CARBAPENEM RESISTANCE IN *ESCHERICHIA COLI* FROM RAW CHICKEN MEAT, CLOACAL SWAB, ENVIRONMENTAL SAMPLES, AND RAW VEGETABLE SAMPLES FROM KOTA BHARU KELANTAN

NURUL SYIFA BINTI SHAID (D17A0030)

DOCTOR OF VETERINARY MEDICINE

SUPERVISOR: DR. ERKIHUN AKLILU WOLDEGIORGIS

CO-SUPERVISOR: PROF. MADYA DR. MAIZAN BINTI MOHAMED

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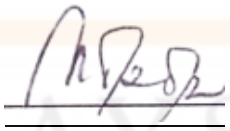
CERTIFICATION

This is to certify that we have read this research paper entitled **'Carbapenem Resistance in *Escherichia coli* from Raw Chicken Meat, Cloacal Swab, Environmental Samples and Raw Vegetable in Kota Bharu Kelantan'** by Nurul Syifa Binti Shaid and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 5436 – Research Project.

Dr. Erkihun Aklilu Woldegiorgis
DVM (AAU), M.Sc. (UPM)
Senior lecturer,
Faculty of Veterinary Medicine,
University Malaysia Kelantan
(Supervisor)



Assoc Prof Dr. Maizan Binti Mohamed
B.Sc (UKM), M.Sc. (UMP)
Ph.D. (St Andrews, UK)
Senior Lecturer,
Faculty of Veterinary Medicine
Universiti Malaysia Kelantan
(Co-supervisor)

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ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 5436 – Research Project.

This study was performed to discover the carbapenem resistance of *Escherichia coli (E. coli)* in the poultry industry and raw vegetables by detecting the Carbapenem Resistance *E. coli* (CREC) in chicken meat samples, cloacal samples, chicken farm environmental samples, and raw vegetable samples. Among 127 samples, only 18 samples were positive for the carbapenemase gene *blaOXA*-48 with 5.521% from raw chicken meat, 3.14% from environmental samples, 1.575% from cloacal sample and 3.937% from raw vegetables sample, and three of raw vegetables were positive for the *blaIMP* gene and all samples were negative for the *blaNDM*-1 gene. These indicates the emergence of CREC in the poultry industry and raw vegetables. This also shows carbapenemase gene *blaOXA*-48 is the dominant carbapenemase gene. Among this positive CREC, two isolates were resistant to Imipenem. The antibiotic resistance susceptibility test results showed that among the CREC, the highest resistance rates were observed against tetracycline (76%) and ampicillin (79%). In conclusion, there is an emergence of CREC in the poultry industry and vegetables, and that can be a possible source of CREC occurrence in humans and there is tetracycline and ampicillin drug resistance that could indicate the improper usage of the antibiotic in poultry industry without the surveillance of authority. The expected outcome may not be achieved because of lack of time for this research, low samples number and few primer used to detect the carbapenamse gene. More primer should be used to detect other Carbapenemase gene such as *blaKPC* and *blaVIM*. The detection of emergence of

CREC indicate a threat to public health and the need for standard precaution to be conducted to control the resistance toward Carbapenem.

***Keywords**: Carbapenem, Escherichia coli, poultry, raw vegetables, cloacal swab, chicken meat, CREC*

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 5436 – Projek Penyelidikan.

Kajian ini dijalankan untuk menentukan rintangan carbapenem *Escherichia coli (E. coli)* dalam industri ayam dan sayur-sayuran mentah dengan mengesan Carbapenem Resistance *E. coli* (CREC) dalam sampel daging ayam, sampel kloaka, sampel persekitaran ladang ayam, dan sampel sayuran mentah. Di antara 127 sampel, hanya 18 sampel yang positif untuk karbapenemase gen *blaOXA*-48 dengan 5.521% dari daging ayam mentah, 3.14% dari sampel persekitaran, 1.575% dari sampel kloaka dan 3.937% dari sampel sayur-sayuran mentah, dan tiga sayur-sayuran mentah positif untuk gen *blaIMP* dan semua sampel adalah negatif untuk gen *blaNDM*-1. Menunjukkan kemunculan rintangan carbapenem dalam *E. coli* dalam industri ayam dan sayur-sayuran mentah. Ini juga menunjukkan gen carbapenemase *blaOXA*-48 adalah gen carbapenemase yang dominan. Di antara CREC positif ini, dua pengasingan menunjukkan ketahanan terhadap Imipenem. Keputusan ujian kerentanan rintangan antibiotik menunjukkan bahawa di kalangan CREC, kadar rintangan tertinggi diperhatikan terhadap tetracycline (76%) dan ampicillin (79%). Kesimpulannya, terdapat kemunculan rintangan carbapenem dalam *E. coli* dalam industri ayam dan sayur-sayuran, dan yang boleh menjadi sumber kemungkinan kejadian CREC pada manusia dan terdapat ketahanan terhadap tetracycline dan ampicillin yang menunjukkan penggunaan antibiotik yang salah dalam industri ayam tanpa pengawasan pihak berkuasa. Hasil dari projek ini yang diharapkan mungkin tidak dapat dicapai kerana kekurangan masa untuk penyelidikan ini, bilangan sampel yang rendah dan sedikit jenis primer yang digunakan untuk mengesan gen

carbapenamse. Lebih banyak primer harus digunakan untuk mengesan gen Carbapenemase lain seperti *blaKPC* dan *blaVIM*. Pengesanan kemunculan CREC menunjukkan ancaman kepada kesihatan awam dan keperluan untuk langkah berjaga-jaga standard dijalankan untuk mengawal rintangan terhadap Carbapenem.

Kata kunci: *Carbapenem, Escherichia coli, ayam, sayur, calitan kloaca, daging ayam, CREC*

1.0 Introduction

Carbapenem resistance to β-lactam drugs may give rise to mechanisms that lead to resistance towards other antibiotics (Ghazali *et al*., 2020). Resistance to carbapenems is a major public health problem, as they are one of the most important antimicrobials for human therapy (WHO, 2017). Carbapenems are one of the most significant antibiotic families, and they are used as a last-resort treatment for severe infections. As a result, the rise of carbapenem resistant in bacteria in livestocks and pets become a serious public health issues (Nordmann *et al*., 2011).

