Detection OF MULTIDRUG-RESISTANT *Klebsiella pneumoniae* IN CHICKEN SAMPLES COLLECTED FROM WET MARKET OF DHAKA CITY

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology

Department of Mathematics and Natural Sciences
Brac University
June 2023

Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing a 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 complete and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted for any other degree or diploma at a university or other institution.
4. We have acknowledged all primary sources of help.
5. We want to request the embargo of our thesis for 24 months from the submission date as we intend to submit our findings to a peer-reviewed journal for publication.

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Abstract

In both humans and animals, antibiotics are often used as a kind of treatment and to prevent illness. Antibiotic resistance may, however, also increase due to broad antibiotic usage. This study aimed to investigate the number of multidrug-resistant (MDR) *Klebsiella pneumoniae* in chicken samples in Dhaka. Chicken cloacal swabs and chicken breast flesh were randomly selected from seven chickens. The obtained cloacal swab and meat samples were inoculated on a HiCrome KPC agar medium for isolation and identification. After the first positive cultures, single colonies were extracted and recognized by their color. Of all the samples, 14 (100%) tested positive for *K. pneumoniae*. The antimicrobial confirmation test found that every 112 isolates of *K. pneumoniae* were an MDR (multi-drug-resistant) bacterium. Amoxicillin (92.6%), cefixime (67.2%), ciprofloxacin (64.9%), tetracycline (100%), and erythromycin (58.25%) are all resistant in antibiotic sensitivity testing. Gentamicin (90%), Meropenem (96%), Piperacillin, and tazobactam (88.5%) antibiotic sensitivity testing observed that *K. pneumoniae* was sensitive to all 112 chicken isolates examined. The proportion of resistant *K. pneumoniae* isolates from 7 chicken antibacterial agents has been confirmed by this investigation. Multidrug-resistant bacteria pose a risk to both human and animal health. Due to Bangladeshi chicken producers continued use of antibiotics without a veterinarian's oversight, there are few effective treatments for the effects of these illnesses.

Keywords: Antibiotic resistance, *Klebsiella pneumoniae*, MDR, chicken, public health

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Adrita Saha Turna, Ashraful Hussain

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List of Acronyms

ESBL	Extended-spectrum beta-lactamase
Hr	Hour
NA	Nutrient Agar
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EMA	European Medicines Agency
AMR	Antimicrobial Resistance
LB	Luria-Betarni
MDR	Multidrug resistant
CLSI	Clinical and Laboratory Standards Institute

Chapter 1

Introduction

Standard antibiotic uses include treating and preventing microbial infections in people and animals. However, extensive antibiotic usage has been linked to increased antibiotic resistance in people and animals (Kempf et al., 2015; Hayati et al., 2019). According to a collaborative study from the European Center for Disease Prevention and Control, the European Food Safety Authority, and the European Medicines Agency (ECDC/EFSA/EMA), animals consume an average of 152 mg/kg of antibiotics per kilogram more often than people (124 mg/kg). CDDEP (2015) estimates that livestock and chicken use almost two-thirds of all antibiotics generated worldwide. Small-scale commercial wet market, which need little capital investment and have been rapidly developing, particularly in rural and semi-urban regions, provide a significant contribution to the expansion of the country's economy. Like other developing nations, these wet markets are often managed by inexperienced, unqualified personnel with no biosecurity expertise (Conan, 2012). Since most of these wet markets lack effective surveillance systems and well-documented monitoring techniques, they cannot detect possible pathogenic microorganisms or other poultry risks that might substantially harm the public's health (Nahar et al., 2014). Given that these bacteria are frequent, naturally occurring microbiome residents, the rise in illnesses brought on by multidrug-resistant (MDR) Enterobacteriaceae bacteria is quite concerning (Navon-Venezia et al., 2017).

