distribution of micro-organisms in beef & mutton samples

Name of organism	Beef (n=20)	Mutton (n=20)
<i>E. coli</i>	0	8
<i>Klebsiella</i>	20	10
No growth	0	2

During the screening process, it was observed that a total of 7 strains exhibited positive for ESBL producing bacteria among the 40 overall meat samples obtained (Table 2) & Five (or 25%) resisted the CAZ and AMC combos in case of beef (All are *Klebsiella*

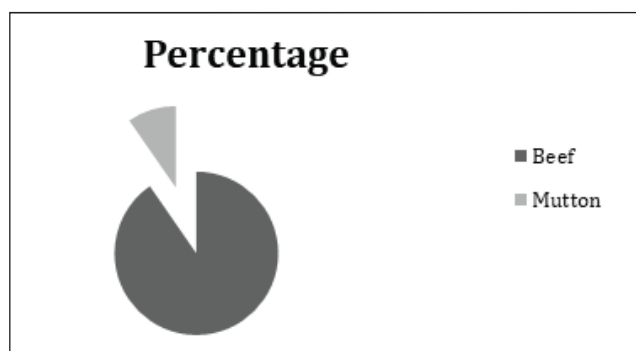


Figure III: This pie chart represents percentage distribution of ESBL producers in animal based foods after Double-disc synergy tests.

Table 2: Double-disk diffusion test results only for ESBL producing bacteria (Zone diameter-mm).

Sample ID	Gram-negative bacteria	CAZ (30µg/ml)	CAZ(30µg/ml) + AMC(30µg/ml)	CTX (30µg/ml)	CTX(30µg/ml) + AMC (30µg/ml)	Results
P3B	<i>Klebsiella spp.</i>	0	25	28	25	ESBL
P7B	<i>Klebsiella spp.</i>	23	25	15	27	ESBL
P9A	<i>Klebsiella spp.</i>	23	23	20	26	ESBL
P9B	<i>Klebsiella spp.</i>	25	27	15	29	ESBL
P10A	<i>Klebsiella spp.</i>	30	30	7	30	ESBL
P10B	<i>Klebsiella spp.</i>	30	30	11	30	ESBL
BaPA	<i>E.coli</i>	22	24	4	20	ESBL

* CAZ = Ceftazidime, CTX= Cefotaxime, AMC= Amoxicillin- Clavulenic acid

Table 3: Shows the antimicrobial drug resistance profile of microorganisms (Klebsiella Species) isolated from beef samples in several Dhaka market locations

Sl No.	Strain name	CAZ (30µg/ml)	CAZ(30µg/ml) + AMC(30µg/ml)	CTX (30µg/ml)	CTX(30µg/ml) + AMC (30µg/ml)	Results
1.	P1A	30	25	35	35	None
2.	P1B	0	0	33	33	None
3.	P2A	26	26	30	28	None
4.	P2B	24	23	27	30	None
5.	P3A	0,	0	27	28	None
6.	P3B	1	25	28	25	None
7.	P4A	0	0	30	30	None
8.	P4B	0	0	30	30	None
9.	P5A	0	0	30	29	None
10.	P5B	0	0	25	20	None
11.	P6A	25	25	27	27	None
12.	P6B	28	28	15	20	None
	P7A					
13.	P7A	25	22	31	32	None
14.	P7B	23	25	15	27	ESBL
15.	P8A	32	30	30	32	None
16.	P8B	25	25	34	34	None
17.	P9A	23	23	20	26	ESBL
18.	P9B	25	27	15	29	ESBL
19.	P10A	30	30	7	30	ESBL
			-			
20.	P10B	30	30	11	30	ESBL

P=Piece of sample, A= Anterior side of the sample, B= Posterior side of the sample; Antibiotics susceptibility zone diameter in mm; On EMB media Klebsiella species purple in color

Tables 3, 4a, and 4b list 40 distinct samples, their sensitivity to cefotaxime (CTX), ceftazidime (CAZ), and clavulanic acid, along with the strain name and abbreviations for each.