Carbapenem resistance Enterobacteriaceae (CRE) is a critical developing issue of antibiotic resistance that has been rising and providing difficulties in treating infections caused by resistant pathogens. Antibiotics known as carbapenems have a wide range of activity against gram-positive and gram-negative bacteria, as well as anaerobic bacteria. (Zhanel *et al*., 2007). Therefore, these antibiotics are typically used as last choice to treat the disease caused gram negative bacteria which are already resistance towards multiple classes of antibiotics. (Zhanel *et al*., 2007; Nordmann *et al*., 2011; Patel and Bonomo, 2013).

Resistance has emerged among the Enterobacteriaceae, particularly *E. coli*, making it difficult to treat infections caused by these bacteria. *E. coli*, which is the most commonly detected CRE bacteria in the family Enterobacteriaceae, is a type of Enterobacteriaceae that has been posed a risk to human and animal's health for a long period (Prestinaci *et al*., 2015). Both clinical and foodborne *E .coli* have been showing an increase in resistance. Resistance among animal isolates increased at a greater rate than resistance among human clinical isolates from 1950 to 2002 (Tadesse *et al*., 2012). Resistance to carbapenems is mediated by several factors such as the loss of

outer membrane porins, production of carbapenemases and overexpressed efflux pumps.

In various countries, including Malaysia, CREC has risen in humans and animals. According to Aklilu *et al.* (2021), 24.36% (19/78) of live chickens in poultry farms in Kelantan were identified as CREC. This is based on phenotypic identifications, of which 17 were carbapenemase genes positive. The identification of CREC in this study indicates that it has emerged as an issue in farm animals, particularly on chicken farms, and demonstrates that these problems do not affect only humans. This has become a subject of concern since these animals spread the disease to humans and highlight the potential dangers to public health by increasing antimicrobial resistance, including CREC in food animals. Although carbapenems are not routinely used in food animal production, including poultry farming, carbapenem resistance in *E. coli* isolates may have coevolved with resistance to other antibiotics that are frequently used to treat resistant strains of bacteria that may also be spread via direct contact, insect vectors, and other animals (Bonardi & Pitino, 2019; Ahmad *et al*., 2018; Poirel *et al*., 2014).

This study aimed to isolate CREC from raw chicken meat, cloacal swabs, environmental samples, and raw vegetables in Kota Bharu Kelantan to determine antimicrobial susceptibility patterns and identify the carbapenemases genes of the isolates.

2.0 Research Problem

In Malaysia, the broiler is one of the growing sectors since most Malaysians are poultry consumers. The increase in chicken consumption is due to the increasing Malaysian population and causing an expansion of the poultry business. One of the reasons for the poultry outbreak is *E. coli* infection. Possible source of the disease could be from the surroundings in which broiler chickens are reared, the litters, or the contaminated feed. To overcome this problem and disease, antibiotics are used in poultry to control bacterial infection and promote growth. Antibiotics are administered to the whole flock instead of to individual birds. This uncontrolled antibiotic usage has led to the emergence of antibiotic resistance including carbapenem resistance in *E. coli* which cause diseases that cannot be cured, thus causing more financial fatalities due to increase in mortality of poultry in the farms. Nevertheless, lack of information has been recorded on the isolation and antibiotic susceptibility of CREC from raw chicken meat, cloacal swab, environmental samples, and raw vegetables in Kota Bharu Kelantan.

3.0 Research Question

3.1 Do carbapenem-resistant *E. coli* present in the vegetables, poultry meat cloacal swab, and environmental samples in Kota Bharu, Kelantan?

3.2 What is the antibiotic susceptibility patterns of *E. coli* isolated from the raw chicken meat, cloacal swab, environmental samples, and raw vegetable in Kota Bharu, Kelantan?

3.3 What are the common carbapenemases encoding genes in the *E. coli* isolates?

4.0 Research Hypothesis

4.1 *E. coli* isolated from vegetables, poultry meat cloacal swabs, and environmental samples in Kota Bharu are resistant to carbapenem antibiotics.

4.2 *E. coli* isolated from the vegetables, poultry meat cloacal swab, and environmental samples in Kota Bharu, Kelantan are susceptible to common antibiotics.

4.3 The carbapenemases encoding genes in the *E. coli* were isolated.

5.0 Research Objective

5.1 To isolate carbapenem resistance *E. coli* from the vegetables, poultry meat cloacal swab, and environmental sample in Kota Bharu, Kelantan.

5.2 To determine the antimicrobial resistance pattern of the carbapenem-resistant in *E. coli* isolated from the vegetables, poultry meat cloacal swab, and environmental sample in Kota Bharu, Kelantan, Malaysia.

5.3 To identify carbapenemases encoding genes in the *E. coli* isolates.

6.0 Literature review

6.1 Characteristics of *Escherichia coli* Species

Escherichia coli is a Gram-negative, facultative anaerobic, rod-shaped coliform bacteria of the genus Escherichia that is typically found in the lower intestine of warm-blooded animals. (Kaper *et al*., 2004). Because of its adaptability and variability, it is widely found in water, soil, and food (Kljujev *et al*., 2012). *E. coli* is frequently motile with flagella and is often have fimbriae. It is also a lactose fermenter bacteria and produces pink colonies on the MacConkey agar. It has characteristic biochemical reactions in biochemical tests, including indole test, urease test, TSI agar, citrate test, and MRVP test. Some of the strains produce green metallic sheen colonies on eosin methylene blue agar, and certain strains of *E. coli* produce hemolytic activity on blood agar. Serotyping is the process of identifying somatic and flagellar antigens that are used to classify organisms into primary phenotypes. Serotyping is a method of bacterial subtyping based on immunologic features of two surface structures: the lipopolysaccharide (LPS), which carries the O-antigen, and the flagella, which contains antigen. Compared to other members of Enterobacteriaceae, *E. coli* contributes for majority of urinary tract infections, newborn meningitis, sepsis, and intestinal problems (Dobrindt *et al*., 2003; Allocati, *et al*., 2013).

