ISOLATION OF β-LACTAM & COLISTIN RESISTANT BACTERIA AND ASSOCIATED β-LACTAMASE & CLR GENE FROM ANIMAL ORIGINATED FOOD

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DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES

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DECLARATION

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I/We have acknowledged all main sources of help.

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ABSTRACT

After the COVID-19 outbreak, the emergence of antimicrobial resistance among bacterial pathogens is another concern for public health. This study was designed to investigate the presence of multi-drug resistant β-lactamase producing and colistin resistant bacteria in raw meat, fish and milk sample collected from the local market.

Carcass of fish (12), chicken (11) and beef samples (10) along with pasteurized milk (10) samples were aseptically collected from the local market of Mohakhali and Karwan Bazar. The samples were processed accordingly and cultured on selective & non-selective media. Single colonies were selected from selective media depending on their colony morphology and cultural characteristics and a series of biochemical tests were conducted for the confirmation of the selected isolates. Subsequently, antibiotic profiling was done through Kirby-Bauer disk-diffusion method for identifying multi-drug resistant bacteria. Finally, Carbapenem & colistin resistant isolates were screened by PCR for detection of the antibiotic-resistant genes. Afterward, the hemolysis pattern of the carbapenem & colistin resistant isolates was observed in blood agar plates.

A total of 400 isolates of gram-positive & gram-negative bacteria were randomly selected from 43 samples by observing distinct colony morphology. Escherichia coli (23.87%) and Klebsiella spp. (21.86%) was the predominant species followed by Pseudomonas spp. (9.05%), Yersinia spp. (8.8%), Shigella spp. (7.03%), Providencia spp. (5.28%), Citrobacter spp. (5.02%), Salmonella spp. (4.77%), Proteus spp. (3.27%), Pasteurella multocida (3.27%), Streptococcus spp. (2.76%), Enterococcus spp. (2.01%), Enterobacter spp. (1.76%) and Moraxella catarrhalis (1.26%). Around 72.56% of isolates were resistant to ampicillin, 50.9% were resistant against 4th generation cephalosporin (cefepime), 12.02% were found to be resistant to carbapenem, 12.63% showed resistance against colistin and 22% isolates resisted at least 4 groups of antibiotics. The presence of bla-NDM gene was detected from 11 isolates and NDM gene was found from 7 isolates followed by bla-TEM gene in 6 isolates, bla-IMP gene in 2 isolates & bla-CTX-M gene in 5 isolates from 48 carbapenem-resistant isolates along with the presence of CLR gene was detected in 19 isolates from 58 colistin resistant isolates which were confirmed by Polymerase chain reaction (PCR) analysis. While observing the hemolysis pattern of 48 carbapenem resistant isolates, 22.91% isolates were alpha hemolytic, 16.66% were β hemolytic. Subsequently out of 58 colistin resistant isolates, 54% showed alpha hemolysis and 46% isolates showed β hemolysis.

The study reveals the alarming emergence of β-lactam & colistin resistant bacteria which is a serious public health concern. Due to horizontal gene transfer, these resistant genes can be shared by pathogens which might be a cause of another pandemic.

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ABBREVIATIONS

ABBREVIATION	ELABORATION
bp	Base pairs
CLSI	Clinical & Laboratory Standards Institute
CoA	Coenzyme A
CTX-M	Cefotaximase-Munich
dH2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
CRE	Carbapenem resistant enterobacteriaceae
ESBL	Extended spectrum β-lactamase
et. al	and others
EtBr	Ethidium bromide
MDR	Multidrug-resistant
mm	Millimeter
NDM	New-Delhi Metallo Beta- Lactamase
OXA	Oxacillinase
IMP	Imipenemase
VIM	Verona imipenamase
PCR	Polymerase chain reaction
pH	Power of hydrogen
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEM	Temoneira

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CHAPTER 1

INTRODUCTION

1.Introduction:

Antimicrobial resistance is an emerging and rapidly evolving phenomenon. The increasing AMR pattern subsequently leads to treatment failure, causing significant morbidity and mortality and additional healthcare costs annually [64,65]. AMR is increasingly reported from all over the world (Nordmann et al., 2011; Mohsin et al., 2017; Khattak et al., 2018; ur Rahman et al., 2018a). This phenomenon is currently observed in all bacterial species including clinically important Gram-negative bacilli (GNB) (Rubin and Pitout, 2014). Gram negative bacilli, "enterobacteriaceae and non-fermenters" are normal inhabitants of the human intestinal microflora (Vaishnavi, 2013); they are responsible for the most common hospital and community acquired infections. The scenario seems even more challenging for developing countries mainly due to unrestricted use of antimicrobials and lack of surveillance programs to monitor emergence of drug resistance (Khan et al., 2010; Mitema 2010; ur Rahman et al., 2018a). The emergence of carbapenemases together with the mcr-1 colistin resistance gene constitutes a global risk to public health [56, 57]. Resistance to carbapenem drugs is quite worrisome as these drugs are thought to be the last resort against MDR bacteria (Meletis,2016). In recent years, extended ESBL and carbapenemase producing Gram negative bacteria have become widespread in hospitals, community settings and the environment. This has been triggered by the few therapeutic options left when infections with these multi-drug resistant organisms occur. The emergence of resistance to colistin, the last therapeutic option against carbapenem-resistant bacteria, worsened the situation. Recently, animals were regarded as potent antimicrobial reservoir and a possible source of infection to humans. Antimicrobial consumption (AMC) in the animal production system is almost double human consumption [66]. In many countries, antimicrobials are widely available to humans and animals without any restriction. Unqualified animal healthcare providers play an important role in using antimicrobials in food-producing animals in developing countries [67]. Exaggerated use of antibiotics in the production facilities of food animals not only for therapeutic purposes but also for growth promotion or prophylaxis purposes is thought to be the crucial factor behind this (Agyare et al., 2019). When bacteria in the guts of animals are exposed to various antimicrobial agents with sub-therapeutic concentrations and frequencies, they acquire resistance to the antimicrobial agents that have been used through selective pressure (Scott et al., 2002). Foods of animal origin act as a vehicle and medium to transmit various resistant microorganism to human population. Transfer can occur by means of residues of antibiotics in food like poultry meat (Jhonson et al., 2007), through the transfer of resistant food-borne pathogens or through the ingestion of resistant strains of the original food microflora and resistance transfer to pathogenic microorganisms (Pesavento et al., 2007). The increased frequency and genetic relatedness of carbapenem and colistin resistant isolates from animal originated food and humans pose a public health threat that urges more prudent use of antimicrobials in livestock farms and aquaculture to avoid the propagation and expansion of resistance to last resort drugs from the animal originated food sources to humans

Carbapenems are a β -lactam group of drugs that were developed in the 1980s. There are three mechanisms by which K. pneumoniae employs carbapenem resistance: (i) enzymatic hydrolysis via carbapenemases enzymes, (ii) overexpression of the efflux pump system and (iii) loss of porin expression [61]. Carbapenemases (i.e., Ambler molecular classes A, B and D β-lactamases) represent the most prevalent mechanism of carbapenem resistance. They hydrolyze a wide variety of β-lactams including penicillins, cephalosporins, monobactams, carbapenems and β-lactamases inhibitors through carbapenemase encoding genes, mainly of class B metallo-β-lactamases (MBL), including imipenem

metallo-β-lactamases (bla-IMP), New Delhi metallo-β-lactamases (bla-NDM) and Verona integron-encoded metallo-β-lactamases (bla-VIM) [60]. At the beginning, nearly all Enterobacteriaceae were susceptible to carbapenems. However, this scenario has changed with the emergence of carbapenem resistant bacteria in the last years. Carbapenems are not approved for use in livestock production anywhere in the world [51]; as a result, animal-feed use is assumed to be rare. Despite of the lack of direct selection pressure, little is known about the prevalence of carbapenems resistant Gram-negative and Gram-positive bacteria, and more specifically carbapenems resistant Enterobacteriaceae, in poultry and livestock populations and their associated environments. Even though there remains a low probability of direct selection, Carbapenem Resistant Gram-Negative Bacteria (CRGNB) have been reported by investigators in few studies. On the other hand, colistin is not only administered in humans, its use has been also described in veterinary medicine. Indeed, it has been suggested that the uncontrolled use of colistin in animals has played an important role in the global emergence of colistin-resistant bacteria (Collignon et al., 2016). The World Health Organization recently added polymyxins to the list of critically important antibiotics used in food producing animals worldwide (Collignon et al., 2016). For many years, colistin resistance was thought to be mainly mediated by chromosomic mutations, with no possibility of horizontal gene transfer. However, the emergence of the mcr-1 plasmid mediated colistin resistance gene (Liu et al., 2016) has thoroughly altered the view of colistin resistance as a worldwide problem (Baron et al., 2016). The current epidemiology of colistin resistance is poorly understood.