Klebsiella pneumoniae is a Gram-negative rod-shaped opportunistic pathogen that is nonmotile, encapsulated, and encased in capsules. According to Fielding et al. (2012) and AlAmmiri et al. (2016), this bacterium is a member of the Enterobacteriaceae family, a facultative anaerobe with a blue colony. Gram-negative bacteria face a significant problem due to the rise in multidrug resistance (Exner et al., 2017). According to Struve et al. (2009), four factors have been postulated for *Klebsiella* pathogenicity, including adhesives, lipopolysaccharide, siderophore, and capsular antigen, the most significant virulence factor in *K. pneumoniae*. In *K. pneumoniae* isolates, multidrug resistance (MDR) cases against more than three antibiotic classes have been documented (Hayati et al., 2019). Notably, Enterobacteriaceae, particularly strains of *K. pneumoniae* isolated from animals, are becoming more common regarding antibiotic resistance (Davis and Price 2016). The formation of *K. pneumoniae* strains carrying different resistance genes has significantly grown during the last several decades due to the widespread use of antibiotics in people, veterinary medicine, and agricultural operations. Antimicrobial drug resistance in *K. pneumoniae* has increased significantly (Wu et al., 2019).

K. pneumoniae is a bacterium that inhabits the intestines of humans. However, if it spreads to other body parts, it can cause pneumonia, bloodstream infections, meningitis, and urinary tract infections. There are numerous *K. pneumoniae* strains. Vesicles surround some cells, while others are not. Researchers have identified 77 varieties of capsular structures. Without capsules, *Klebsiella* bacteria are less infectious than those with capsules. Humans are the primary Source carriers of *K. pneumoniae* in the environment, but most humans will not become infected. Those with compromised immune systems due to medications or medical conditions are at a greater risk. Researchers report that this form of bacteria is more prevalent in specific populations, including those of Chinese descent and those with alcohol use disorder. The disease is more likely to cause certain infections in some people. In the United States, for example, *K. pneumoniae* is the most common cause of hospital-acquired pneumonia. Meanwhile, *Klebsiella* rarely causes meningitis in Western regions. In Taiwan, however, *K. pneumoniae* infection is the primary cause, accounting for 25–40% of adult cases of bacterial meningitis. Liver abscesses can cause *K. pneumoniae* meningitis in some individuals. The abscess bacteria can spread from the liver to the central nervous system.

Additionally, catheters and medical equipment can transmit *K. pneumoniae* to the urinary tract, circulation, and incisions.

Antimicrobial resistance is increasingly recognized as a worldwide health issue and is often brought on by the misuse of antibiotics in human and animal treatment (Pitout & Laupland, 2008; Widodo et al., 2020). Antimicrobial resistance (AMR) is the term used to describe the ability of germs to resist antimicrobial medications that are initially responsive. It is a normal process aided by abusing antibiotics excessively (Riwu et al., 2020). By creating enzymes that can break down antibiotics, creating resistant metabolic pathways, and altering their antimicrobial agent receptors, gram-negative bacteria have achieved this resistance (Okonko et al., 2009; Wibisono et al., 2020). There are two different kinds of antibiotic resistance mechanisms in *K. pneumoniae*. One method is the expression of ESBL, which confers monobactam and cephalosporin resistance in bacteria. The expression of carbapenemase is the second pathway, which aids in developing resistance to all obtainable β-lactams (Riwu et al., 2020). It has been discovered that the Extended Spectrum Beta-Lactamase (ESBL) gene, made up of the SHV, TEM, and CTX-M genes encoded by plasmids, affects *K. pneumonia's* resistance to several antibiotics (Ahmed et al., 2014). Antimicrobial resistance has also evolved in *Klebsiella spp.*, which is concerning in human medicine (Lynch et al., 2013). This antimicrobial treatment failure unharmed people and animals (Guardabassi et al., 2004).

K. pneumonia's antibiotic sensitivity has yet to be well investigated, particularly in chicken. Due to the need to maintain the rate at which Broiler chickens' body weight increased, their usage of antimicrobials was greater than that of laying hens. At the same time, fewer antimicrobials are required to keep laying chickens from producing eggs (Wibisono et al., 2020). In order to exactly supply the policies that will have the most significant effect, given the distinctions between the kinds, it is vital to comprehend how the incidence of resistance changes (Brower et al., 2017). This study investigated the number of multidrug-resistant (MDR) *K. pneumoniae* isolates from Dhaka's wet market.

Chapter 2

Methods & Materials

2.1 Sample Collection and Processing

This study was conducted in Dhaka, Bangladesh, at BRAC University's Department of Mathematics and Natural Sciences. The collection of samples spanned four months, beginning in November 2022 and ending in March 2023. Seven different chickens raised on wet markets in Dhaka gave a total of fourteen samples. Each sample comprised a cloacal swab and a piece of breast meat.