E. coli is a commensal bacteria found in the intestine and is found in the normal flora of animals. Extraintestinal infections or diseases of the mammary gland or urinary system are common with *E. coli*. *E. coli* colonizes animals' intestines shortly after birth from the environment and does not usually cause disease in the intestine. However, infection from drinking contaminated water or direct contact with animals infected with *E. coli* can cause pathogenic *E. coli*. Pathogenic *E. coli* have virulence

strains that cause disease in mammalian species Serogroups, pathogenicity mechanisms, clinical signs, and virulence factors classify these pathogenic *E. coli* (Kaper *et al*., 2004). Enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) are the six types of intestinal pathogens (Nataro & Kaper, 1998; Kaper *et al*., 2004).

6.2 *Escherichia coli* in Raw Poultry Meat

E. coli is the most common microorganism that can cause food poisoning (Akbar *et al*., 2014). In underdeveloped nations, *E. coli* is responsible for 25% of newborn diarrhea (WHO, 2000). Pathogens such as Salmonella, *E. coli*, and Campylobacter can be transmitted through poultry meat, red meat, sweets, and eggs (Hughes *et al*., 2007).

Akbar *et al.* (2014) reported that out of 152 poultry meat samples tested to check for *E. coli* presence, 25% (38/152) were contaminated, with three *E. coli* O157 strain samples contribute for 2% (3/152). In a comparable study in Washington, D.C., USA, Zhao *et al.* (2001) found a 38.7% frequency of *E. coli* in chicken meat. In the study by Akbar *et al*. (2014), antibiotic susceptibility and resistance patterns of *E. coli* isolates, including *E. coli* O157, were evaluated against nine routinely used antibiotics. Nine of the thirty-eight isolates (23.7%) were resistant to the antibiotic streptomycin, while 63.2 % (24/38) were resistant to ciprofloxacin. Ampicillin resistance was detected in 92.1 % (35/38) of the *E. coli* isolates, whereas tetracycline resistance was reported in the same percentage. Ampicillin and tetracycline resistance were found to be the most common. Twelve of the thirty-eight *E. coli* isolates (31.6%) tested positive for sulfamethoxazole/trimethoprim. In contrast, 47.4% (18/38) tested positive for

gentamicin, 39.5% (15/38) tested positive for chloramphenicol, and 31.6% (12/38) tested positive for nalidixic acid, and 15.8% (6/38) tested positive for kanamycin. Streptomycin and kanamycin were more active than the other antibiotics employed in the study against 38 different *E. coli* isolates from poultry flesh. The survey outcome indicates its presence in food products is an issue. Still, drug resistance among these common viruses is a more significant worry for food safety and public health. Most regularly used antibiotics were ineffective against *E. coli* isolates from the poultry meat.

Adeyanju and Ishola (2014) conducted another study to examine the prevalence and level of *E. coli* contamination in poultry meat, ground beef, and beef on the market, as well as to assess retail meat and meat preparation compliance with Turkish food codex standards and consumer health risk. In the study, 90 out of the 168 samples tested (53.6%) were contaminated with *E. coli*, including poultry meat, ground beef, and beef. *E. coli* was discovered in 49 (87.5%) of the samples of poultry meat, 27 (48.2%) of the samples of minced beef, and 14 (25%) of the samples of beef. The study concludes that retail meats and handling and processing in unsanitary and unsanitary conditions may pose significant health risks to humans. The levels of *E. coli* infection in three distinct meat groups were significantly different ($p<e<0.05$). In addition, the rates of *E. coli* in ground beef and beef were identical throughout the experiment; only *E. coli* in poultry products had a greater frequency ($p<e<0.05$).

6.3 *Escherichia coli* in Raw Vegetable

Fresh produce consumption has risen significantly due to many nutritional and functional reasons (Liu, 2003). The average amount of fresh produce consumed per person in the United States has increased by 25% in the last 30 years (Castro-Rosas *et al*., 2012). Pathogenic *E. coli* in raw fruits and vegetables in the field can be due to inadequately composted manure, contaminated water, wildlife, and contaminated harvesters. It can also happen during food preparation, such as washing, cutting, soaking, and packing (Castro-Rosas *et al*., 2012).

Escherichia coli with virulence genes was discovered in 35.3% of vegetable salads, according to Toe *et al*. (2018). The distribution for the virulent gene was 21.2% enterotoxigenic *E. coli* (ETEC), 4.9% enteropathogenic *E. coli* (EPEC), 0.7% Shiga toxigenic *E. coli* (STEC), and 7.5% enteroaggregative *E. coli* (EAC) (EAEC). According to the study, vegetable salads sold in collective catering in Abidjan are susceptible to *E. coli* pathovar infection. The study concluded the presence of possibly pathogenic *E. coli* strains, including ETEC, EAEC, EPEC, and STEC, in vegetable salads that already in packaging form that offered in collective catering in Abidjan was emphasized.

Another study by Waturangi *et al*. (2019), using multiplex PCR, revealed that 7.69% of the 65 salad vegetable samples were positive, while 11.11% of the 63 fruit samples were positive. Among these, 55 (72.37%) of the 76 isolates tested positive for aggR (EAEC), 12 (15.79%) tested positive for eae (EPEC), and 9 (11.84%) tested positive for elt (ETEC). Antibiotic resistance testing revealed 83.33% of the isolates were positive for multiple antibiotic resistance. Ampicillin (22.22%), Ciprofloxacin (11.11%), Gentamycin (33.33%), Kanamycin (38.89%), Streptomycin (55.56%),

Trimethoprim (16.67%), and Polymyxin B are all show resistance (61.11%). This concludes that regardless of the low incidence of pathogenic *E. coli*, pathogenic *E. coli* was found in a wide range of salad vegetables and fruits sold in Jakarta due to contamination from the soil, and water during planting, harvesting, and distribution. The antimicrobial sensitivity test also suggests that most *E. coli* in Jakarta's salad vegetable and fruit samples are multi-drug resistant.