Table 4 (a): Strain abbreviations & their meanings

Strain name	Full form	Strain name	Full form
T	Thigh	B	Belly
Ta	Tail	Lu	Lungs
H	Head	C	Chest
L	Liver	Ba	Back
LA	Leg anterior	LB	Leg back

Table 4 (b): Shows the antimicrobial drug resistance profile of microorganisms isolated from mutton samples in several Dhaka market locations

Sl No.	Strain name	Organism	CAZ (30 µg/ml)	CAZ(30µg/ml) + AMC(30µg/ml)	CTX (30µg/ml)	CTX(30µg/ml) + AMC (30µg/ml)	Results
1.	LBPA	<i>E. coli</i>	0	0	25	27	None
2.	BaPA	<i>E. coli</i>	0	0	4	20	ESBL
3.	BPA	<i>E. coli</i>	0	0	0	0	None
4.	BPB	<i>E. coli</i>	0	0	0	0	None
5.	LuPA	<i>Klebsiella spp.</i>	0	0	20	15	None
6.	LuPB	<i>E. coli</i>	0	0	25	25	None
7.	LAPA	<i>Klebsiella spp.</i>	0	0	0	0	None
8.	LAPB	<i>Klebsiella spp.</i>	0	0	0	0	None
9.	LPA	<i>Klebsiella spp.</i>	0	0	0	0	None
10.	LPB	<i>E. coli</i>	0	0	0	0	None
11.	CPA	<i>Klebsiella spp.</i>	0	0	13	13	None
12.	CPB	<i>Klebsiella spp.</i>	0	0	22	22	None
13.	TaPA	<i>E. coli</i>	0	0	0	0	None
14.	TaPB	<i>E. coli</i>	0	0	0	0	None
15.	HPA	<i>Klebsiella spp.</i>	0	0	22	22	None
16.	HPB	<i>Klebsiella spp.</i>	0	0	20	20	None
17.	TPA	<i>Klebsiella spp.</i>	0	0	0	0	None
18.	TPB	<i>Klebsiella spp.</i>	0	0	0	0	None
19.	LBPB	Nil	0	0	0	0	None
20.	BaPB	Nil	0	0	0	0	None

Table 5: Value of the purity and concentration determination of the dsDNA (Thermo Fisher Nano Drop Micro volume UV-vis Spectrophotometer was used to measure optical density after supernatant was collected in 7 brand-new Eppendorf tubes based on the sample size.)

Sample Number	Sample ID	A260/280	Concentration(ng/ul)
.....	Blank	0.000	
01.	P38	1.88	42.1
02.	P7B	1.92	58.7
03.	P9A	1.94	54.6
04.	P9B	1.87	58.8
05.	P10A	1.88	63.3
06.	P10B	1.88	68.6
07.	BaPA	1.95	19.5

Using the reverse and forward primer pairs , using a boiled suspension of bacterial cells and the β -lactamase genes (blaVIM,,blaDHA) were cycle conditions were only slightly altered^{10,11} . discovered by PCR. The DNA template was created

Table 6: PCR conditions for VIM primer¹⁰

Segment	PCR steps	Temperature (°C)	Time (in minutes)	No. of cycles
1	Initial denaturation	94.0	05.00	01
2	Denaturation	94.0	00.30	35
	Annealing	57.5	00.30	
	Extension	72.0	00.30	
	Final Extension	72.0	05.00	
3				01

Table 7: PCR conditions for DHA primer¹¹

Segment	PCR steps	Temperature (°C)	Time (in minutes)	No. of cycles
1	Initial denaturation	94.0	05.00	01
2	Denaturation	94.0	00.30	35
	Annealing	60.0	00.30	
	Extension	72.0	00.30	
3	Final Extension	72.0	05.00	01

Table 8: Information of short DNA sequence (primers) that were used during the experiment