In Bangladesh, commercial chicken and aquaculture industries are expanding day by day to meet the increasing demand for animal-source nutrition for humans. Both sectors are playing a significantly important role in the food value chain. Commercial chicken and fish farms perform intensive operations to increase production and minimize disease prevalence. Many types of drugs, including antimicrobials, vitamins, minerals, and antimicrobial growth promoters are extensively used in commercial chicken, cow farm and aquaculture production sectors [68, 69, 70, 71, 6, 72]. Many farmers in Bangladesh are less aware of the negative impact of excessive, irrational, and prophylaxis use of antibiotics in animals, and aquaculture. Inadequate veterinary healthcare facilities, insufficient monitoring and regulatory services on antibiotic usage, high occurrence of diseases, and malpractices by unqualified veterinary healthcare providers (quack, drug sellers, and animal feed dealers) contributed a crucial role in the increased and misusage of antibiotics in animal health sectors [72]. As a result in the last few years, carbapenem and colistin resistant bacteria have gradually appeared in animals and food. In Bangladesh, many studies reported antibiotic resistance in the commercial layer chicken in Bangladesh [6, 2, 4]. Colistin use in broiler production was not uncommon. There was an evidence of E. coli isolates that carried colistin-resistant mcr-1 genes and some of them showed resistance against tetracycline, and Beta-Lactam antibiotics [21,73]. In Bangladesh, a study reported 37.5% of the layer farms used colistin during the chicken production cycle and bacterial isolates detected from fecal samples of layer chicken showed resistance against colistin [21]. Frozen chicken meat samples were also tested positive for ESBL producing E. coli and Methicillin-resistant Staphylococcus aureus (MRSA) [2, 76, 26]. The E. coli isolated from chicken meat samples were found resistant against multiples antibiotics, such as oxytetracycline, amoxicillin, ampicillin, trimethoprim–sulfamethoxazole, pefloxacin, tetracycline, and carbapenems [78]. S. aureus showed wide-ranging resistance against cefoxitin, nalidixic acid, ampicillin, oxacillin, colistin, amoxicillin–clavulanic acid, amoxicillin, penicillin-G, cloxacillin, oxytetracycline, and cefixime [77] The occurrence of antibiotic-resistant bacteria in fish and freshwater was not uncommon in Bangladesh [74, 75]. In Bangladesh, 3,079 metric tons of poultry manure is produced per day and 50% of this is directly used in aquaculture [79]. For example, detectable amount of oxytetracycline residues was traced in 25% Pangas

fish samples from Sylhet Sadar [80]. Antibiotic resistance of E. coli and Salmonella spp. in foods including meat (poultry), milk and milk products (both raw and pasteurized) [15,81], were also found by some studies conducted in Bangladesh. Some studies also demonstrated the presence of the residue of different antibiotics (ciprofloxacin, enrofloxacin, amoxicillin, doxycyline, oxytetracylcine, and tetracycline) in poultry meat [83], milk, eggs [82] and fishes [80] as well. In a study conducted in Bangladesh E. coli isolated from beef sample were highly sensitive to ciprofloxacin, gentamicin and neomycin and 50% of the total isolates of E. coli from different animal originated food were multi-drug resistant.[2]

Minimal information is available to describe the antibiotic usage practices in food-producing animals in Bangladesh. On the other hand, several studies were carried out to examine antibiotic resistance. Antibiotic resistance, particularly in commercial chicken production, was explored more than other animal production sectors, including aquaculture and livestock but the role of different kind of animal originated food product in transmission of carbapenem-and colistin co-resistant isolates and the molecular characterization of these resistant gene is poorly explored. In our study we tried to focus on molecular characterization of carbapenem and colistin resistant gram positive and gram-negative bacterial isolates found from different kind of animal originated food. As these types of animal originated food (Chicken, meat, fish & milk) are consumed by people on a regular basis so it is important to study the resistance pattern of the last defense antibiotic in the bacterial isolated harbored by the animal because horizontal gene transfer can occur to human by consumption of such contaminated food which pose a threat to public health.

1.1 Objectives:

In this study our results demonstrate that diverse organisms with various carbapenemase and colistin resistant genes are widespread in animal originated foods (poultry, beef, fish & milk) in local market of Dhaka, highlighting the need for promoting proper food hygiene and effective measures to prevent further dissemination. The specific objectives of this study are as follows:

1. Screening of collected isolates by disk diffusion to detect the carbapenem and colistin resistant strains.

2. Checking the hemolysis pattern of the resistant isolates.

3. Detection of NDM, bla-NDM CTX-M, TEM and CLR genes by PCR.

CHAPTER 2

MATERIALS & METHODS

2. Materials & Methods:

2.1 Sample Collection & Processing:

Chicken meat (11), beef (10), fish (12) and packet milk (10) samples were collected from the local market of Mohakhali & Kawran bazar of Dhaka, Bangladesh from August to November 2021. The samples were immediately transported to the laboratory after being stored in sterile collection bags containing ice and further processing was done. The homogenization process for chicken liver, beef, and fish samples were carried out by taking 10 grams of sample and 90 ml of sterile distill water. All the samples were homogenized at 400 rpm for 15 minutes and filtered. After that the filtrate along with milk samples (both diluted to two-fold) were plated through spread plate method in both selective and non-selective media then incubated for 24 hours at 37 degrees centigrade.

2.2 Microbiological analysis of the isolates:

From the non-selective media, the total viable count of each dilution was determined and distinct colonies were randomly selected depending on colony morphology and phenotypic characteristics from each selective media. Following selective and non-selective media were used in this experiment.

Table 2.1: Name of the selective & non-selective media

Selective Media	Non-selective media
1. Eosine methylene blue Agar	1. Nutrient Agar
2. MacConkey Agar	
3. Cetrimide Agar	
4. Xylose lysine deoxycholate Agar	

Selected isolates from selective media were streaked on nutrient agar (NA) for pure culture.

2.3 Biochemical confirmation of the isolates:

All the selected isolates were subjected to a series of biochemical tests for the identification of different species of bacteria. Following biochemical tests were conducted for bacterial identification:

Table 2.2: Name of the conducted biochemical test

Name of the conducted biochemical test
1. Triple sugar iron test
2. Indole test
3. Methyl Red test
4. Voges-Proskauer test
5. Citrate test
6. Motility test
7. Urease Test
8. Catalase test
9. Oxidase test
10. Gram Staining

2.4 Antibiotic Susceptibility Testing

The antibiotic susceptibility of 10 different antibiotics from 9 distinct groups was tested on Muller Hinton agar using the conventional Kirby-Bauer disk diffusion method. In brief, after adjusting the turbidity of bacterial suspension equivalent to 0.5 McFarland standard, test suspension was inoculated on to Mueller–Hinton agar plates, and then antibiotic disks were placed and incubated at 37◦C for 18–24 h. Ciprofloxacin (CIP, 5 mg), cefepime (FEP,30mg), ampicillin (AMP, 10 mg), imipenem (IMI, 10 mg), azithromycin (AZM, 15mg), piperacillin tazobactam (TZP, 15 mg), gentamycin (CN, 10mg), colistin (CT, 10mg), meropenem (MRP, 10mg), Amikacin (AK , 30mg) were used for the assay. The results of the antimicrobial susceptibility test were interpreted according to the guidelines of Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2018).

2.5 Screening of Carbapenem & Colistin Resistant isolates:

Based on the antibiotic susceptibility test result the isolates which showed resistance against meropenem and imipenem were listed as carbapenem resistant isolates and resistance against colistin was observed. Further molecular gene detection and pathogenicity characterization was performed on the carbapenem and colistin resistant isolates.

2.6 Pathogenicity Characterization in Carbapenem & Colistin Resistant isolates:

To check the pathogenicity of the carbapenem and colistin resistant isolates, the pure culture of the isolates was streaked on Blood agar to observe the hemolysis pattern.

2.7 Molecular Detection of Carbapenem & Colistin Resistance Genes & Genetic Characterization:

2.7.1 DNA Isolation**:** Genomic DNA of the selected isolates were extracted via boiling method. For the process of boiling method, the isolates were cultured in Luria Bertoni broth and incubated overnight at 37 C. 700 microliter of the bacterial culture was transferred to eppendorf tubes and was centrifuged at 3000 rpm for 10 minutes. The pellet was washed with 300 microliter phosphate buffer and was centrifuged at 13000 rpm for 5 minutes. The pellet was then suspended in 200 microliter TE buffer and subjected to boiling at 100 degree C in a water bath for 15 minutes, followed by cold shock of 10 minutes. Then cell suspension was then centrifuged at 13000 rpm for 5 minutes which lead to the precipitation of the cell debris. The final supernatant was collected which contained the DNA, it was then visualized by loading in agarose gel electrophoresis.

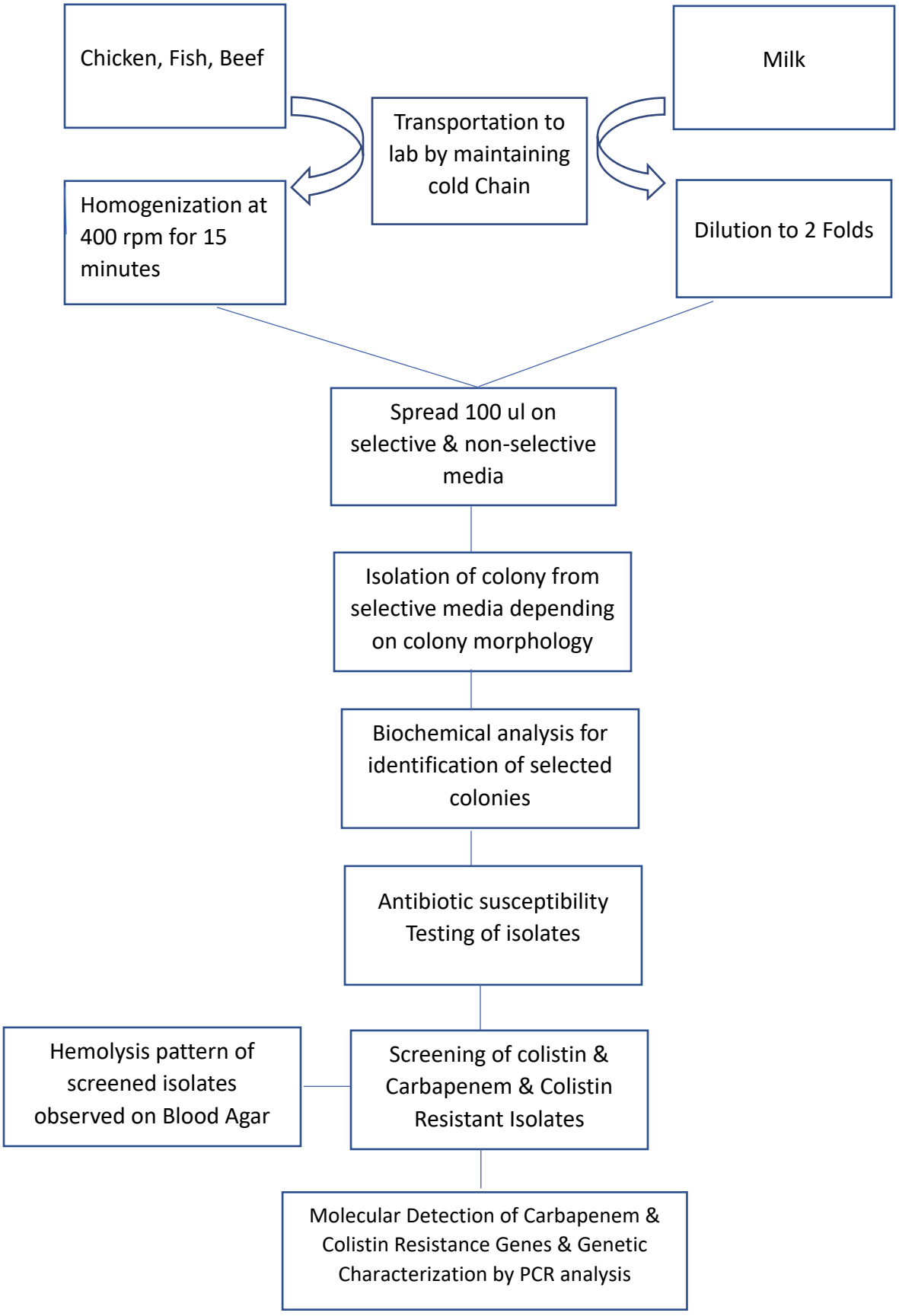
2.7.2 PCR Amplification of resistance genes: The presence of particular gene was determined by performing the polymerase chain reaction. PCR amplification was carried out for beta-lactamase gene of the family NDM, bla-NDM, bla-CTXm, bla-TEM, bla-IMP, bla-SHV and for colistin resistance gene CLR using following pair of primers.