6.4 *Escherichia coli* from the Cloacal Swab and Environmental Samples

Azad *et al*. (2019) reported that all cloacal swabs from broiler chickens in Bangladesh tested for *E. coli* were positive. All the bacteria were similarly resistant towards ampicillin, streptomycin, tetracycline, erythromycin, ciprofloxacin and trimethoprim-sulphamethoxazole, however the bacteria were most susceptible to colistin sulfate (73.5%), subsequently gentamicin (49.5%) and levofloxacin (17%). The comparatively high multi-drug-resistant levels among *E. coli* isolates in this investigation can be related to Bangladesh's widespread usage of antimicrobial drugs. Kevin (2021) found that 80 samples were obtained from two local marketplaces, and *E. coli* was isolated from 126 (70%) of the samples using standard bacteriological procedures. The total prevalence in the Kasubi market and Kalewe market were 84.4% and 55.6% respectively. *E. coli* was found in 51.7% of feed, 70% of litter, and 88.3% of cloacal swab samples, respectively. The study found that the isolates were susceptible to gentamicin (83.4%) and ciprofloxacin (64.5%), while tetracycline (73.8%), ampicillin (70.6%), chloramphenicol (66.7%), and nalidixic acid (66.7%) were the most resistant antibiotics (56.3%). These data confirm a significant increase in antimicrobial resistance in *E. coli*, most likely because of antibiotics as feed additives for growth promotion and inappropriate antibiotic use for disease prevention

and treatment in poultry. In addition, the findings imply that chicken litter and feed are significant sources of *E. coli* infection in birds.

6.5 Carbapenem Antimicrobials

Antibiotics such as penicillins, cephalosporins, monobactams, and carbapenems are among the most widely used antibiotics in the world. They all have the same beta-lactam ring and work by attaching to and inactivating the penicillin-binding proteins (PBPs) that help bacteria construct their cell walls. Carbapenems are bactericidal and beta-lactam antimicrobials are used to destroy bacteria producing extended spectrumase (ESBL) that causing severe infections (Hawkey *et al*., 2012). Consequently of the developing resistance to cephalosporin antibiotics in the *Enterobacteriaceae* group, antibiotic drug including meropenem, doripenem, imipenem ertapenem, panipenem, and biapenem are in use worldwide. The increase of carbapenem-destroying lactamases has recently emerged as a new resistance mechanism, leaving limited therapeutic choices (Patel & Bonomo, 2013).

Carbapenems are the antibiotic from beta-lactam family and have similar in structure to penicillins. The mode of action of carbapenem is by breaking down bacteria's cell walls and attaching to enzymes known as penicillin-binding proteins (PBPs) (Mouton *et al*., 2000). The primary inhibitory series is comprised of PBPs 1a, 1b, 2, and 3. The subsequent deadly effect is the deactivation of an inhibitor of autolytic enzymes inside the cell wall, which ultimately results in the demise of the bacteria (Sumita *et al*., 1995).

6.6 Emergence of Carbapenem Resistance in *Escherichia coli*

Carbapenems are one of the most significant antibiotic families, and they are used as a last-resort treatment for severe infections. The spread of CRE is currently

one of the critical public health threats worldwide (Sherchan *et al*., 2015), as carbapenems are among the last treatment options for severe infections caused by multidrug-resistant strains producing extended-spectrum β-lactamases (ESBLs). The reduction of outer membrane porins, the formation of carbapenemases, and the overexpression of efflux pumps all contribute to carbapenem resistance (Bhardwaj *et al*., 2015). Increased carbapenem use has come from the increased frequency of Gram-negative bacteria expressing extended-spectrum-lactamase enzymes, resulting in the emergence and expansion of carbapenemase-producing *Enterobacteriaceae* (Muller *et al*., 2018). Carbapenemases are β-lactamases that hydrolyze almost all beta-lactam antibiotics. These bacteria found in drainage and wastewater have the potential to pollute the environment and disseminate resistance genes to a wide range of species (Picao *et al*., 2013). Three different types of carbapenemases that were recognised are class A carbapenemase (KPC), class B metalloenzymes, and class D enzymes (OXA-48 type) (Nordmann *et al*., 2017).

6.7 Typing of Carbapenem-Resistant Gene in *Escherichia coli*

Typing is necessary to detect the carbapenem-resistant gene in *E. coli*. In 2014, in a hospital in Xinxiang, Henan, China , Liang *et al*. (2018) explored the prevalence of CREC and the process of carbapenem resistance occur in *E. coli*. Three carbapenem-resistant genes were picked from the study: *blaNDM*, *blaVIM*, and *blaKPC* from the study, *blaIMP*, and *blaOXA*-48 from the study, as well as other resistance genes such as *blaOXA*-1 (Queenan & Bush, 2007; Oliver *et al*., 2002). The sequencing analysis revealed *blaNDM*-1 and *blaNDM*-5 variations, with two strains showing 100% identity with the published *blaNDM*-1 gene sequence and the other two

strains belonging to *blaNDM*-5. The carbapenem-resistant genotypes *blaNDM*-1 and *blaNDM*-5 were the most frequent in Xinxiang, Henan.

7.0 Materials and Methods

7.1 Sample (*Escherichia coli* Isolate)

The *Escherichia coli* have been previously isolated from raw chicken meat samples, raw vegetable samples, and environmental samples, including the poultry feed, litter, cloacal swab, raw chicken meat, and raw vegetable purchased from the local supermarket in various locations in Kota Bharu were used for this research.