Target genes primer	Primer sequence	Amplicon size(bp)	Reference
blaVIM F	5'-GATGGTGTGGTTCGCATA-3'	490	Naas et al.,2011
blaVIM R	3'-CGAATGCGCAGCACCAG-5'		
blaDHA F	5'-AACTTTCACAGGTGTGCTGGGT-3'	500	Viveiros et al.,2012
blaDHA R	3'-CCGTACGCATACTGGCTTTGC-5'		

Table 9: Volume measurement used during PCR for all the samples

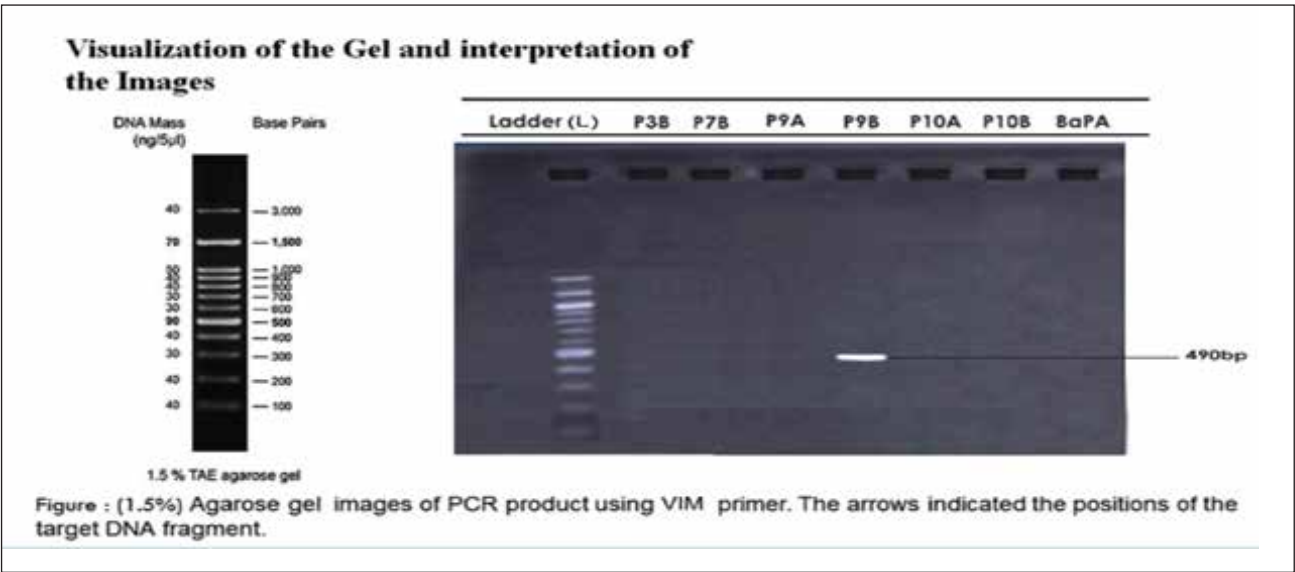
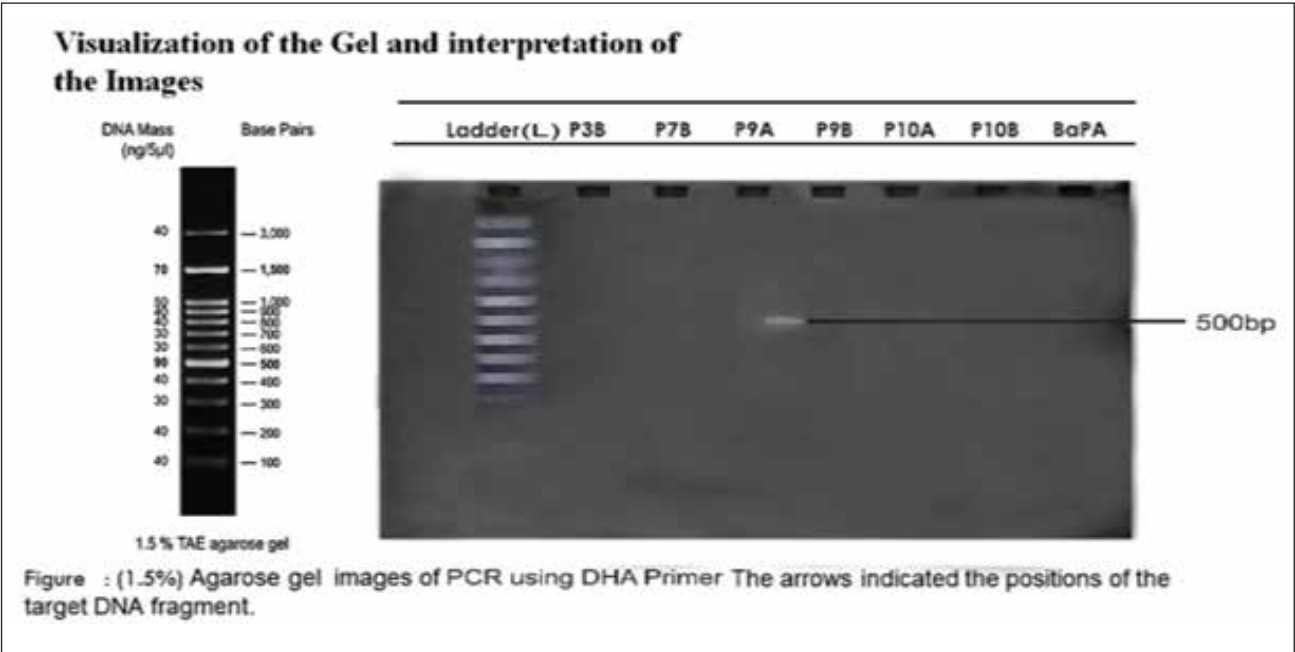
Sample name	QIAGEN Hot Star Taq Master Mix(ul)	Forward primer(ul)	Reverse primer(ul)	DNA(ul)	Nuclease Free wate(ul)	Total(ul)
P3B	6	1	1	4	1	13
P7B	6	1	1	4	1	13
P9A	6	1	1	4	1	13
P9B	6	1	1	4	1	13
P10A	6	1	1	4	1	13
P10B	6	1	1	4	1	13
BaPA	6	1	1	4	1	13
Negative control	6	1	1		1	9
Extra	6	1	1		1	9
Total	54	9	9	28	9	