Table 2.3: The oligonucleotide primers set as forward and reverse

Primer	Primer sequence	PCR Condition	Number of Cycle	Amplicon Size
NDM	NDM-F: 5'-GGTTTGGCGATCTGGTTTTC-3' NDM-R: 5'-CGGAATGGCTCATCACGATC-3'	95°C for 5 minutes 94°C for 30 seconds 58°C for 30 seconds 72°C for 30 seconds 72°C for 7 minutes	36	264
bla-NDM	bla-NDM-1- F: 5'ACCGCCTGGACCGATGACCA-3' bla-NDM-1- R: 5'-GCCAAAGTTGGGCGCGGTTG-3'	95°C for 7 minutes 94°C for 30 seconds 58°C for 30 seconds 72°C for 30 seconds 72°C for 7 minutes	36	264
bla-IMP	bla-IMP-F: 5′- GAAGGCGTTTATGTTCATAC-3′ bla-IMP-R: 5′- GTATGTTTCAAGAGTGATGC-3′	95°C for 5 minutes 95°C for 45 seconds 60°C for 45 seconds 72°C for 1 minute 72°C for 8 minutes	35	587
bla-CTX-M	bla-CTX-M F: 5'-ACGCTGTTGTTAGGAAGTG-3' bla-CTX-M R: 5'-TTGAGGCTGGGTGAAGT-3'	94°C for 3 minutes 94°C for 60 seconds 58°C for 30 seconds 72°C for 60 seconds 72°C for 10 minutes	36	857
bla-TEM	bla-TEM F: 5' AAAATTCTTGAAGACG-3' bla-TEM R: 5' TTACCAATGCTTAATCA-3'	94°C for 3 minutes 94°C for 30 seconds 50°C for 30 seconds 72°C for 2 minutes 72°C for 10 minutes	35	980
CLR	CLR F: 5'CGGTCAGTCCGTTTGTTC-3' CLR R: 5'CTTGGTCGGTCTGTAGGG-3'	94°C for 7 minutes 94°C for 30 seconds 58°C for 90 seconds 72°C for 60 seconds 72°C for 10 minutes	36	309

4 microliter of DNA template, 12 microliter of PCR master mix (Thermofisher), 5 microliter of nuclease-free water 2 microliter of forward and 2 microliters of reverse primer were used in a 25-microliter reaction mixture. The PCR master mix contained equal amount of dNTP, Mgcl2, and Taq polymerase. The PCR was carried out on the sample under suitable and different conditions for each gene primer. Following PCR condition was used for each primer respectively. The PCR products were analyzed by electrophoresis with 1.2% agarose gel concentration. The gel was stained with ethidium-bromide and visualized under UV transilluminator.

Fig 2. 1: An overview of followed protocol



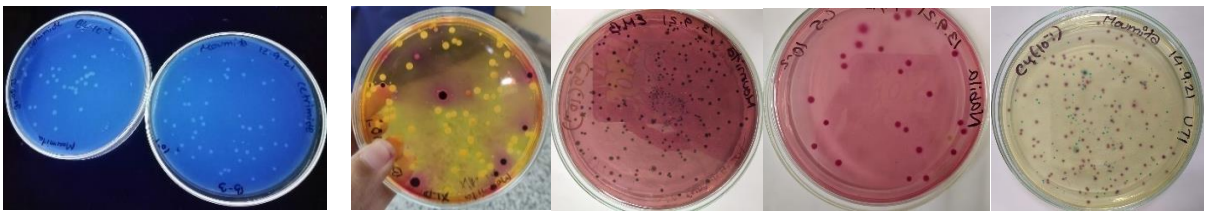
CHAPTER 3

RESULTS

3. Result:

3.1: Isolated bacteria from animal originated food:

From 43 samples of animal originated food which included 11 chickens, 10 beef, 12 fish and 10 pasteurized packet milk samples, 400 isolates were obtained. The isolates were selected randomly depending on their phenotypic characteristics from 5 selective media named Cetrimide, EMB, MacConkey, UTI and XLD where the sample were speeded.



1.Cetrimide Media 2. XLD media 3. EMB media 4. MacConkey media 5. UTI media

Fig. 3. 1: Growth of specific bacterial colonies on different selective media

Figure 3.1 shows the growth of distinct bacterial colonies on different selective media which were grown after incubation of 37-degree C.

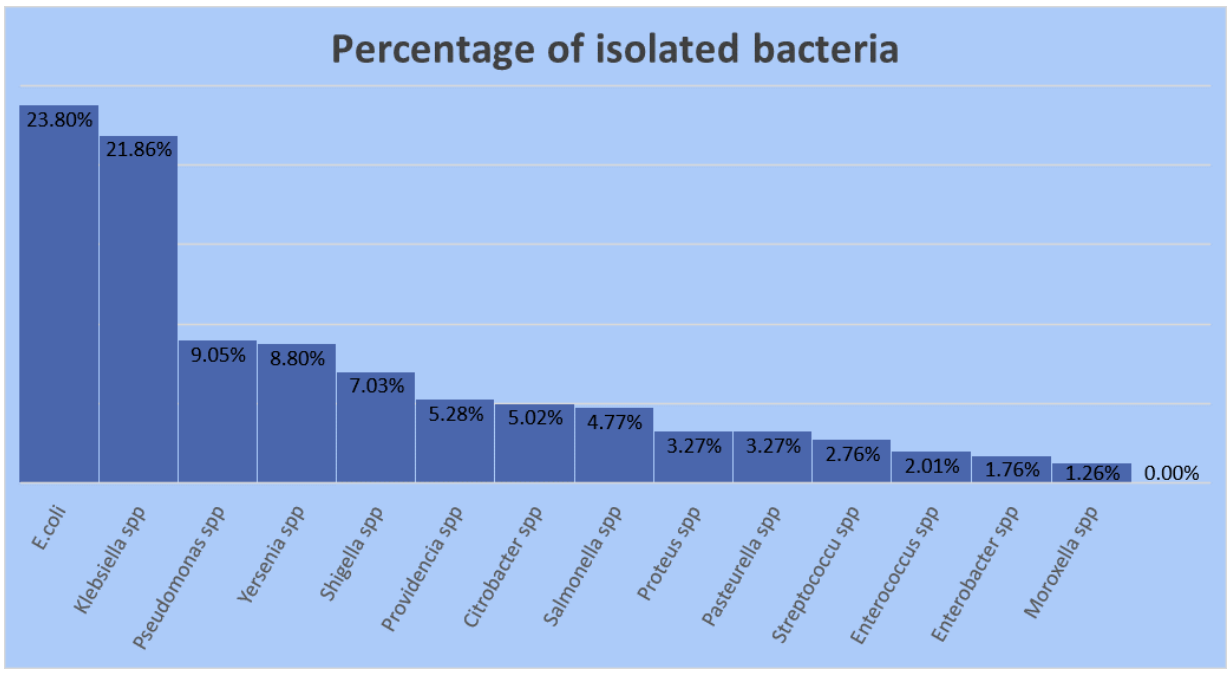


Fig. 3. 2 :Percentage of isolated bacteria from animal originated food sample

A total of 400 isolates were biochemically identified, of which 23.87% were Escherichia coli, 21.86% were Klebsiella spp. 9.05% were Pseudomonas spp. 8.8% were Yersinia spp. 7.03% were Shigella spp. 5.28%

were Providencia spp. 5.02% were Citrobacter spp. 4.77% were Salmonella spp. 3.27% were Proteus spp. 3.27% were Pasteurella multocida, 2.76% were Streptococcus spp. 2.01% were Enterococcus spp. 1.76% were Enterobacter spp. (1.76%) and 1.26% were Moraxella catarrhalis which were shown in the figure 3.2

3.2: Antibiotic Susceptibility Test:

Antibiotic susceptibility test shows the details of antibiotic resistance of the isolates. To check the antimicrobial susceptibility pattern of isolated bacteria from beef, chicken meat, milk and fish antibiotic disk diffusion method was performed. To compare the collected result CLSI guideline was followed.

