ORIGINAL ARTICLE

# Antibiotic Resistant *Escherichia coli* Isolated from Faecal Samples of Cow at Livestock Farm in Kedah, Malaysia

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## ABSTRACT

**Introduction:** *Escherichia coli* is a typical microflora found in the intestines of livestock, but regular exposure to antibiotics puts them under selection pressure to acquire antibiotic resistance. This study aimed to identify and characterise the antibiotic resistance profile of *E. coli* isolated in cow faeces collected from Tobiar Modern farm located in Kedah, Malaysia. **Materials and methods:** The antibiotic susceptibility test (AST) was conducted to assess the sensitivity of *E. coli* isolates to tetracycline (30 µg/mL), erythromycin (15 µg/mL) and ampicillin (10 µg/mL) using disk diffusion method followed by Minimum Inhibitory Concentration (MIC) assay. For molecular identification of selected resistant isolates, 16S rDNA gene sequencing was carried out. **Results:** Six (Isolates A1, P1, P2, P3, P4, and P5) out of 30 isolates were identified as *E. coli* based on their colonial morphological characteristics. The tests for catalase, indole, MR, TSI, and lactose fermentation all yielded positive results for the isolates, whereas the tests for oxidase, citrate, and VP yielded negative results. All six isolates were found to be erythromycin resistant. The Isolate P4 was observed as a multidrug resistant (MDR) bacterial strain since it exhibited resistance to all tested antibiotics. The MDR Isolate P4 is identified as *E. coli* strain LWY24 using molecular identification with a 99.7% identity rate. **Conclusion:** This study offers important preliminary information on the incidence of antibiotic-resistant bacteria (ARB) on this particular local livestock farm. This data is useful for developing plans to reduce the prevalence of ARB in livestock.
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## INTRODUCTION

*Escherichia coli* is a Gram-negative, glucose-fermenting, and facultative bacterium from the Enterobacteriaceae family (1). Within the livestock setting, transmission of *E. coli* to humans can occur through indirect or direct contact with an animal, contaminated water and food, and person-to-person contact among workers (2). In susceptible individuals, it can cause a wide range of health issues such as haemolytic-uremic syndrome and bloody diarrhoea (3). As the guts of the livestock is an ecological niche for *E. coli*, frequent exposure to antibiotics puts them under increased selection pressure to acquire antibiotic resistance (AR) (4). According to the United States Department of Agriculture (USDA), antibiotics are helpful in treating and preventing diseases or epidemics when used at therapeutic doses (5). Additionally, subtherapeutic use of antibiotics can accelerate market weight increase while requiring less feed by increasing nutrient absorption and reducing the growth of competing bacteria (6). However, antibiotic overuse or misuse in animal feed results in the development of bacterial multidrug resistance, subsequently lowering the therapeutic efficacy of routinely used antibiotics in human and veterinary treatment (3). AR occurs when bacteria develop resistance to antibiotics, allowing them to multiply and grow even in the presence of antibiotics.

According to the Ministry of Health Malaysia (MOH), ampicillin resistance in E. coli has been found to remain as high as 68.6% in Malaysia, although the rate of resistance in *E. coli* to other various antibiotics such as cefotaxime, gentamicin, and ciprofloxacin decreased in 2020 compared to 2019 in local clinical settings (7). However, this is still a cause for concern, as ampicillin are frequently used as a first-line antibiotic to treat infections like pneumonia, ear infections and *E. coli* infections (8).

Antibiotic resistance can be defined as the capability of the bacterium to proliferate and evolve in the presence of an antibiotic (9). Commonly, illnesses become untreatable and sometimes lethal without effective antibiotics, causing risks to public health and food security (9). Bacteria can resist the action of antibiotics due to intrinsic (natural) or acquired resistance mechanisms. Intrinsic resistance is the natural innate ability of a specific bacteria to resist certain antibiotics (10). Gram-negative bacteria possess intrinsic resistance against specific antibiotic compounds because it has an outer membrane layer that is not permeable for the antibiotic to pass and reach target sites within the cell (10). An acquired resistance mechanism is when the bacteria acquire resistance genes through chromosomal mutation or horizontal gene transfer (conjugation, transduction, and transformation). An acquired resistance mechanism traits include conformational changes of the target site, ability to extrude antibiotic molecules via efflux pump, changes in the permeability of the cell wall that decrease antibiotic uptake and degradation or enzymatic modification of the antibiotics (10).