During the collection process, sterile cotton swab sticks were used to get cloacal swabs. When the collection was complete, the cotton swab sticks were immediately placed for transfer into a falcon filled with sterile saline water. The flesh from the chicken breast piece is placed on a clean foil paper and then wrapped. All of the samples were collected and transported to the laboratory as quickly as possible while preserving the integrity of the cold chain.

Figure 1: Sample collection from chicken

2.2 Isolation & Identification of *Klebsiella pneumoniae*

A sterile knife is used to chop the chicken breast flesh into multiple little pieces, and gloves are used to prevent contamination. The total weight of the chicken breast meat will be around 10 grams. After that, these 10 grams are poured into a beaker that contains 90 milliliters of distilled water. The sample was then processed via a digital homogenizer to make it more consistent. Before that, the digital homogenizer is cleaned with hot and soapy water to make it sterile. On the other hand, the cloacal sample was transferred to 10 milliliters of LuriaBetarni (LB) broth for bacterial growth at 37°C for four hrs.

Once the glass test tubes have been cleaned, they should be put on a rack to dilute ten times in a row. When the tenfold serial dilution process is done, clearly mark each test tube with its amount of diluted solution. Use a micropipette to evenly put one ounce of the diluted solution into each sterilized tube. Transfer 100 milliliters in the volume of the cloacal sample using a micropipette into the test tube. Mix. After multiplying by 10, this is the first dilution. When doing a second dilution by a factor of ten, use a micropipette that has been recently fitted with a fresh, sterile tip. Please proceed with the ten-fold dilution process until we reach the last tube, representing the fourth time. Similarly, the growth in LB broth may also be done as a serial dilution up to a factor of 10^-4.

After that, both dilution samples were transferred to a HiCrome KPC agar plate, where they were spread out and then re-incubated at 37°C for 24 hrs. It was done to isolate *K. pneumonia* specifically. Following the incubation period, the individual metallic blue colonies were picked up (Figure 1). After that, it was streaked on NA (Nutrient Agar) plates to produce isolate colonies and incubated at 37°C for 24 hrs.