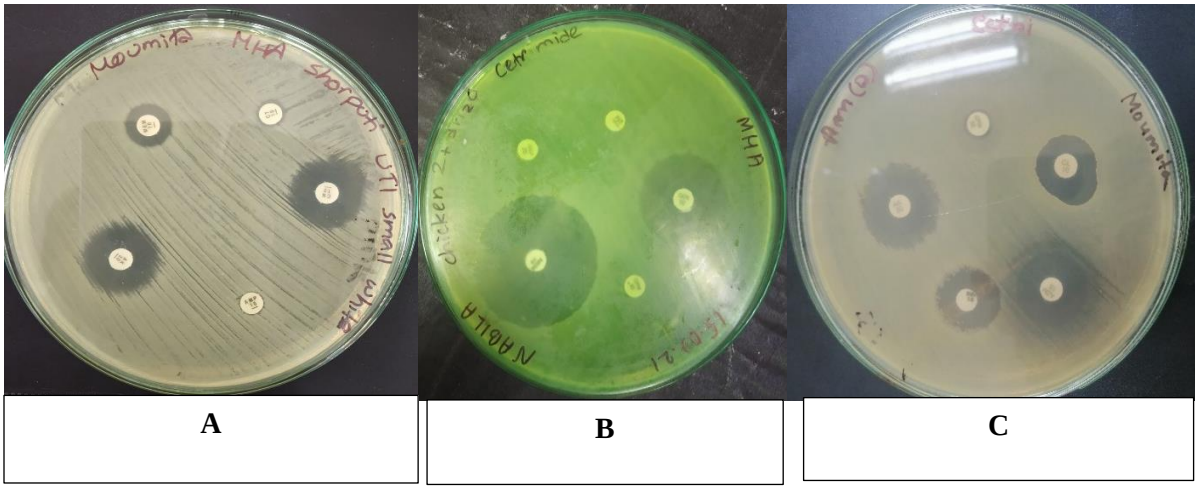


Fig. 3. 3 : Disk Diffusion method performed to check antimicrobial susceptibility pattern in selected isolates collected from chicken, beef, fish and milk

Fig. 3.3 A: shows antimicrobial susceptibility pattern of the bacterial isolate collected from cultured fish. The isolate shows resistance to ampicillin, colistin, meropenem and shows sensitivity to gentamycin. Fig. 3.3 B: shows the antimicrobial susceptibility pattern of the of bacterial isolate collected from chicken. The isolate shows resistance to ampicillin, piperacillin tazobactam, cefepime and shows sensitivity to, meropenem and gentamycin. Fig. 3.3 C: shows antimicrobial susceptibility pattern of the bacterial isolate collected from pasteurized packet milk. The isolate shows resistance to cefepime, imipenem and shows sensitivity to amikacin, colistin, azithromycin.

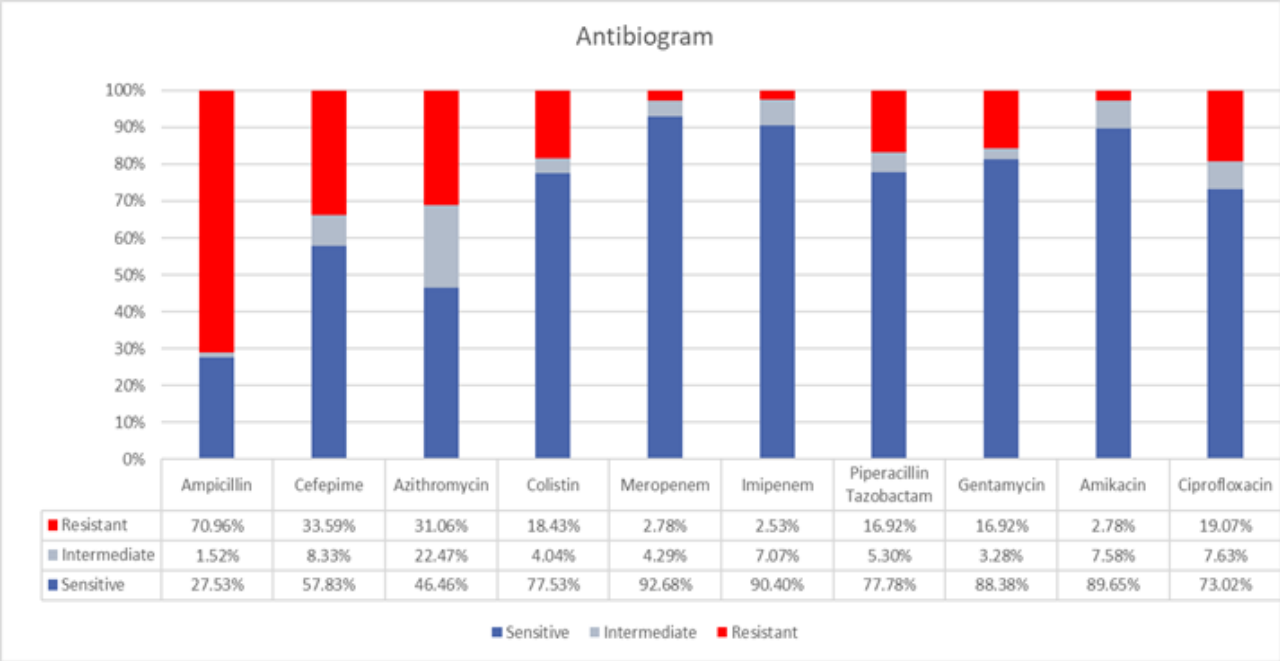


Fig. 3. 4 : Antimicrobial Susceptibility Pattern of isolated bacteria from animal originated food

Figure 3.4 shows the details of total antibiotic resistance percentage of isolates where around 72.48% of isolates were resistant to ampicillin, 41.92% were resistant against 4th generation cephalosporin (cefepime), 9.6% were found to be resistant to imipenem, 7.07% were resistant to meropenem, 22.47% showed resistance against colistin, 27.14% were resistant to ciprofloxacin, 10.36% to amikacin, 53.53% were to azithromycin, 11.61% were to gentamycin, 22.22% were to piperacillin/tazobactam 22.22% were to piperacillin/tazobactam and 22% isolates resisted at least 4 groups of antibiotics.

Table 3. 1: Resistance percentage of antibiotics in different animal originated food sample

Sample	Ampicillin	Cefepime	Gentamycin	Piperacillin Tazobactam	Imipenem	Meropenem	Ciprofloxacin	Amikacin	Azithromycin	Colistin
Chicken	75.43%	15.78%	22.80%	32.45%	12.3%	8.77%	37.71%	17.54%	66.66%	14.65%
Beef	82.22%	100%	7.77%	31.11%	4.44%	8.8%	38.8%	3.33%	43.2%	21.50%
Milk	73.3%	38.5%	12.3%	27.7%	12.3%	10.8%	27.7%	13.8%	50.8%	6.06%
Fish	64%	28.8%	4.8%	4%	9.6%	4%	8%	7.2%	44%	20%

Table 3.1 shows that bacteria isolated from different animal originated food provide different type of resistance pattern against specific group of antibiotics.

3.3 Distribution of Carbapenem & Colistin Resistant isolates in animal originated food:

All samples of beef, chicken, fish & milk analyzed in our study harbored resistance against carbapenem and colistin.

Table 3. 2: Details of selected carbapenem resistant isolates from different animal originated food types

Name of the isolates	Number of isolates	Sample (Number of total isolates = 400)			
		Chicken (116)	Fish (125)	Milk (66)	Beef (93)
Klebsiella spp.	16	4	7	0	5
E. coli	8	1	6	0	1
Pseudomonas spp.	6	2	2	1	1
Pasteurella multocida	3	0	2	0	1
Salmonella spp.	3	3	0	0	0
Shigella spp.	2	1	1	0	0
Yersinia spp.	2	0	0	1	1
Proteus spp.	2	0	1	0	1
Enterobacter spp.	2	2	0	0	0
Enterococcus spp.	1	1	0	0	0
Streptococcus spp.	1	1	0	0	0
Moraxella catarrhalis	1	0	0	0	1
Citrobacter spp.	1	0	1	0	0
Total =	48	15	20	2	11

Table 3.2 shows that *Klebsiella spp. E. coli. Pseudomonas spp*. is the predominant carbapenem resistant isolates and highest carbapenem resistance was found in fish sample.

Table 3. 3: Details of selected colistin resistant isolates from different animal originated food types

Name of the isolates	Number of isolates	Sample (Number of total isolates = 400)			
		Chicken (116)	Fish (125)	Milk (66)	Beef (93)
Klebsiella spp.	15	3	8	0	4
Shigella spp.	9	3	2	0	4
Yersinia spp.	8	1	2	2	3
Pseudomonas spp.	8	2	4	1	3
E. coli	7	1	2	0	0
Salmonella spp.	6	4	2	0	0
Providencia spp.	5	1	2	1	1
Proteus spp.	3	2	0	0	1
Citrobacter spp.	2	0	0	0	2
Enterococcus spp.	1	0	1	0	0
Pasteurella multicoda	1	0	1	0	0
Enterobacter spp.	1	0	1	0	0
Total =	66	17	25	4	20

Table 3.3 shows that *Klebsiella spp., Shigella spp., Pseudomonas spp.* is the predominant colistin resistant isolates and highest colistin resistance was found in fish sample.