Based on the data from the Centers for Disease Control and Prevention (CDC), antimicrobial resistance (AMR) gives rise to at least 2,049,442 infections and 23,000 mortality cases in the United States (11). Resistant bacteria that cause infections keep rising yearly, with an estimated 50,000 people dying each year in the United States and Europe (12). The WHO has recognized *Acinetobacter baumannii, Pseudomonas aeruginosa,* and Enterobacteriaceae as the highest priority pathogens that must be controlled to prevent AR (13). The WHO has devised a global action plan to reduce the risk of AR. One of the focuses is by implementing proper antibiotic use to control and eliminate antibiotic-resistant infections. This includes raising public knowledge and understanding of AR, especially among healthcare staff, people involved in food and livestock production, also those engaging in aquaculture. AR must be addressed through surveillance, monitoring and research to provide knowledge on antimicrobial concerns and the possible threat of AR, as stated by the WHO (9).

Antibiotics have been introduced to be used in animal feeds for almost 50 years (1). The applications include as a growth promoter, treatment for sick animals and for eliminating or inhibiting the occurrence of infectious disease. Typically, the β-lactams such as penicillin, cephalosporin, and carbapenems are used in ruminants to prevent diseases such as anthrax, streptococcal mastitis and keratoconjunctivitis (14). Moreover, tetracyclines, streptomycin and bacitracin are commonly used as additives in feed for livestock in the attempt to increase growth rate efficiency. Other than that, polymyxins are used in calves to prevent colibacillosis and salmonellosis (15). Consequently, the bacteria in the livestock may develop resistance after receiving antibiotics for an extended period. These bacteria multiply rapidly within the livestock and can spread to other animals through interactions, leading to the formation of other sub-population or ecological niches of antibiotic-resistant bacteria (16). For example, faecal matters of livestock frequently carry resistant bacterial pathogens that can be easily spread to the soils within the sheds (15).

An AR is a global public health problem that needs to be tackled. This study provides baseline data regarding antibiotic resistance in a specific livestock farm in Kedah. Understanding the circumstances, variables, and practices that lead to antibiotic resistance is essential as it can assist in identifying places or areas where action is needed to improve the quality and safety of livestock meat and dairy products from this farm. Moreover, selected resistant *E. coli* strains that may be linked to zoonotic disease and foodborne infections will be identified.

## MATERIALS AND METHODS

### Bacterial Isolation and Identification

The raw material used in this research study were cow faecal samples collected from Tobiar Modern Farm. It is a local cattle farm in Pendang, Kedah with approximately 120 number of cows. Serial dilution and spread plate technique was performed to isolate bacteria from the faecal samples. Selective media (Eosin Methylene Blue (EMB) agar and MacConkey agar) were used for the isolation of *E. coli*, followed by Gram staining and various biochemical assays including catalase, citrate, Methyl Red and Vogues-Proskauer (MRVP), oxidase and triple sugar ion (TSI) test. Glycerol stocks of positive isolates were prepared and kept at -80°C for future usage.

### Antibiotic Susceptibility Testing (AST)

The AST was conducted using disk diffusion assay following the Clinical Laboratory Standards Institute (CLSI) guidelines. The first step was made with the preparation of inoculum, in which the suspension was adjusted to a 0.5 McFarland turbidity standard. A prepared inoculum suspension was applied to Mueller Hinton Agar (MHA) using a sterilised cotton swab. To ensure a uniform dispersion of inoculum on the MHA, the swab was streaked four times while rotating the plate by 60°C each time. Paper discs with a known concentration of antibiotics; gentamicin (10 µg), ampicillin (10 µg),

erythromycin (15 µg) and tetracycline (30 µg) were placed on the agar surface using sterile forceps. Each disc was placed within equal distances from the other disc and pressed to the agar's surface to prevent disc displacement. The plate was inverted and incubated with agar side up at 37°C overnight (16 to 18 hours). The size of the inhibition zone (IZ) was then measured and described in accordance with recommendations provided by the Clinical Laboratory Standards Institute (17).