7.2 Isolation and Identification of *Escherichia coli*

The glycerol stock samples were cultured on MacConkey agar by taking the sample using a sterile wire loop and streaking the bacteria on agar and incubated under 37°C for 24 hours. After overnight incubation, a pink colony which indicates lactose fermenter bacteria was chosen and streaked on EMB agar by taking a colony of bacteria from the MacConkey agar using a sterile wire loop and cultured it on EMB agar and incubated for 24 hours under 37°C. A green metallic sheen colony presumptively confirms the isolation of *E. coli*. The isolated colony on the EMB was cultured in nutrient agar to maintain the *Escherichia coli* for other purpose including gram staining and biochemical test. The biochemical tests included are TSI, Citrate, Urease, SIM, MRVP, Catalase, and Oxidase. Gram stain was conducted to classify the colony of bacteria into shape and gram-negative or positive.

7.3 Antimicrobial Susceptibility Testing (AST)

Kirby Bauer techniques will be used to determine antimicrobial susceptibility and drug resistance patterns. A single colony from the nutrient agar plate was inoculated to a test tube which contain 10mL of 0.9 % normal saline. After that, the turbidity of the 0.5 McFarland standard and the sample was compared, and a bacterium

lawn was swabbed uniformly across Mueller-Hinton Agar (MHA). The antibiotic disc was placed on the inoculated agar with sterilized forceps. The plates were then incubated for 24 hours at 37°C. The zone of inhibition was measured and interpreted using the (CLSI, 2022) standards. Antibiotics used in this study were Ceftazidime (CAZ30), Ciprofloxacin (CIP5), Amoxicillin and clavulanate acid (AMC30), Cefotaxime (CTX30), sulfamethoxazole/ trimethoprim (SXT25), aztreonam (ATM30), Nalidixic acid(NA30), Ceftazidime (CAZ30), Tetracycline (TE30), Ampicillin (AMP10), Chrolamphenicol (C30), and Carbapenem antibiotics which are Meropenem (MEM10) and Imipenem (IMP10).

7.4 Carbapenem Inactivation Method (CIM) Test

A suspension was created by suspending colony of bacteria from the nutrient agar by using inoculation loop into 400 µL of sterile distilled water. A 10 µg meropenem disc was then put in the suspension. The suspension was then incubated for at least two hours at 35°C. A suspension of OD595 1.25 (corresponds to a McFarland value of 0.5) smeared uniformly with a sterile cotton swab was used to inoculate the Mueller-Hinton agar plate with the indicator strain. Following incubation, the disc was removed from the suspension and set on a Mueller-Hinton agar plate inoculated with susceptible *E. coli* indicator strain (ATCC 29522), which was then incubated at 35° C.

7.5 Detection of Carbapenem-Resistant *Escherichia coli* by Polymerase chain reaction

7.5.1 DNA Extraction

Genomic DNA was extracted by using the boiling method. Two colonies from nutrient agar were taken using inoculating loop into a 1.5mL microcentrifuge tube containing 1000µl of NaCl. The tube was centrifuged (12000 rpm) for 5 minutes. The supernatant was discarded, and the pellet was suspended in 500µL nuclease water and was vortex vigorously. The tube was then boiled in a 100°C water bath for 15 minutes and immediately cooled afterward for 10 minutes. Then, the tube was centrifuged again at 12000 rpm for 5 minutes. Lastly, 480µL of the supernatant was moved into another 1.5 ml microcentrifuge tube using a micropipette and kept at -20°C conditions.

7.5.2 DNA Amplification

PCR reaction mixture was prepared in 1.5ml microcentrifuge tube before adding the samples. 45µl of the reaction mixture was then aliquoted into PCR tubes. PCR Mastermix (Promega, USA) that was used to prepare the reaction mixture. The reagent concentration and and volumes for a single PCR reactions are contain 25µl PCR Master mix, three µl forward primers, three µl reverse primers, 14 µl sterile nuclease free water. 5 µl DNA template was added to each tube. The following primer sequences (Table 1) were used to confirm *Escherichia coli* species and detect carbapenemases genes in *E. coli* (Poirel *et al*., 2011).

Table 1. List of primers for confirmation of *E. coli* species and to detect the presence of carbapenemases genes

Primer	Sequence* (5'-3')	Gene	Product size (bp)
PhoA-F	GTGACAAAAGCCCGGACACCATAAATGCCT	*phoA*	903
PhoA-R	TACACTGTCATTACGTTGCGGATTTGGCGT		
NDM-1-F	GGTTTGGCGATCTGGTTTTC	*blaNDM-1*	621
NDM-1-R	CGGAATGGCTCATCACGATC		
OXA-48-F	GCGTGGTTAAGGATGAACAC	*blaOXA-48*	438
OXA-48-R	CATCAAGTTCAACCCAACCG		
IMP-F	GGAATAGAGTGGCTTAAYTCTC	*blaIMP*	232
IMP-R	GGTTTAAYAAAACAACCACC		

The following thermal cycling conditions were used for amplification of *E. coli* gene: Pre-denaturation at 94°C for 2 mins, then 35 cycles: 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and 72°C for 10 mins of final elongation.

The following thermal cycling conditions were used for amplification of Carbapenem resistant genes: Pre-denaturation at 95°C for 5 min, then 30 cycles: 95°C for 45 sec, 56°C for 1 min, 72°C for 2 min, and 72°C for 5 min of final elongation (Candan & Aksöz, 2017).

Electrophoresis of PCR products was done in a 1.8 % agarose gel at 100 V for 45 minutes in 1xTBE (89 mM Tris, 89 mM Boric Acid, and 2 mM EDTA) containing Midori green, and images were acquired using Gel Doc™ EZ Imager (Vio-Rad, USA).