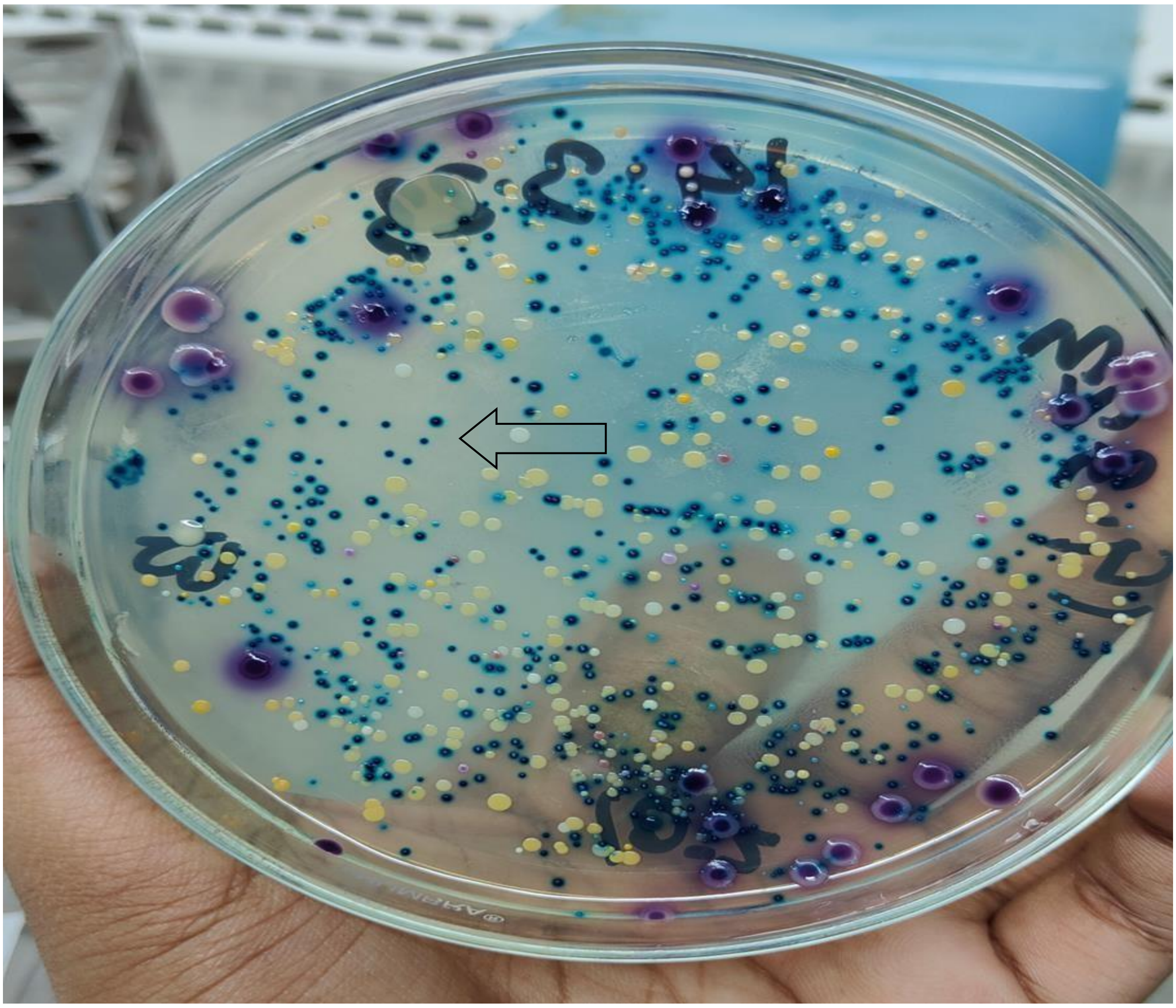

Figure 2: *Klebsiella pneumoniae* on KPC agar; grey arrow is metallic blue colony

2.3 Antimicrobial Susceptibility Testing

The disc diffusion by the Kirby-Bauer technique on Mueller-Hinton Agar was used to assess the antibiotic susceptibility of all 112 isolated *K. pneumoniae* bacteria (Effendi et al., 2019). Amoxicillin (Amx 30 µg), Cefimixe (Cfm 10 µg), Ciprofloxacin (Cip 5 µg), Gentamicin (Gen 10 µg), Meropenem (Mrp 10 µg), Piperacillin and tazobactam (Pit 75 µg), Tetracycline (TE 30 µg), Erythromycin (E 15 µg) were all present on each disk utilized in the disc diffusion test. A 0.5 McFarland standard was applied to the culture turbidity. After dipping the sterile cotton swab into the suspension was uniformly applied to the Mueller-Hinton Agar surface

(Geta et al., 2019). The inoculation plates were covered with antibiotic discs and then incubated at 37°C for 16–18 hours. The millimeter-scale zone widths of inhibition were classified as sensitive, intermediate, and resistant (Table 1) (CLSI 2020). Following the Clinical and Laboratory Standards Institute (CLSI) recommendations, samples were evaluated for antibiotic resistance.

Antimicrobial class	**Antimicrobial agent**	**Critical value**		
		Sensitive	**Intermediate**	**Resistant**
Aminoglycoside	Gentamicin	≥15	13–14	≤12
Fluoroquinolone	Ciprofloxacin	≥26	22-25	≤21
Macrolide	Erythromycin	≥13	-	≤12
Tetracyclines	Tetracycline	≥15	12–14	≤11
Cephalosporin	Cefixime	≥21	18-20	≤17
Carbapenem	Meropenem	≥23	20-22	≤19
Penicillin Combination	Piperacillin and Tazobactam	≥21	18-20	≤17
Penicillin	Amoxicillin	≥17	14-16	≤13

Table 1: Critical value for each antibiotic disks

Chapter 3

Result

3.1 Distribution of *Klebsiella pneumoniae* in different chicken samples

The results of the isolation and identification of 7 chickens with seven cloacal swabs and seven pieces of chicken meat from wet markets revealed that 14 (100%) of the samples contained *K. pneumoniae* (Table 2). The distribution of samples exhibiting growth on KPC Agar took place in Dhaka. On KPC Agar, positive samples of *K. pneumoniae* appear metallic blue with colonies.