**Minimum Inhibitory Concentration (MIC)**

The experiment was carried out in 96-well microtiter plates. Each well received a total of 100 µL of medium (MHB), with the exception of Column 12, which received 200 µL as a sterility control. A total of 100 µL bacterial suspension was added in Column 11 as a growth control. After that, 100 µL of antibiotic was added to Column 1, and a dilution step was taken. Then 100 µL of mixture from Column 1 was transferred to Column 2, and the dilution was followed until it reached Column 10. Then, 100 µL of bacterial suspension was added to Column 1 to 10. The same steps were repeated for other antibiotics. The 96-well microtiter plate was incubated for 18 to 24 hours at 37 °C. Lastly, turbidity was observed as an indicator of the bacterial growth. The MIC was defined as the lowest concentration of the antibiotics that inhibited growth of bacteria (18).

**Molecular Identification of Resistant Isolates**

Genomic extraction was conducted using DNeasy Blood and Easy Tissue Kit (Qiagen, USA) following the protocol provided. The 16S rDNA gene amplification was conducted using BioMix Red (Bioline, UK). The universal primer pair 27F and 1492R was used with the following thermal profile: initial denaturation at 94oC for 2 min, 30 PCR cycles (94°C, 1 min; 55°C, 1 min; 72°C, 30 sec; 1 cycle of 4 min at 72°C and preservation at 4°C). The PCR amplicons were sent for sequencing and obtained 16S rDNA gene sequences were analysed by using BLAST nucleotide search program (BLASTn).

## RESULT

**Isolation and Identification of *E. coli***

Faecal samples of cows were collected from two different areas within the same holding pen at the sampling site. From the nutrient agar plate, a total of 30 isolates were picked and reinoculated onto selective and differential media, which are the MacConkey and EMB Agar. A total of 15 colonies from each plate that were observed to show the colonial morphological characteristics of *E. coli* (greyish white, large, thick, moist, and translucent discs) were picked and reinoculated onto MacConkey Agar. After 24 hours of incubation, only six out of 30 bacterial isolates showed pink coloured colonies; one isolate derived from sample 1 and denoted as A1, while another five isolates derived from sample 2 and denoted as P1, P2, P3, P4 and P5 (Fig. 1). For validation, all

**Fig 1: Colony morphology of bacterial isolate A1 originated from sample 1. A1 showing pink-coloured colonies on MacConkey Agar, indicating that the bacteria are able to ferment lactose. All other positive isolates from sample 2 (Isolates P1, P2, P3, P4, and P5) show the same colonial morphology.**

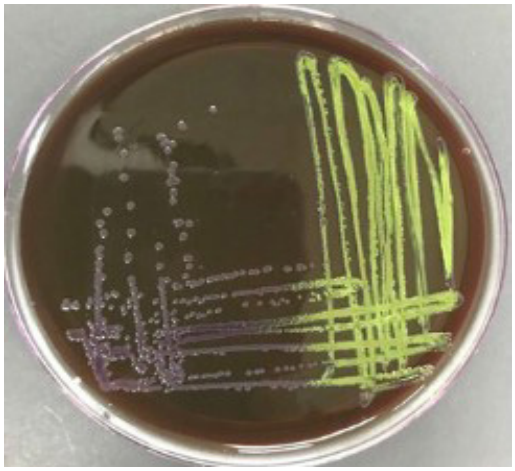

**Fig 2: Colony morphology of bacterial isolate A1 originated from sample 1. A1 showing dark blue-black and metallic green sheen-coloured colonies on Eosin Methylene Blue (EMB) Agar, indicating the bacteria are able to ferment lactose. All other positive isolates from sample 2 (Isolates P1, P2, P3, P4 and P5) show the same colonial morphology.**

six isolates were reinoculated onto EMB agar. All six isolates (Isolate A1, P1, P2, P3, P4 and P5) produced green metallic sheen-coloured colonies on EMB agar (Fig. 2).