Table 10: Genotype of beta-lactamase Genes

Sample ID	Food origin	Bla genes	Genotype
P3B	Beef	-	-
P7B	Beef	-	-
P9A	Beef	+	blaDHA
P9B	Beef	+	blaVIM
P10A	Beef	-	-
P10B	Beef	-	-
BaPA	Mutton	-	-

Figure IV: PCR assays for the detection of genotypes from phenotypically ESBL positive isolates using DHA primer. Lane P9A gene (500bp) positive isolates. Lane L represents the molecular weight marker (100bp DNA Ladder, Promega).

Figure V: PCR assays for the detection of genotypes from phenotypically ESBL positive isolates using VIM primer. Lane P9B gene (490bp) positive isolates. Lane L represents the molecular weight marker (100bp DNA Ladder, Promega)



Discussion

In the Dhaka metropolitan area, specifically in the neighborhood markets of Dhaka, a total of 40 animal-based foods were assessed to assess ESBL production. According to the Dhaka Tribune, red meat accounts for more than 50% of all meat consumption in Bangladesh. Additionally, it is believed that food obtained from the local markets in Dhaka may harbor pathogens capable of causing food borne illnesses. The published article¹² brought attention to the potential health risks associated with retail beef due to inadequate sanitation and hygiene practices during