Types of samples	**Number of samples**	**Number of *Klebsiella pneumoniae* positive sample (%)**
Cloacal Swab	7	7 (100%)
Meat	7	7 (100%)
Total	**14**	**14 (100%)**

Table 2: *Klebsiella pneumoniae* positive from different chicken samples

3.2 Antimicrobial Resistance Profile

The antimicrobial resistance profile of eight antibiotics against the *K. pneumoniae* isolates is shown in Tables 3 and Figure 2 by CLSI 2020 criteria. All 112 *K. pneumoniae* isolates tested mostly resistant to erythromycin, tetracycline, amoxicillin, ciprofloxacin, and cefixime. Contrarily, all of the isolates, respectively, showed the highest sensitivity to gentamicin, meropenem, piperacillin, and tazobactam.

Antimicrobial class	**Antimicrobial agent**	**Percentage of isolates**		
		Sensitive	**Intermediate**	**Resistant**
Aminoglycoside	Gentamicin	90%	1%	9%
Fluoroquinolone	Ciprofloxacin	0%	35.1%	64.9%
Macrolide	Erythromycin	27.46%	14.29%	58.25%
Tetracyclines	Tetracycline	0%	0%	100%

Cephalosporin	Cefixime	24.2%	8.6%	67.2%
Carbapenem	Meropenem	96%	0%	4%
Penicillin Combination	Piperacillin and Tazobactam	88.5%	4.8%	6.7%
Penicillin	Amoxicillin	5.3 %	2.1%	92.6%

Table 3: Antibiotic resistance profile of isolates

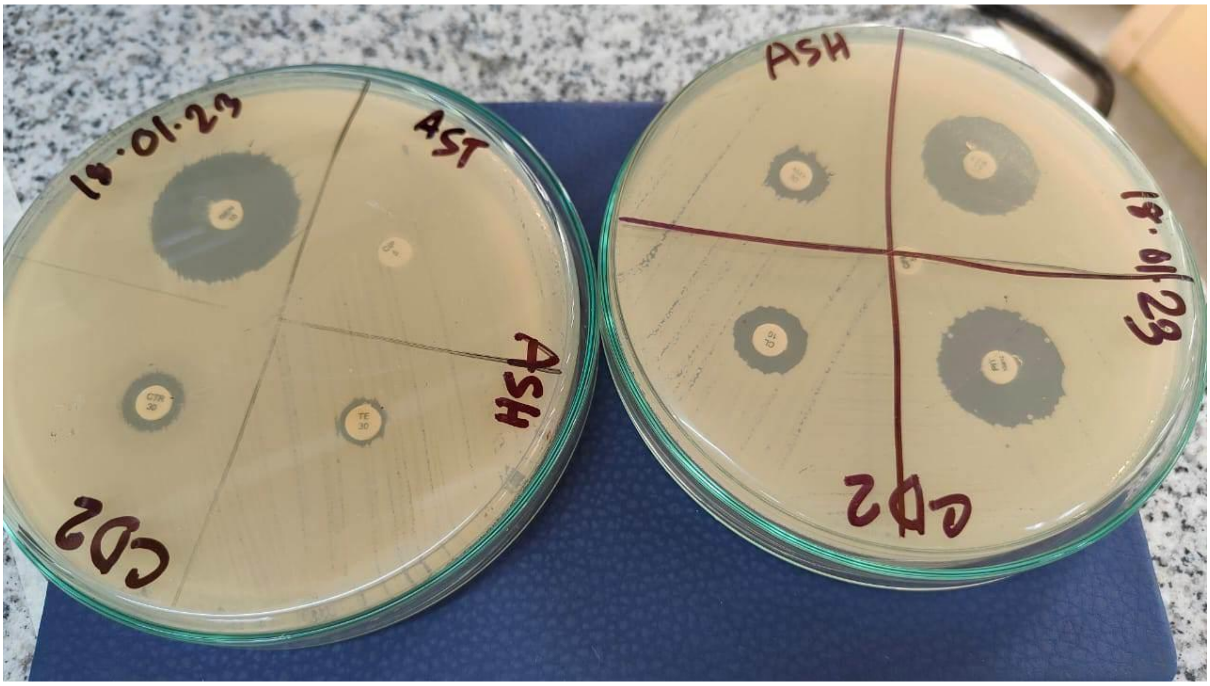

Figure 3: zone and no zone area in MHA pates.