**Biochemical characterisation**

All presumptive *E. coli* isolates denoted as Isolates A1, P1, P2, P3, P4 and P5 were subjected to various biochemical tests and showed positive reactions for catalase, Methyl Red (MR), and Triple Sugar Ion (TSI) tests but negative reactions for citrate, oxidase and Voges-Proskauer (VP) tests.

**Antibiotic Susceptibility Testing (AST)**

AST was carried out on all six isolates to screen for any antibiotic-resistant strain among the presumptive *E. coli* isolates originated from the faecal samples. All isolates were diluted in MHB until the optical density matched the McFarland standard before being swabbed

on the MHA. Disk diffusion, also known as Kirby Bauer, was conducted. The antibiotics used in this study are ampicillin (10 µg), erythromycin (15 µg) and tetracycline (30 µg), while gentamicin (10 µg/mL) is used as a positive control. The results for the AST is shown in Table I.

**Table I: Mean (± standard error) of diameter of inhibition zone (mm) of all isolates (A1, P1, P2, P3, P4 and P5) against tetracycline, ampicillin, and erythromycin.**

| Isolates | Antibiotics | Symbol | Disk Potency (µg) | Zone of Inhibition (mm) Mean ± Std. error | Interpretation |
|---|---|---|---|---|---|
| A1 | Tetracycline | TE | 30 | 26.00 ± 0.577 | S |
| | Ampicillin | AMP | 10 | 16.67 ± 0.333 | I |
| | Erythromycin | ERM | 15 | 0 | **R** |
| P1 | Tetracycline | TE | 30 | 25.00 ± 1.000 | S |
| | Ampicillin | AMP | 10 | 15.67 ± 0.333 | I |
| | Erythromycin | ERM | 15 | 0 | **R** |
| P2 | Tetracycline | TE | 30 | 27.67 ± 0.333 | S |
| | Ampicillin | AMP | 10 | 13.67 ± 0.333 | I |
| | Erythromycin | ERM | 15 | 0 | **R** |
| P3 | Tetracycline | TE | 30 | 26.00 ± 0.577 | S |
| | Ampicillin | AMP | 10 | 19.67 ± 0.667 | S |
| | Erythromycin | ERM | 15 | 0 | **R** |
| P4 | Tetracycline | TE | 30 | 0 | **R** |
| | Ampicillin | AMP | 10 | 0 | **R** |
| | Erythromycin | ERM | 15 | 0 | **R** |
| P5 | Tetracycline | TE | 30 | 26.67 ± 0.333 | S |
| | Ampicillin | AMP | 10 | 23.67 ± 0.333 | S |
| | Erythromycin | ERM | 15 | 0 | **R** |

Note. "R": Resistance. "S": Sensitive. "I": Intermediate.

### MIC and Molecular Identification of MDR Isolate P4 Isolate

MIC measures the effect of decreasing antibiotic concentration over a predetermined duration on the amount of microbial population suppression to assess the antibacterial effectiveness of different antibiotics (18). MIC results are interpreted based on the CLSI criteria for *E. coli*. According to the result, Isolate P4 was interpreted as resistant to all antibiotics. Table II shows the MIC range for the MDR Isolate P4 against erythromycin, tetracycline, and ampicillin. Molecular identification using 16S rDNA gene amplification and sequencing revealed that the MDR Isolate P4 is homologous to *E. coli* strain LWY24 with percentage identity of 99.7% and 0.0 e-value.