processing and handling. *Escherichia coli* (*E. coli*) is thought to spread through meat because of the people and tools used to process it, such as knives and cutting boards, as well as poor hygiene and sanitation practices¹³. The transmission of contamination during the slaughtering process may occur through various means, including the hands of the slaughter men, water, and equipment utilized. Among the 40 samples derived from the red meat, 95% belong to the family Enterobacteriaceae, which supports the study in Ghana¹⁴ where 65.0% Enterobacteriaceae was found in

food samples. Among all the strains, 100% and 50% of *Klebsiella* species were found in beef and mutton samples, respectively, however, this study strongly aligns with the investigations in Athens¹⁵. In this study, a number of strains (17.5%) showed phenotypic positivity for making the ESBL enzyme. This is different from other studies, which found conflicting results (45.9%) for this phenomenon, which was seen in China¹⁶. Five (or 25%) resisted the CAZ and AMC combos in case of beef (All are *Klebsiella* species). Regarding the mutton, there were 20 that were resistant to CAZ+AMC (100%), and 12 were resistant to CTX+AMC (60%). The mutton findings closely match the Nigerian study¹⁷ where 91 samples (82.7%) were resistant to AMC.

A variety of enteric illnesses, including diarrhea and endocarditis, as well as infections of the skin, soft tissues, joints, bones, eyes, respiratory tract, and urinary tract, are caused by the Enterobacteriaceae family. Consequently, it is now a growing problem. We only got 40 pieces of meats, so the sample size was really small. The sample was not collected from throughout the nation but rather from different parts of Dhaka city. The weight, size, and nutritional value of the meats are not taken into account in this investigation. We are unable to utilize human samples due to a lack of resources and time. The World Health organization has designated several Enterobacteriaceae superbugs because of their increased production and spread of Carbapenamaeses and ESBL.

Based on the genotype confirmation, it has been observed that the genotypes *bla_{VIM}* and *bla_{DHA}* are accountable for a proportion of 2.5% and 2.5% respectively. A recent study conducted in Pakistan observed that among individuals producing carbapenemase, the *bla_{VIM}* gene was found to be the most prevalent at 47.7%¹⁸. These findings present some discrepancies when compared to our research. Furthermore, an additional survey conducted in northern and eastern Europe¹⁹ revealed a prevalence of 2.4% for the *bla_{DHA}* genotype among ESBL isolates, which is consistent with the findings of our investigations.

Conclusion

This study shows that the ESBL-producing Enterobacteriaceae found in raw meat in Dhaka, Bangladesh, have high antibiotic resistance. The rising rate of antibiotic resistance is highlighted by the need to enact policies to rationalize antibiotic usage and

build a national resistance surveillance system. These policies will help local communities produce antibiotic therapy guidelines. The most significant degree of public health and laboratory techniques must be significantly advanced to stop the spread of bacteria resistant to medications.

Acknowledgements

I would like to express my gratitude to the faculty, lab officer, seniors, juniors, and staff at North South University for their unwavering support, as well as the professors of BAMC Microbiology department for their direction.

Conflict of Interest

The authors declare no conflict of interest.

Financial Disclosure

This research received no external funding

Authors' contributions

Fariha RSH, Saha R, Jabeen I conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript. Saha S, Mahmuda H, Khan NM contributed to the analysis of the data, interpretation of the results and critically reviewing the manuscript. Fariha RSH involved in the manuscript review and editing. All authors read and approved the final manuscript.

Data Availability

Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

Ethical approval for the study was obtained from the Institutional Review Board. All methods were performed in accordance with the relevant guidelines and regulations.

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How to cite this article: Fariha RSH, Saha R, Jabeen I, Saha S, Mahmuda H, Khan NM. Isolation and Phenotypic Analysis of ESBL-Producing Enterobacteriaceae in Red Meat from Dhaka City: Antibiotic Resistance Profiles and Molecular Genotyping. Bangladesh J Med Microbiol, 2025;19(1):26-34

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Article Info

Received: 7 September 2024

Accepted: 2 October 2024

Published: 1 January 2025

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