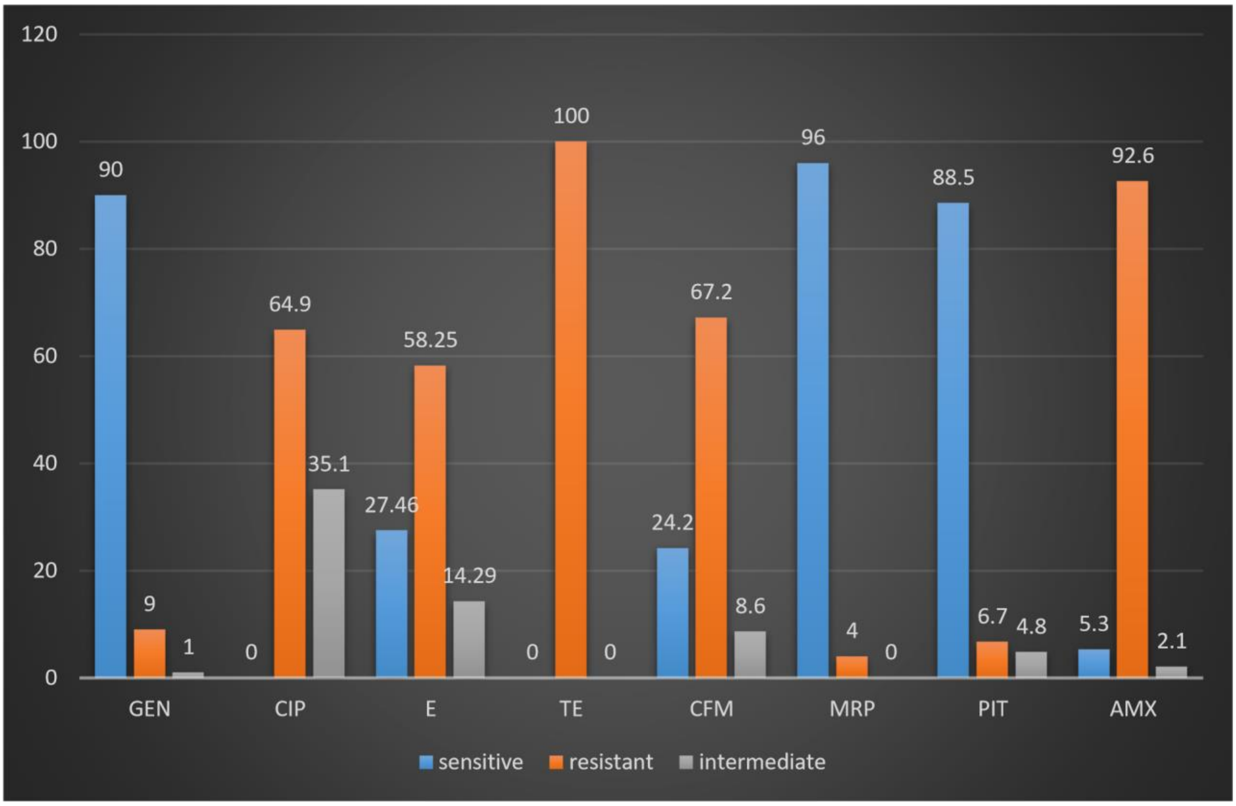

Figure 4: Percentage of antimicrobial resistance *Klebsiella pneumoniae*

3.3 Multiple Drug resistance (MDR)

Multiple-drug-resistant (MDR) isolates display resistance to at least three antibiotic categories. In this study, at least three antibiotic-resistant groups were detected in all 112 isolated *K. pneumoniae* colonies.

Chapter 4

Discussion

All (112) of the isolates of *K. pneumoniae* that underwent the antimicrobial confirmation test were MDR bacteria. *K. pneumoniae* infections that were multidrug-resistant required longer treatment times and were more challenging to treat. It is anticipated that the antibiotic resistance pattern will serve as a guide for choosing the best antibiotic for a given situation. Antibiotic resistance may result from the broad use of antibiotics without careful monitoring (Hayati, 2019). Antibiotics were once simple to get and could be administered without veterinarian oversight. In Bangladesh, up to 94.16% of chicken producers continue to use antibiotics without a veterinarian's supervision.