7.6 Data Analysis

The data were then recorded and analyse by using descriptive analysis. The data were entered into Microsoft Excel spreadsheet for statistical analysis. The data were sorted and checked for consistency and duplication. The data focused on sets of samples that showed result of the prevalence of CREC and the antimicrobial resistance and antimicrobial susceptibility. We have classified the result of prevalence of CREC and the antimicrobial susceptibility test as resistance, susceptible and intermediate and categorized the antimicrobials into their classes and further identified the antimicrobial that show highest resistance. Classes of antimicrobials included were Ceftazidime (CAZ30), Ciprofloxacin (CIP5), Amoxicillin and clavulanate acid (AMC30), Cefotaxime (CTX30), sulfamethoxazole/ trimethoprim (SXT25), aztreonam (ATM30), Nalidixic acid(NA30), Ceftazidime (CAZ30), Tetracycline (TE30), Ampicillin (AMP10), Chrolamphenicol (C30), and Carbapenem antibiotics which are Meropenem (MEM10) and Imipenem (IMP10). Prevalence of CREC were compared between four the different isolate samples which are cloacal samples, raw chicken meat, raw vegetable and environmental samples. Descriptive statistics for association between AMR and prevalence of CREC with different samples was performed (Elmi *et al*., 2021).

8.0 Results

8.1 Bacterial Isolation and Identification

All 127 samples of chicken meat, cloacal swab, raw vegetable, and environmental samples from the glycerol stock isolated on the MacConkey agar show pink colony growth as in figure 1, which indicates lactose fermenting bacteria. The colony from the MacConkey agar was isolated on EMB agar. All the bacteria isolated show green metallic green sheen as in figure 2, which confirms the isolation of *E. coli*.

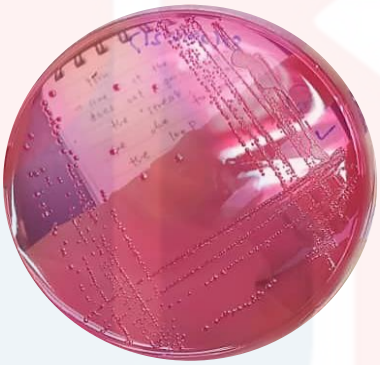


Figure 1: Pink colonies on MacConkey agar

Figure 2: The green metallic sheen colonies of *E. coli* on EMB agar

The isolated colony on the EMB were subcultured on nutrient agar to maintain the *Escherichia coli* for other purposes, including gram staining and biochemical test.

Upon gram staining of the isolated colony on the nutrient agar, gram-negative bacilli bacteria were observed under magnification of x1000, as shown in figure 3.

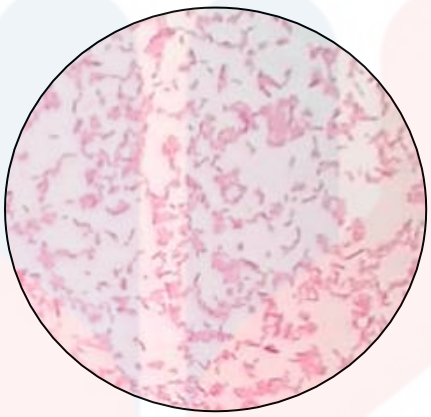


Figure 3: gram-negative bacilli bacteria under x1000 magnification

The biochemical test show result of positive catalase test, negative coagulase test, negative oxidase test, negative indole rest, positive methyl red test, negative VP test, acid/ acid and gas production in TSI test, negative urease test, and negative citrate test, no H_2S production and motile in SIM test.

8.2 Molecular Detection of *E. coli* gene (*phoA*)

All the 127 samples that were confirmed as *E. coli* were subjected to molecular confirmation of *E. coli* targeting the *pho*A gene using monoplex PCR. All the samples were confirmed positive for the *E. coli* species-specific gene.

8.3 PCR results for Carbapenemase genes in *Escherichia coli*

As for the detection of the Carbapenemase gene (*blaNDM*-1), all the isolates were confirmed to be negative for this gene and for the detection of Carbapenemase genes (*blaIMP* and *blaOXA*-48), 18 samples were positive for *blaOXA*-48, and three of them were also positive *blaIMP* carbapenemase genes. Raw chicken meat samples showed the highest positive results with seven samples were positive for carbapenemase gene, *blaOXA*-48 (figure 4 and table 2).

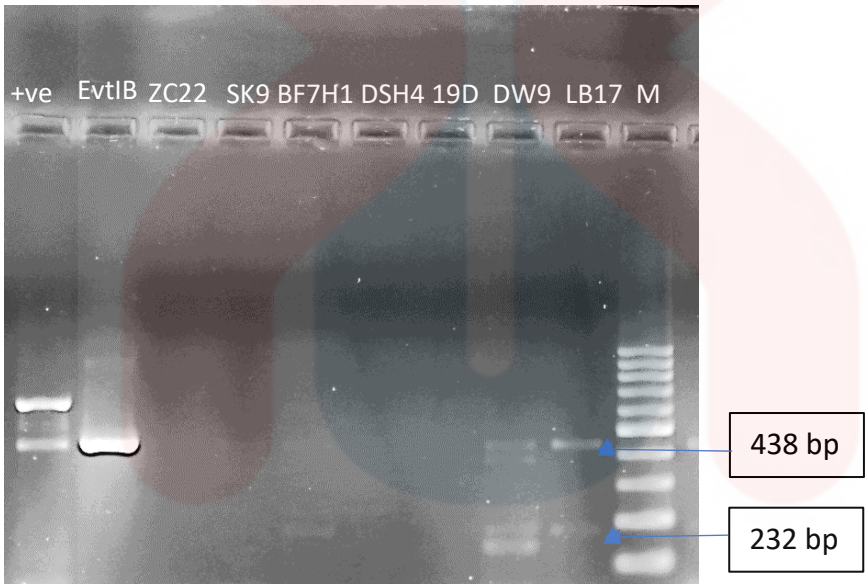


Figure 4: 18 positive results for carbapenemase gene (*blaOXA*-48) at approximately 434 bp and three positive results positive for carbapanemase gene (*blaIMP*) at approximately 232 bp.

Keys: +ve = positive control for Carbapenemase gene (*blaOXA*-48) and (*blaNDM*-1), M= DNA ladder 100bp (Marker)

Table 2: Occurrence of Carbapenemase genes in the *E. coli* isolates from the samples.