3.4: Hemolysis pattern of Carbapenem Resistant isolates :

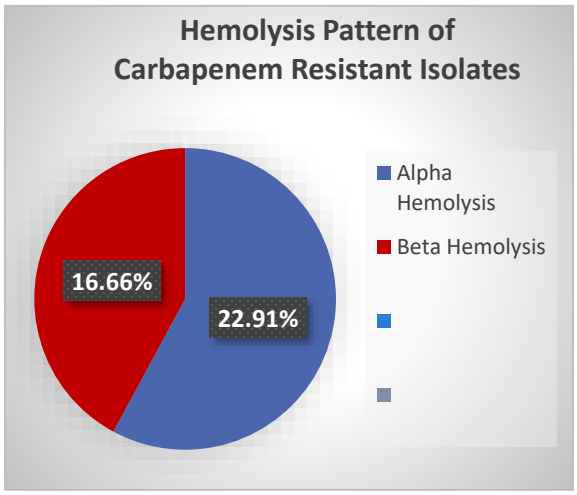


Fig. 3. 5: Percentage of hemolysis pattern of carbapenem resistant isolates

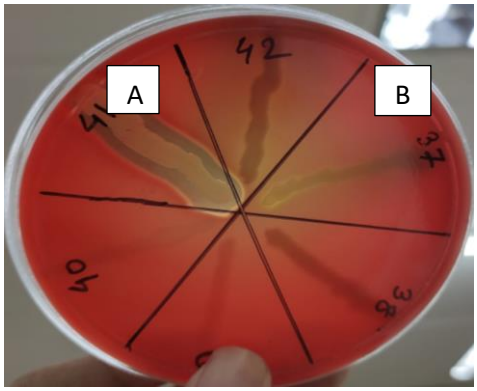


Fig. 3. 6: Hemolysis pattern on blood agar

Organism Name	Sample	Hemolysis Pattern	
		Alpha hemolysis	Beta hemolysis
Pseudomonas spp.	Fish	1	0
	Milk	1	0
	Beef	1	1
	Chicken	1	1
Klebsiella spp.	Fish	0	0
	Milk	0	0
	Beef	1	2
	Chicken	0	0
E. coli	Fish	0	3
	Milk	0	0
	Beef	0	0
	Chicken	0	0
Salmonella spp.	Fish	0	0
	Milk	0	0
	Beef	0	0
	Chicken	2	0
Yersinia spp.	Fish	0	0
	Milk	0	0
	Beef	0	1
	Chicken	0	0
Streptococcus spp.	Fish	0	0
	Milk	0	0
	Beef	0	0
	Chicken	1	0
Citrobacter spp.	Fish	1	0
	Milk	0	0
	Beef	0	0
	Chicken	0	0
Pasteurella multicoda	Fish	1	0
	Milk	0	0
	Beef	0	0
	Chicken	0	0

Table 3.4: Details of hemolysis pattern in carbapenem resistant isolates of animal originated food types

Figure 3.5 shows that alpha hemolysis is higher in carbapenem resistant isolates than beta hemolysis.

Table 3.4 shows that *pseudomonas spp.* was the predominant isolate which exhibited the highest hemolysis pattern followed by *Klebsiella spp*. and *E. coli*.

In figure 3.6, A is *Pseudomonas spp*. of beef sample which exhibited Beta hemolysis and B is *Salmonella spp.* of chicken sample which exhibited alpha hemolysis.

3.5: Hemolysis pattern of Colistin resistant isolates:

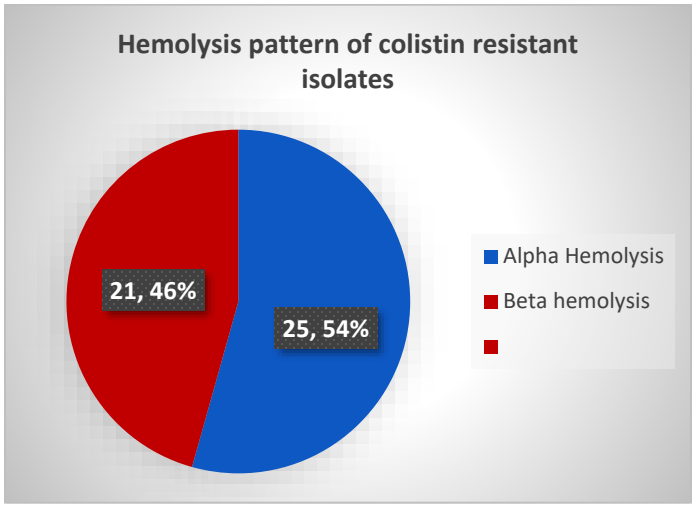


Fig. 3. 7: Percentage of hemolysis pattern of colistin resistant isolates

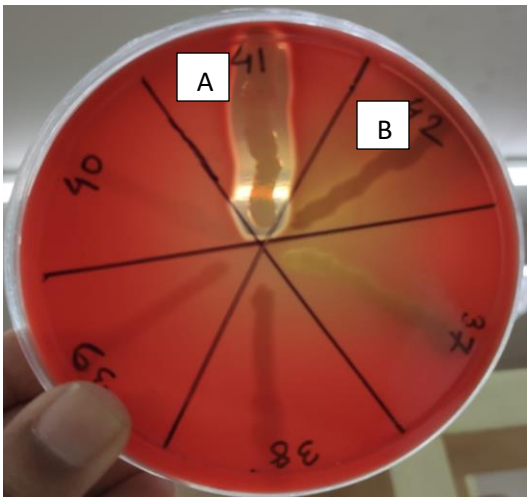


Fig. 3. 8: Hemolysis pattern on blood agar

Organism Name	Sample	Hemolysis Pattern	
		Alpha hemolysis	Beta hemolysis
Shigella spp.	Fish	1	0
	Milk	0	0
	Beef	0	3
	Chicken	0	3
Pseudomonas spp.	Fish	2	1
	Milk	0	0
	Beef	1	1
	Chicken	1	1
Klebsiella spp.	Fish	2	1
	Milk	0	0
	Beef	3	0
	Chicken	0	0
Yersinia spp.	Fish	1	0
	Milk	1	1
	Beef	0	1
	Chicken	0	1
Salmonella spp.	Fish	1	1
	Milk	0	0
	Beef	0	0
	Chicken	2	0
E. coli	Fish	1	0
	Milk	1	0
	Beef	0	2
	Chicken	0	0
Proteus spp.	Fish	1	0
	Milk	0	0
	Beef	1	0
	Chicken	1	1
Citrobacter spp.	Fish	0	0
	Milk	0	0
	Beef	2	0
	Chicken	0	0
Providencia spp.	Fish	0	1
	Milk	0	0
	Beef	0	0
	Chicken	0	1
Enterobacter spp.	Fish	0	0
	Milk	0	0
	Beef	1	0
	Chicken	0	0
Pasteurella multicoda	Fish	1	0
	Milk	0	0
	Beef	0	0
	Chicken	0	0

Table 3.5: Details of hemolysis pattern in colistin resistant isolates of animal originated food types

Figure 3.7 shows that alpha hemolysis is higher in colistin resistant isolates than beta hemolysis.

Table 3.5 shows that *Shigella spp*. was the predominant isolate which exhibited the highest hemolysis pattern followed by *Pseudomonas spp., Klebsiella spp.* and *Yersinia spp.*

In figure 3.8, A is *Yersinia spp*. of milk sample which exhibited Beta hemolysis and B is *Klebsiella spp.* of fish sample which exhibited alpha hemolysis.

3.6: Identification of antibiotic resistant gene through PCR:

All 48 carbapenem resistant isolates of animal originated food sample were further characterized for the presence of carbapenemase-encoding genes including bla-NDM, NDM, bla-IMP and results are shown in figure 3.8. Briefly, our results revealed that bla-NDM was predominant and identified in 11 (22.91%) isolates. This was followed by NDM carried by 7/48 (14.58%) isolates while two was was harboring bla-IMP gene. The presence of ESBl gene (bla-CTXm, bla-TEM) in the carbapenem resistance isolates were also analyzed and genetic characterization was conducted. Six isolates out of 48 carbapenem resistant isolates (12.5%) carried bla-TEM gene and four isolates (8.33%) carried bla-CTXm gene. All 66 colistin resistant isolates of animal originated food sample were further characterized for the presence of colistin -encoding gene named CLR carrying mcr-1 gene and results are shown in figure 5. Out of 66 colistin resistant isolates 20 isolates (30.30%) showed resistance against CLR gene.

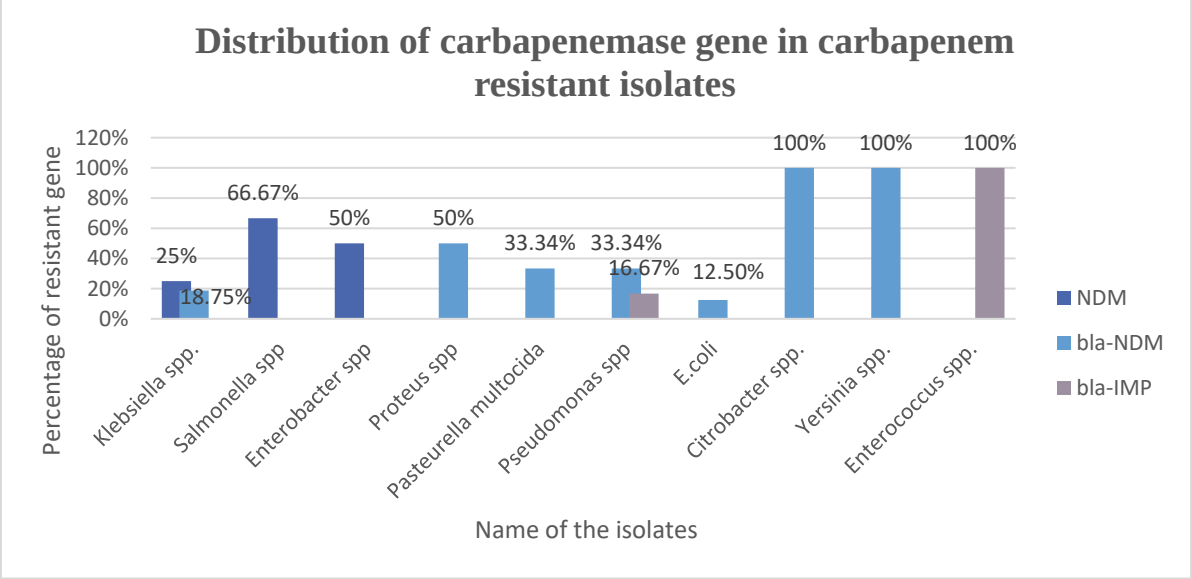


Fig. 3. 9: Distribution of carbapenemase gene in carbapenem resistant isolates

Figure 3.9 shows that only *Klebsiella spp.* isolates harbored both NDM and Bla-NDM gene and *Pseudomonas spp.* isolates harbored both bla-NDM and bla-IMP gene. *Citrobacter spp., Yersinia spp.* and *Enterococcus spp.* had 100% of harboring carbapenemase resistant gene and *E. coli* isolates had the lowest percentage.