All (112) of the isolates were resistant to amoxicillin (92.6%), 58.25% against erythromycin, 100% against tetracycline, 64.9% Ciprofloxacin, and 67.2% against cefixime. According to Rashed et al. (2013) and Effendi et al. (2018), *Klebsiella spp.* exhibited increased amoxicillin resistance (90%). *Klebsiella spp.* did not support this conclusion. It shows resistance to amoxicillin. All studied aminoglycosides, quinolones, tetracyclines, trimethoprim, cotrimoxazole, chloramphenicol, and nitrofurantoin showed that *Klebsiella spp.* were natively susceptible to penicillin or intermediately sensitive to it (Stock & Wiedemann, 2001). These have been observed to substantially contribute to the formation of multidrug-resistant chicken isolates, whether administered alone or in combination with two or more ABs (McEwen & Fedorka-Cray, 2002). The total of 112 isolates is 90% sensitive to gentamicin, 96% to meropenem, and 88.5% to piperacillin and tazobactam.

Both animals and general people are at risk from the occurence of multidrug-resistant microorganisms. There may be few choices for treating specific illnesses. The only rules governing the use of antibiotics in food animal production for domestic use demand that antibiotics be removed before food animal products are processed (Brahmachari et al., 2013).

Additionally, the usage of an antibiotic, such as colistin, that is no longer used due to toxicity was prompted by multidrug-resistant bacteria (Fard, 2004). Building surveillance systems and monitoring chickens were two actions that may be implemented. Breeders must also practice better biosecurity. According to evidence, the pollution of soil, surface water, and groundwater resources near farms engaged in intensive broiler-raising operations may also be caused by antibiotic residues in manure (FAO, 2008). Since antimicrobial efficacy is a resource and obligation shared by all countries, policy steps should be implemented immediately to protect it (Ganguly et al., 2011). In order to avoid polluting the air, land, and water and having detrimental effects on human health, litter and manure waste must be handled appropriately in intensive production systems (Thyagarajan, 2014).

Unfortunately, no reliable statistics or information are available about using antimicrobials in chicken (Rahmahani et al., 2020). The use of antimicrobials in wet market is a well-known accelerator for developing resistance in bacteria (Wibisono et al., 2020). Compared to companion animals, the livestock industry uses antimicrobials too often. The intimate relationship between pets and people increases the danger of AMR because it creates possibilities for both commensal and pathogenic transmission in two different ways (Pomba et al., 2017). Cefotaxime, ceftazidime, and aztreonam are oxyimino-based β -lactams that may be hydrolyzed by the β-lactamase known as ESBL (Putra et al., 2019). Most *K. pneumoniae* create non-ESBL β-lactamases based on SH, such as SHV, while specific Escherichia coli make TEM, another non-ESBL β-lactamase. Ampicillin can be digested by enzymes like SHV-1 and TEM-1, whereas oxyimino cephalosporins like ceftriaxone, cefotaxime, and ceftazidime cannot. These antibiotics have been carefully crafted to work against the hydrolysis caused by these bacterial enzymes (Karanika et al., 2016; Putra et al., 2020).

This research investigates the frequency of resistance in chickens that may have used antibiotics. Due to these restrictions, the critical comparison in our research is the resistance profile of several wet markets that influence the development of resistance. Furthermore, limiting the use of antibiotics in food animal production can reduce the occuerce of antimicrobial resistance without hurting productivity, as shown by the experience of Denmark's poultry and pork industries (Levy, 2014).

Chapter 5

Conclusion

Overall, our investigation supports the conclusion that all 14 samples of chickens are positive for *K. pneumoniae* by showing metallic blue colonies on HiCrome KPC agar. This research also revealed that chicken 112 isolates had the most significant levels of resistance to the antibiotics Amoxicillin (92.6%), Cefixime (67.2%), Ciprofloxacin (64.9%), Tetracycline (100%), and Erythromycin (58.25%). All chicken 112 isolates *K. pneumoniae*, tested positive for antibiotic sensitivity to Gentamicin (90%), Meropenem (96%), Piperacillin, and tazobactam (88.5%). Multidrug-resistant microorganisms provide a risk to both human and animal health. Since chicken producers in Bangladesh continue to use antibiotics without a veterinarian's supervision, the effects of these disorders are limited and make treatment options more difficult. Adopting policy and veterinary oversight is crucial for illness
treatment and animal husbandry in Bangladesh.

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