Carbapenemase genes	Raw vegetables (n=28)	Raw chicken meat (n=35)
blaOXA-48	2 (1.575)	7 (5.512)
blaIMP+blaOXA-48	3 (2.362)	0

Carbapenemase genes	Cloacal (n=32)	Environmental (n=32)
blaOXA-48	2 (1.575)	4 (3.15)
blaIMP+blaOXA-48	0	0

8.4 Antibiotic Susceptibility Test (AST)

Escherichia coli isolated from the raw chicken meat, cloacal swab, and environmental samples showed the highest resistance towards Ampicillin (AMP10) which is 95.65%, Tetracycline (TE30), which is 95.65%, Chloramphenicol (C30) which is 93.48%, Sulfamethoxazole/ trimethoprim (STX25) which is 90.22% followed by Nalidixic acid (NA30) which is 79.35%. Compared with the three types of samples mentioned above, raw vegetable samples showed a lower percentage of antibiotic resistance. Particularly in carbapenem antibiotics, there are two isolates showing resistance towards Imipenem (IMP10); one is isolated from the raw chicken meat while the other is isolated from cloacal swab. There is no resistance towards Meropenem (MEM10) observed. Meanwhile, the isolates from the raw chicken meat showed the highest susceptible result towards Imipenem (84.78%), Meropenem (90.22%), Aztreonam (83.70%) and Ceftazidime (72.83%) and isolates from

vegetable sample showed higher susceptible result towards more different antibiotic compare to the other group of samples (Table 3 & 4).

Table 3: Antimicrobial susceptibility patterns of *E. coli* isolated from raw vegetables and raw chicken meat.

Antibiotic discs	**Raw Vegetable (n=28)**		**Raw Chicken Meat (n=35)**	
Concentration - µg	R (%)	S (%)	R (%)	S (%)
CAZ (30µg)	0	25(89.29)	5 (14.29)	27 (77.14)
MEM (10µg)	0	26 (92.86)	0	29 (82.86)
C (30µg)	5 (18.52)	22 (78.57)	32 (91.42)	3 (8.57)
CIP (5µg)	11 (40.74)	6 (21.43)	32 (91.42)	2 (5.714)
IMP (10µg)	0	21 (75)	1 (2.857)	27 (77.14)
AMP (10µg)	7 (25.93)	11 (39.29)	33 (94.29)	2 (0.057)
AMC (30µg)	1 (3.704)	21 (75)	4 (11.42)	19 (54.29)
TE (30µg)	6 (20.22)	21 (75)	33 (94.29)	2 (0.057)
CTX (30µg)	3 (10.71)	15 (42.86)	12 (34.29)	10 (28.57)
SXT (25µg)	3 (10.71)	22 (78.57)	30 (85.71)	5 (14.29)
ATM (30µg)	1 (3.57)	25 (89.29)	4 (11.42)	30 (85.71)
NA (30µg)	1 (3.57)	20 (71.43)	27 (77.14)	4 (11.42)

Table 4: Antimicrobial susceptibility patterns of *E. coli* isolated from cloacal swab and environmental samples.

Antibiotic discs	**Cloacal swab (n=32)**		**Environmental (n=25)**	
Concentration - µg	R (%)	S (%)	R (%)	S (%)
CAZ (30µg)	2 (6.25)	24 (75)	3 (12)	16 (64)
MEM (10µg)	0	30 (93.75)	0	24 (96)

C (30µg)	29 (82.86)	1 (3.125)	24 (96)	0
CIP (5µg)	29 (82.86)	1 (3.125)	25 (100)	0
IPM (10µg)	1 (3.125)	26 (81.25)	0	25(100)
AMP (10µg)	30 (93.75)	0	25 (100)	0
AMC (30µg)	5 (15.63)	16 (50)	8 (32)	9 (36)
TE (30µg)	30 (93.75)	0	25 (100)	0
CTX (30µg)	7 (21.88)	18 (56.25)	11 (44)	8 (32)
SXT (25µg)	28 (87.5)	2 (6.25)	25 (100)	0
ATM (30µg)	1 (3.125)	28 (87.5)	3 (12)	19 (59.38)
NA (30µg)	23 (71.88)	1 (3.125)	23 (92)	1 (4)

9.0 Discussion

Escherichia coli is bacteria commonly found in the chicken's intestine that can cause gastrointestinal diseases such as diarrhea and can lead to food poisoning in humans that consume the infected poultry meat. The carbapenem resistance in *E. coli* is present in poultry even though the carbapenem antibiotic is not commonly used in the poultry industry. This could be caused by the evolution of resistance to other antibiotic resistance genes in the poultry because of the usage of different antibiotics in chicken. CREC in chicken is a problem for public health as consuming the chicken meat with carbapenem resistance potentially lead to antibiotic resistance in humans.

In this study, 14.17% (18/127) of the *E. coli* isolates were positive for *blaOXA*, and three of the samples were positive for both *blaOXA* and *blaIMP*. The most dominant gene-positive for the carbapenemase gene is *blaOXA*, while all samples were negative for *blaNDM*. Among this carbapenem resistance sample, only two isolates show resistance toward Imipenem and no resistance toward the Meropenem. This can happen due to absence of gene resistance expression that does not show resistance to the antibiotic clinically. The unexpressed gene, resulting in susceptible result in AST because of the low level of Carbapenem minimum inhibitory concentration (MIC), are encoded by genes that are horizontally transferable by plasmids or transposons and are commonly associated with genes encoding for other resistance determinants (Meletis, 2015). In addition, a study by Quale *et al*. (2006) showed that has been demonstrated that they have a weak capability for carbapenem hydrolysis, and their excessive production may result in carbapenem resistance together with decreased outer-membrane permeability or overexpression of the efflux pump, which indicate that the carbapenem gene detection can indicate the potential of the gene to be fully expressed and to be clinically resistant to carbapenem antibiotics. These differences can be

attributable to the tests' various degrees of selective capability. When compared to MIC analysis and PCR, disc diffusion antimicrobial susceptibility testing is generally the least accurate method. A combination of molecular and culture-based techniques is necessary for accurate monitoring of CRE from animals because both phenotypic and molecular detection and characterization of CRE have their limits (Köck *et al*., 2018).