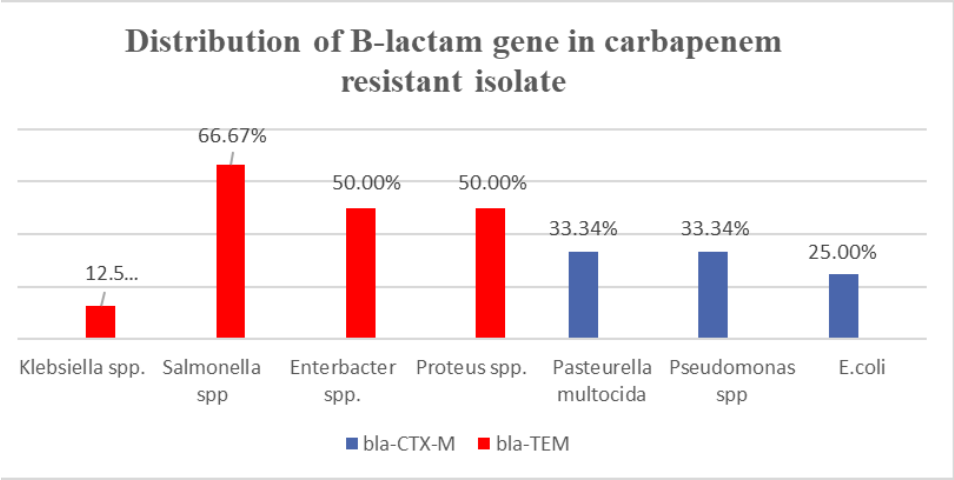


Fig. 3. 10: Distribution of B-lactam gene in carbapenem resistant isolates

Figure 3.10 shows that there were no isolates that harbored both bla-CTX-M and bla-TEM gene. *Salmonella spp.* had 66.67% of harboring bla-TEM gene and *Klebsiella spp.* isolates had the lowest percentage. *Pseudomonas spp.* and *Pasteurella multocida* had 33.34% of harboring bla-CTX-M gene and *E. coli* isolates had the lowest percentage.

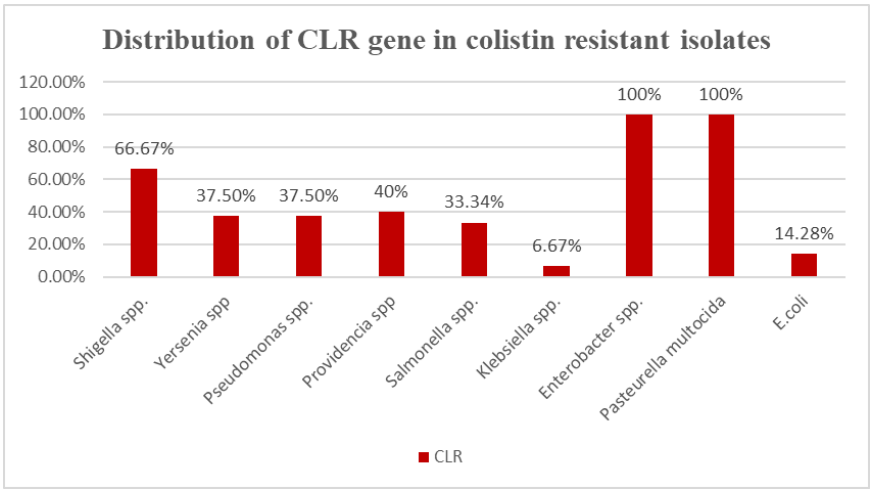


Fig. 3. 11: Distribution of CLR gene in colistin resistant isolates

Figure 3.11 shows that Enterobacter spp. and Pasteurella multocida had 100% of harboring CLR gene. After that, Shigella spp. had the highest percentage (66.67%) of carrying CLR gene, followed by Providencia spp., Yersenia spp., Pseudomonas spp. and Salmonella spp. Klebsiella spp. had the lowest percentage.

Table 3.6: Distribution of B-lactam genes in different bacteria isolated from different animal originated food sample

Organism	Distribution of B-lactam genes																			
	NDM				bla-NDM				bla-IMP				bla-CTX-M				bla-TEM			
	B	F	C	M	B	F	C	M	B	F	C	M	B	F	C	M	B	F	C	M
Klebsiella spp.	1	3	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Salmonella spp.	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
Enterobacter spp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Proteus spp.	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Pasteurella multocida	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Pseudomonas spp.	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	0	0	0
E. coli	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Citrobacter spp.	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yersinia spp.	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus spp.	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0

B = Beef; F = Fish; C = Chicken; M= Milk

Table 3.6 shows that NDM gene was prevalent in *Klebsiella spp.* isolated from fish sample and bla-NDM was prevalent in *Salmonella spp.* isolated from fish sample too. On the other hand, the table shows that bla-IMP was prevalent in *Pseudomonas spp.* and *Enterococcus spp.* which were isolated from fish and chicken sample respectively. According to the table, bla-TEM was prevalent in in *Salmonella spp.* isolated from fish and bla-CTX-M was prevalent in *Pseudomonas spp.* and *E. coli* which were isolated from fish, chicken and beef sample. Only one Yersinia spp. isolate found from milk sample carried the bla-NDM gene while rest of the carbapenem resistant isolates did not carry any other B-lactam resistant gene.

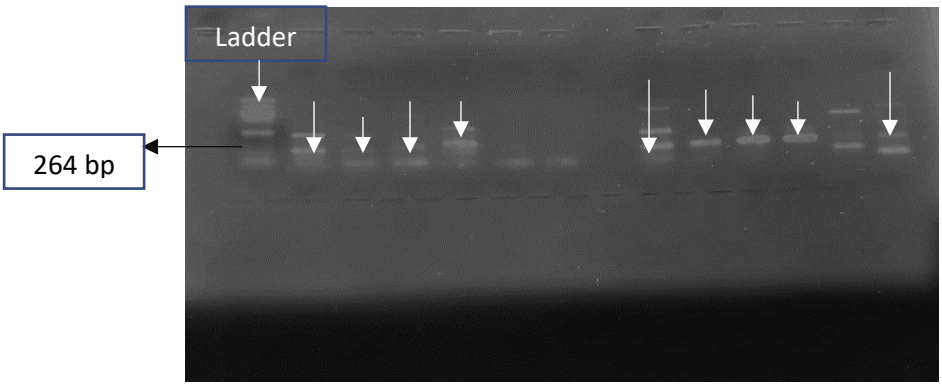


Fig. 3. 12: Gel electrophoresis of 100bp ladder and PCR product of bla-NDM

Fig. 3.12 shows the gel electrophoresis result of blaNDM-1 gene. The gel shows nine bands of different isolates which were collected from beef, fish, chicken and milk sample. The arrowed isolates were positive for bla-NDM. The isolates showed the band at 264 bp which confirms the presence of the bla-NDM gene.

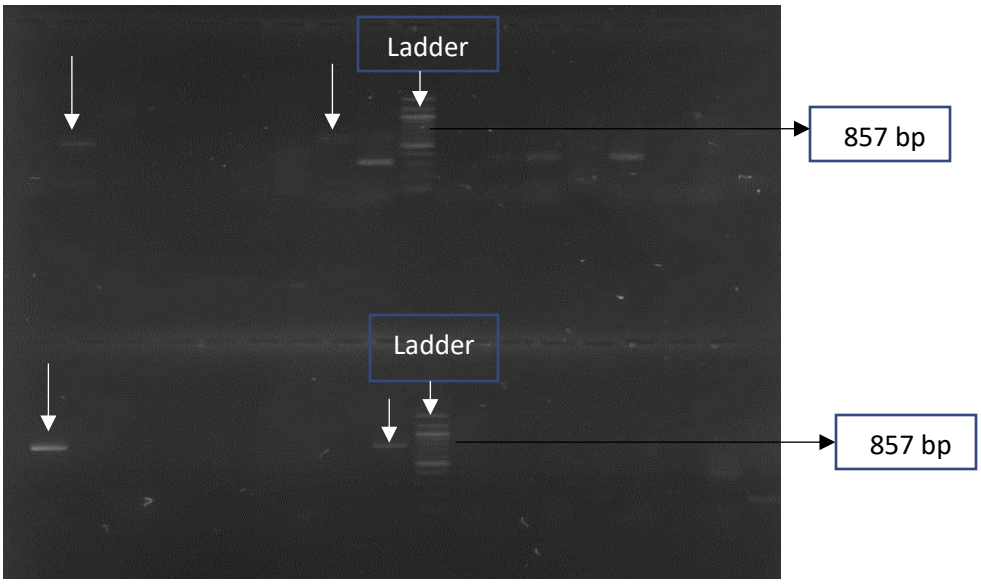


Fig. 3. 13: Gel electrophoresis of 100bp ladder and PCR product of CTX-M

Fig. 3.13 shows the gel electrophoresis result of bla-CTX-M gene. The gel shows four bands of different isolates which were collected from beef, fish and chicken sample. The arrowed isolates were positive for bla-CTX-M. The isolates showed the band at 857 bp which confirms the presence of the bla-NDM gene.

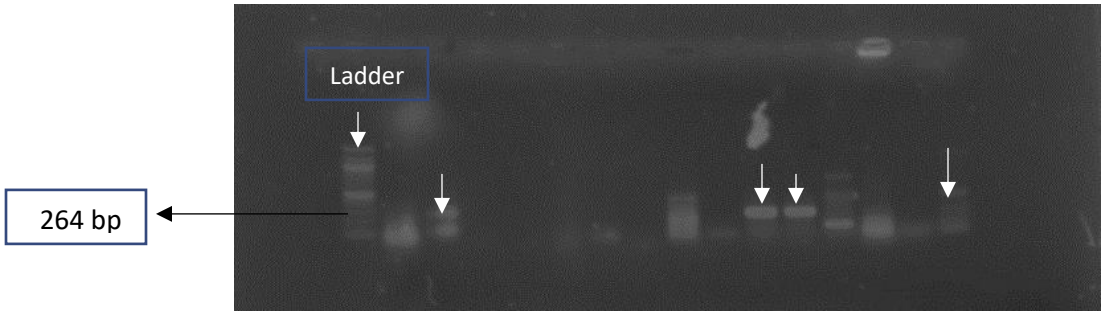


Fig. 3. 14: Gel electrophoresis of 100bp ladder and PCR product of NDM

Fig. 3.14 shows the gel electrophoresis result of NDM gene. The gel shows four bands of different isolates which were collected from beef, fish and chicken sample. The arrowed isolates were positive for NDM. The isolates showed the band at 264 bp which confirms the presence of the NDM gene.