Escherichia coli isolated from the raw chicken meat, cloacal swab, and environmental samples showed the highest resistance toward Ampicillin (95.65%) and Tetracycline (95.65%). This is supported by a study done by Racewicz *et al*. (2022) in Poland that showed *E. coli* isolates were the most resistant to tetracyclines (96.6%) and penicillins (100%). In another study by Akbar *et al*. (2014), Ampicillin resistance was detected in 92.1% (35/38) of the *E. coli* isolates, whereas tetracycline resistance was reported in the same percentage. Ampicillin and tetracycline resistance were found to be the most common. Another study by Ghazali *et al.* (2020) in Terengganu, Malaysia showed 100% of the cloacal samples were resistance toward penicillin antibiotic. Meanwhile, the isolates from the raw chicken meat showed the highest susceptible result towards Meropenem (90.22%), Imipenem (84.78%), Aztreonam (83.70%) and Ceftazidime (72.83%) and isolates from vegetable sample showed higher susceptible result towards more different of antibiotic compare to the other group of samples. This is consistent with a study by Mgaya *et al*. (2021) stated that Cefotaxime, and particularly imipenem, which are not frequently used, were less resistant (Table 3 & 4).

These data might indicate antibiotics as feed additives for growth promotion and inappropriate antibiotic use for disease prevention and treatment in poultry. In

addition, the findings imply that chicken litter and feed are significant sources of *E. coli* infection in birds.

In comparison to these three samples, vegetable samples showed fewer samples of *E. coli* exhibiting resistance toward tetracycline (20.22%) and ampicillin (25.93%). This is supported by a study done by Richter *et al*. (2020) in South Africa, which showed that *E. coli* from the vegetable samples with resistance to ampicillin (38.81%) and tetracycline (19.40%). The presence of pathogenic *E. coli* in raw fruits and vegetables in the field can be due to inadequately composted manure, contaminated water, wildlife, and contaminated harvesters. It can also happen during the preparation, such as washing, slicing, soaking, and packing of food (Castro-Rosas *et al*., 2012). These results reveal the need of monitoring fresh fruits and vegetables production in both formal and informal marketplaces, since these products can be a source of multidrug-resistant bacteria with antibiotic resistance and virulence genes, posing a health concern to human.

All the isolates that were positive for carbapenemase gene in PCR is negative for CIM. This is contradicted with a study done by Zwaluw *et al*. (2015), which reveals 70 out of 283 *Enterobacteriaceae* (24.7%) were shown to carry a carbapenemase encoding gene by PCR and all PCR-positives (100%) were shown to produce carbapenemase by the CIM. Weak expression of carbapenemase gene would result in low-level carbapenemase activity around the detection-threshold of CIM (Zwaluw *et al*., 2015), explaining the contradiction between the two test performed with this isolate as well as the AST test in this study which show no carbapenem resistance.

10.0 Conclusion

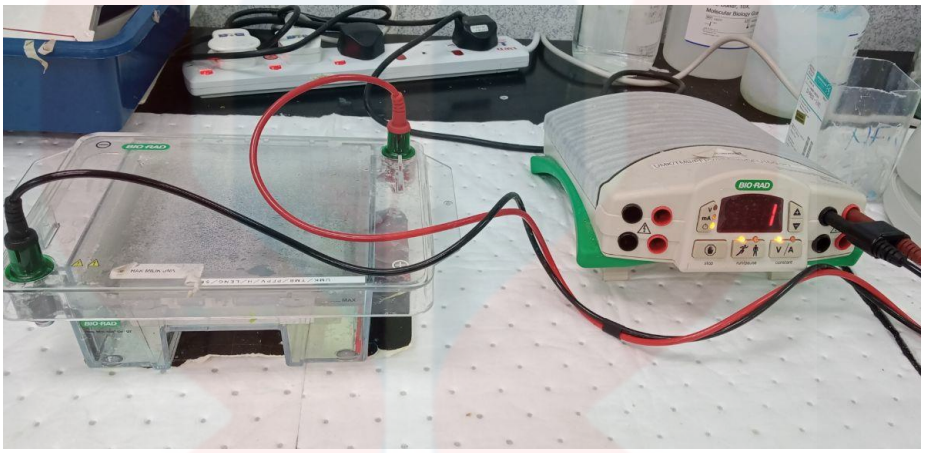
Carbapenem resistance was discovered in a poultry farm in Kota Bharu, Kelantan, Malaysia. According to this study, 18 (14.17%) of the 127 *E. coli* isolates tested positive for the *blaOXA*-48 carbapenemase gene, and three (2.36%) of the isolates tested positive for *blaIMP*, indicating that the *blaOXA*-48 gene is the most dominant gene among the positive isolates, with just two showing resistance to Imipenem. This suggests that carbapenem resistance is evolving in chicken farms, even though carbapenem antibiotics are not routinely used in the poultry sector, posing a public health risk because carbapenem antibiotics are considered the last line of antibiotic defence. Most isolates have high resistance toward Ampicillin and Tetracycline, which indicates the possibility of unsupervised antibiotic usage for disease prevention and treatment, growth promoter in poultry farms, and the vegetable can be contaminated during the preparation to a retail shop.

11.0 Recommendation

To learn more about the prevalence of carbapenem resistance in *E. coli* in Malaysia, more research with a larger number of samples can be performed, which could aid in the monitoring of the establishment of this antibiotic resistance and, in turn, contribute to the control of carbapenem resistance. Other than that, antibiotic usage should also be monitored. The veterinarian should educate the owner about the dangers of improper antibiotic use and keep the farmer up to speed on Malaysia's prohibited antibiotics. Food safety standards must be strengthened throughout the raw vegetable supply chain, from the farm to the retail store. Surveillance of fresh vegetable production is also necessary to identify possible causes of contamination that influence the prevalence and spread of antibiotic resistance.

12.0 Appendices

Appendix A- Electrophoresis procedure



Appendix B- MacConkey agar preparation

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