Table 3.7: Distribution of colistin resistant genes in different bacteria isolated from different animal originated food sample

Organism	Distribution of Colistin resistant genes			
	CLR			
	Beef	**Fish**	**Chicken**	**Milk**
Klebsiella spp.	1	0	0	0
Salmonella spp.	0	0	2	0
Enterobacter spp.	1	0	0	0
Yersinia spp.	0	1	1	1
Pasteurella multocida	0	1	0	0
Pseudomonas spp.	1	2	0	0
E. coli	1	0	0	0
Shigella spp.	2	1	3	0
Providencia spp.	0	1	1	0

Table 3.7 shows that CLR gene was prevalent in *Shigella spp.* isolated from chicken sample followed by beef sample. Only one Yersinia spp. isolate found from milk sample carried the CLR gene. The table also shows that CLR gene was most prevalent in the bacterial isolates found from chicken sample followed by beef and fish sample.

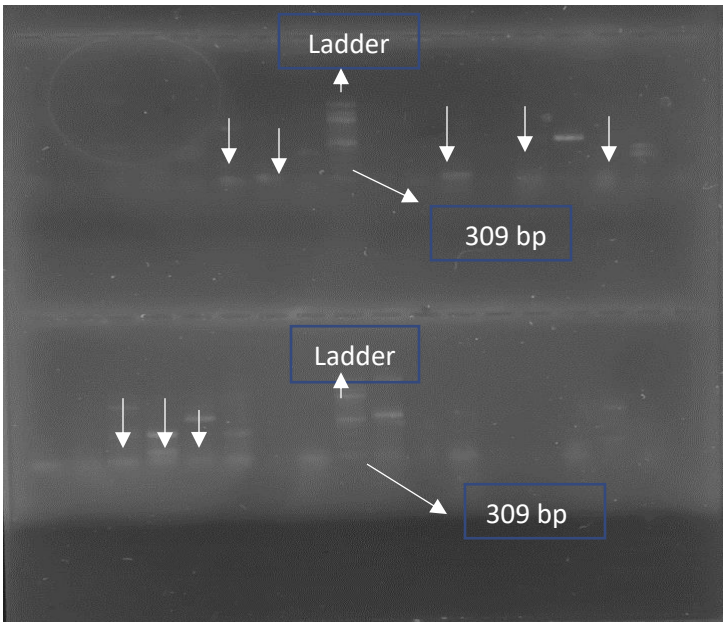


Fig. 3. 15: Gel electrophoresis of 100bp ladder and PCR product of CLR

Fig. 3.15 shows the gel electrophoresis result of CLR gene. The gel shows eight bands of different isolates which were collected from beef, fish and chicken sample. The arrowed isolates were positive for CLR. The isolates showed the band at 309 bp which confirms the presence of the CLR gene

CHAPTER 4

Discussion

Discussion:

The increasing prevalence of antimicrobial resistance created serious problems for treatment of bacterial infections and continue to be a major challenge for public health worldwide. The high emerging of multidrug resistance is aggravated by the use of antibiotics without restrictions in agriculture, especially in food animals. The spread of antibiotic resistant bacteria from chicken or animal samples in general poses public health risk and increases the likelihood to spread in the human population. Analysis of foods Of livestock and aquatic origin such as fish is expected to yield diverse species of bacteria and we identified 12 genera of both gram-positive and gram-negative bacteria through biochemical identification. In this study, we aimed to investigate carbapenems & colistin resistance among gram- negative and gram-positive isolates from chicken, fish, milk & beef. The emergence of colistin resistance is a global public health concern, since this antibiotic is the last defense line against carbapenem-resistant isolates. So far, there is only one published data on colistin and carbapenem resistance in ESBL isolated from chickens and their environment in the same geographical region (Elmonir.et. al, 2021). There is no specific published data on colistin and carbapenem resistant gene which focused on total four animal originated food sample (poultry chicken, fish, milk & beef) simultaneously.

In this study, *Escherichia spp.* were the most frequently isolated bacterial species (23.80%) from all four samples. Rahman et al. reported the prevalence of *E. coli* in foods of animal origin was 37.86% which is higher than our findings as in our study the prevalence of *E. coli* in animal originated food was 23.86% (Figure 1). The prevalence of *E. coli* was 64.4% in the poultry meat in our study which is lower than the result obtained by Mandal et al. who reported *E. coli* (76%) being prevalent in poultry chicken in Bangladesh but higher than the results obtained by Chika et al. who reported *E. coli* (30.3%) as the most prevalent organisms isolated from cloacal swabs of poultry birds. Chika et al. found *Klebsiella* species & *Pseudomonas aeruginosa* (34.88%, 32.7% respectively) in abundance in poultry chicken but in our study the prevalence of *Klebsiella spp*. and *Pseudomonas spp*. was 12.9% and 8.77 respectively in poultry meat. In our study the prevalence of other bacterial species in poultry meat were also observed which were in quite low percentage than *E. coli* and the prevalence of gram-negative bacteria is higher than gram positive bacteria. Similar result was also overserved in our other three animal origin food sample where the percentage of *E. coli* & *Klebsiella spp*. was the most prevalent. In beef sample the prevalence of *E. coli* and Klebsiella spp. was 23% and 13% respectively and in milk sample the prevalence was 17% and 15% which is lower than the study reported by Rahman et al. because the prevalence of *E. coli* was 70% in beef and 29.63% in milk. Asem et al. reported E. coli as the predominant species isolated from seafood, followed by *Klebsiella oxytoca, K. pneumoniae*, and *Citrobacter diversus* which resembles with our study as the prevalence of *E. coli* & *Klebsiella spp*. (20.17% and 28%) was also predominant in our fresh water fish but the prevalence of *Klebsiella spp.* is higher than *E. coli.* This variation in prevalence with current study might be due to the variation in the selection of sample and isolating a wide range of bacterial species instead of focusing on specifically one. There is no specific study in Bangladesh which focused on a wide range of gram positive and gram-negative bacteria found in different type of animal originated food types.

In the antimicrobial susceptibility study, we used 10 antibiotics of nine different classes. The challenge was not negligible as four (meropenem, cefixime, colistin, and ciprofloxacin) of the antibiotics tested in this study are classified by the World Health Organization as extremely important antibiotics in human medicine, and the other six (levofloxacin, ampicillin, amikacin, gentamicin, imipenem, azithromycin) are classified as highly important antibiotics (World Health Organization, 2017). In our study the highest resistance was observed against ampicillin followed by 4th generation cephalosporin, azithromycin and ciprofloxacin (Figure 3.3) in animal origin of food. The study of Rowshan et.al reported 55% prevalence of Extended-Spectrum Beta-Lactamase (ESBL)-producing E. coli in broiler ceca and feces at households, farms, and live poultry markets where majority (71%) of the ESBL-producing E. coli isolates showed

resistance against fluoroquinolones and cefepime, followed by sulfonamides (65%) and aminoglycosides (31%) but in our study the isolated bacteria of poultry meat collected from local market showed maximum resistance in ampicillin (75.43%), followed by azithromycin (66.66%), Ciprofloxacin (37.71%) and Piperacillin tazobactam (32.45%) (Table 1). Contamination in the beef liver by antibiotic-resistant bacteria with resistance against ciprofloxacin (40%) and ampicillin (6.67%) was also reported by Rana et.al which differed from our studies as the resistance of ampicillin (82.22%) was very higher in beef respectively 100% resistance was observed in 4th generation cephalosporin 38.8% in ciprofloxacin and 43.2% in azithromycin. (Table-3.1). In our study predominant bacteria found in cultured fish of local market showed highest resistance in ampicillin (64%) followed by azithromycin (44%), cefepime (28.8%) & colistin (28.8%) differs with the study by Farazana et. al (2018) as they found 100% resistance in Penicillin and cephalosporin but they did not overserve any carbapenem resistance like our study shows resistance pattern in meropenem (4%) & imipenem (9.6%). The resistance pattern observed in the predominant bacteria found in pasteurized milk showed highest resistance in ampicillin (73.3%) followed by azithromycin (50.8%), cefepime (38.5%) and piperacillin tazobactam (27.7%) which resembles with the study of Marjan et.al as the highest resistance was also found against ampicillin followed by ciprofloxacin cephalosporin in different pathogenic isolates of milk and milk products in their study. Although limited numbers of samples from different animal originated food samples were analyzed in our study, the results clearly point to the contamination of Livestock and fresh water fish with multiple antibiotic-resistant.

The overall percentage of Carbapenem resistant among Gram negative bacteria was 95.84%. This rate is much higher than the prevalence rate of 59% obtained from Spain and higher than that obtained from Qatar (2.2%) . A study by Gregg et al., (2018), showed greater than 50% CRE in any of the isolates obtained from poultry meat, while Gernot et al., (2014) showed no CRE in any of the isolates obtained from Chicken Meat in Austria but in our study, we found 12.93% carbapenem resistant isolates from poultry chicken. In our study 12.3% imipenem resistant isolates obtained from pasteurized packet milk which resemble with the study by Marajan. et. al (2014) as they found 10.5% resistance in imipenem. Total 11 carbapenem resistant isolates were found from 10 beef samples in our study which is higher than the study by Qianhui et. al as they found only 3 carbapenem resistant isolates from 60 beef sample from 2016-2018. The percentage of total carbapenem resistant isolates were 11.82% in beef sample (imipenem- 4.4%, meropenem-8.8%) and 11.55% in pasteurized packet milk which is lower than other two animal origin food sample (chicken & fish) of our study (Table-3.1). The total percentage of carbapenem resistant isolates in fish sample is 16% (Table-3.2) of our study which is higher than the study by Sugawara et. al as they found 12.5% carbapenem resistant isolates in fish. In this study prevalence of carbapenem resistance was observed in *Klebsiella spp* and *E. coli* (Table-3.2) which is similar to other studies (Ballot.et.al; 2019, Perovic.et.al; 2018, Grundmann.et.al; 2017). The occurrence of Carbapenem resistant bacteria in livestock and seafood has been also reported in African, American, Asian, and European countries. Two studies investigated the transmission of CRE between animals and exposed humans [19]. Differences in the prevalence of carbapenem resistant bacteria in different country in different animal originated food is visible in this study. The reasons for these differences may be due the time each country started using carbapenems in clinical practice and regulations and restrictions imposed on antibiotic use in animal farm. The prevalence differences may also be attributed to the geographical location, climatic circumstances, environmental contamination, sample types, differences in breed, management systems and growth conditions.

Colistin is considered one of the last-resort reserved antibiotics for humans. However, colistin is used in animals for therapeutic, prophylactic, and growth promotion purposes (Catry.et.al, 2015). In Bangladesh, a study reported 37.5% of the layer farms used colistin during the chicken production cycle and bacterial isolates detected from fecal samples of layer chicken showed resistance against colistin (Islam.et.al, 2020). In another study conducted in Bangladesh, Hashem and colleagues reported *E. coli* isolates that were 100%

susceptible to colistin sulphate. The rate of susceptibility is only 77.53% among our isolates in all of our animal originated food sample. In our study the highest colistin resistance was overserved in beef (21.50%), followed by fish (20%) and chicken (14.67%) and lowest colistin resistance was observed in milk (6.06%). The study by Islam. et. al (2020) reported highest colistin resistance in *Proteus spp*. followed by *Klebsiella spp., Shigella spp., Salmonella spp.* isolates from poultry chicken but in our study highest colistin resistance was found in *Klebsiella spp.* (22.72%), *Shigella spp*. (13.63%), *Yersinia spp*. (12.12%) and *Pseudomonas spp.* (12.12%) in all animal originated food sample. This can reflect either the abuse of colistin sulphate, or the acquisition of colistin resistance genes while integrating other antibiotic resistance genes, if these determinants are located on the same mobile genetic element.

In the study, hemolysis pattern was observed in the screened carbapenem & colistin resistant isolates to check the pathogenicity. No study has been found in Bangladesh which reported the pathogenicity of antibiotic resistant isolates. In our study 22.91% carbapenem resistant isolates were alpha hemolytic and 16.66% isolates were β hemolytic. (Figure-3.4) In total 4 sample the highest hemolysis pattern was observed in fish followed by chicken, beef and milk. In carbapenem resistant isolates Pseudomonas spp. (6) was predominant followed by *Klebsiella spp*. (3), *E. coli*, (3) *Salmonella spp*. (2), *Yersinia spp*. (1), *Streptococcus spp*. (1), *Citrobacter spp.* (1), *Pasteurella multocida* (1) (Table- 3.4). In this study 54% colistin resistant isolates were alpha hemolytic and 46% isolates were beta hemolytic (Figure-3.6). The highest hemolysis pattern was observed in beef followed by fish, chicken and milk. In colistin resistant isolates Shigella spp. (7) was predominant followed by *Pseudomonas spp.* (7), *Klebsiella spp.* (6), *Yersinia spp.* (5), *Salmonella spp.* (4), *E. coli* (4), *Proteus spp*. (4), *Citrobacter spp.* (2), *Providencia spp*. (2), *Enterobacter spp*. (1), *Pasteurella multicoda* (1) (Table-3.5). The resistant isolates which showed alpha and β hemolysis in blood can be designated as potential pathogen which can cause infectious diseases among animal and human.

The emergence of metallo- β lactamases, ESBl and colistin resistance genes in poultry, livestock and fish production facilities may influence the spread of resistant bacteria among animals and humans. In the poultry production system, one study reported the isolation of *K. pneumonia* and *K. oxytoca* harboring NDM metallo beta-lactamases (Abdallah et al., 2015). Another study described the identification of *K. pneumoniae* carrying OXA-48, NDM and KPC type carbapenemases. Isolated strains were recovered from the liver, lungs, and trachea of broiler chicken (Hamza et al., 2016). Predominance of NDM and bla-NDM producing *Klebsiella spp*. was identified in our study where three NDM and three bla-NDM producing *Klebsiella spp.* isolates were found from cultured fish and only one NDM harboring *Klebsiella spp.* was detected in beef. From poultry meat, we have found one *Enterobacter spp.* which harbored NDM gene and bla-TEM gene respectively and one *Enterococcus spp*. isolate which harbored bla-IMP gene. Bla-NDM positive one *Pasteurella multocida* isolate were also identified in beef sample. The study by Sonia et. al (2020) reported that all the isolated of Salmonella spp. found from frozen chicken meat in their study were positive for the bla-TEM gene, 2.7% were positive for bla-CTX-M-1, and 20.3% for bla-NDM-1 whereas in our study 66.67% Salmonella spp. isolated from poultry meat sample were positive for bla-TEM and bla-NDM. In this study, prevalence of bla-CTX-M gene was identified in *E. coli, Pseudomonas spp*. and *Pasteurella multocida* in only fish and beef sample. In another study conducted in India, it was reported that bla-NDM-5 carbapenemase gene was found in one E. coli isolated from milk samples obtained from mastitic cow (Ghatak.et.al, 2013) whereas our study reported bla-NDM harboring Yersinia spp. and bla-imp harboring Pseudomonas spp. found in pasteurized packet milk. E. coli and K. pneumoniae are the most common enterobacteria harboring TEM-type β-lactamases, but their occurrence in other bacterial species is increasingly being reported. Some of these include *Enterobacter aerogenes, Morganella morganii, Proteus mirabilis, Proteus rettgeri*, and *Salmonella enterica* (Bradford.et.al, 2001). In our study, two *Klebsiella spp.* isolate from fish and chicken respectively and one *Proteus spp.* isolate from fish harbored

bla-TEM gene. Identification of NDM in isolates of different bacteria recovered from meat, milk and fish is alarming suggesting that NDM has been equally widespread both among animal- as well as human. NDM gene is considered to be originated from Indian continent particularly India, Pakistan and Bangladesh and spread to other parts of the world quite speedily (Poirel et al., 2010; Habeeb et al., 2013).

In a Swiss study, the mcr-1 gene was detected in 25.8% of retail poultry meat (chicken and turkey) from Germany (28 samples) and Italy (5 samples). The mcr-1 gene has not been found in any of a set of chicken and turkey meat samples from Switzerland, Denmark, Austria, and Hungary (Zurfluh et al., 2016). In Brazil, the presence of *E. coli* with mcr-1 was detected in 19.5% of the tested retailed chicken meat and liver samples (Monte et al., 2017). This study identified mcr-1 at a higher frequency in *Salmonella spp.* than in *E. coli*. In a Bangladeshi study by Islam. et. al showed that approximately one-third of the isolates from poultry chicken droppings carried the hazardous mcr-1 gene which resembles with our study as 30.30% gram-negative colistin resistant bacteria isolated from different animal origin food sample harbored plasmid mediated mcr-1 gene. In our study, the prevalence of mcr-1 mediated *Shigella spp.* and *Yersinia spp.* was higher in different food sample but the percentage of mcr-1 mediated *Enterobacter spp.* and *Pasteurella multocida* was 100% in food samples. Highest mcr-1 mediated gram-negative bacteria was isolated from chicken meat, (7) followed by fish (6), beef (6) and milk (1) in this study. It would be interested to further investigate the genetic background of these genes, plasmid types, and plasmid sizes, and whether all these genes are carried on the same or different plasmids.

Altogether, our data report on the presence of β -lactam and colistin resistant gene suggesting that poultry meat, beef, fish, milk might be a source of these genes. While exploring the risk factors for MDR bacterial infection in broiler chickens, Beef and fish was the potential risk factors namely ‘use of antibiotics without prescription of veterinarians’ were identified. One of the major concerns is over-the-counter sale of antibiotics without prescription that promote irrational use, overuse, and misuse of antibiotics in the animal health as well as human health sectors in most of the developing countries including Bangladesh (Hassan et al., 2021; Kalam et al., 2021; Kumar et al., 2013; Masud et al., 2020). Subsequently, indiscriminate use of antibiotics without prescription contributes to the development and spread of antimicrobial resistance (McEwen & Collignon, 2018; Singer et al., 2003). The high prevalence of multidrug-resistant bacteria and antibiotic residues in fish samples indicate frequent and indiscriminate use of antibiotics in aquaculture. The use of medicated feed is widespread in aquaculture. Avoiding overuse of feed is thus a relatively straightforward way to reduce environmental contamination with antimicrobial residues and resistant bacteria, since up to 30% of feed is unconsumed (Cabello et al. 2013). Inappetence is a typical symptom of infections in aquatic animals, so pro-viding medicated feed can be of questionable benefit unless very carefully managed (Ranjan et al. 2017). The presence of B-lactamase and colistin resistance gene in pasteurized packet milk indicated toward inadequate farm conditions, operational errors such as faulty pasteurization, and personnel related contamination which are important in terms of packet milk safety.

CHAPTER 5

Conclusion

Conclusion:

The dissemination of ESBL, carbapenemase, and colistin resistant gram-negative & gram-positive bacteria in food producing animals brought into question the real efficacy of antibiotic administration in animals in terms of treatment, prophylaxis and growth promotion. Antibiotics usage in commercial chicken, cow-farm and aquaculture production sectors was extensive in Bangladesh. Non-therapeutic usage of antibiotics in commercial chicken, cow and fish has raised significant concerns about the development of antibiotic resistance. Since antibiotic resistance is a multi-faceted problem, Bangladesh needs well-coordinated efforts through the One Health approach to combat antibiotic resistance. Government should develop and implement strict guidelines urgently for the use of antimicrobial agents in food animals. Comprehensive antibiotic administration monitoring systems can be helpful to minimize the emergence of antibiotic resistance. An extensive awareness program for farmers is crucial to reduce the unnecessary use of antibiotics in healthy chickens and cow. Intensive awareness training program for farmers, feed dealers and drug sellers may be helpful to raise awareness on good farm practices, standard biosecurity practices and their benefit, personal hygiene and the prudent use of antibiotics. Adequate laboratory diagnostic facilities need to be established at the central to the root level to help veterinarians and animal farmers for the prudent use of antibiotics. Finally, more research is required to generate more specific data on antibiotic usage in animal sectors and detect the emergence of antibiotic resistance in animals and humans.

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