**Table II: The MIC range for P4 isolates against the erythromycin, tetracycline, and ampicillin.**

| Isolate | Antibiotic | Breakpoints | |
|---|---|---|---|
| | | MIC range, (µg/mL) | Category |
| P4 | Ampicillin | 4-8 | R |
| | Tetracycline | 8-16 | R |
| | Erythromycin | 16-32 | R |

Note. "R": Resistance. "S": Sensitive. "I": Intermediate.

## DISCUSSION

Based on the morphological characteristics, six isolates were observed to produce bright, pink-coloured colonies surrounded by an intense yellow zone due to fermentation of lactose when grown on the selective MacConkey Agar. Thus, it is deduced that these six isolates are Gram-negative bacteria and lactose fermenters. When grown on EMB, green metallic sheen-coloured colonies were observed which is a typical morphological characteristics of *E. coli* on EMB. The result of biochemical tests match the general biochemical profile of *E. coli* and this result is also in line with a previous study by (19). Based on this, we can deduce that all isolates are *E. coli*.

According to the AST result in Table I and Fig. 3, Isolates A1, P1, P2, P3, and P5 were observed to be susceptible to tetracycline. High susceptible rates to tetracycline in these five isolates were in line with the finding reported by (20) that found 100% (n=4) of *E. coli* from faecal samples of the cow in Bali is susceptible to tetracycline (20). In this study, the low prevalence of tetracycline resistance among the bacterial isolates recovered from faecal samples of cows may be due to reduced and cautious usage of antibiotics by the farm management. Reportedly, the farm have significantly lowered the antibiotic usage for the last six years and has opted to

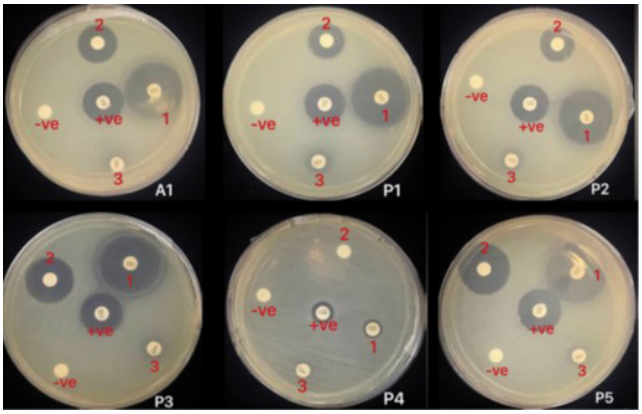


**Fig 3: Inhibition zone of presumptive E. coli isolates (Isolates A1, P1, P2, P3, P4 and P5) against tetracycline, ampicillin, and erythromycin.**

supplement with vitamins and probiotics instead. This is similar to a study conducted in a dairy farm in Japan, whereby from prudent usage of antibiotics, a decrease number of resistant *E. coli* strains was observed (21). Therefore, it can be suggested that a cautious antibiotic regimen implemented into farms will lead to a significantly lower and controlled resistance towards antibiotics. On another note, only one isolate (Isolate P4) was observed to be resistant to tetracycline. Tet genes, such as *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G) genes, are specifically stated to be the most common genes in *E. coli* that confer resistance to tetracycline via efflux pump activity (22). Since these genes are commonly found in mobile genetic elements such as

conjugative transposon, this tetracycline resistant trait may be acquired via horizontal gene transfer (HGT) (10). Furthermore, as tetracycline is commonly utilized in both human and animal medicine, as well as growth enhancer in food-producing animals, this can contribute to the development of resistant due to the selective pressure (7).

The Isolate P4 is also observed to be resistant to ampicillin with a mean diameter size of less than 13 mm. Since ampicillin is categorised as a β-lactam antibiotic, the production of the β-lactamases is the common resistance mechanism employed by Gram-negative bacteria against ampicillin and other β-lactam antibiotics (23). There are three main mechanisms of antibiotic resistance against ampicillin: enzymatic degradation by β-lactamases, efflux pump regulation of β-lactam and target modification of the penicillin-binding proteins (PBPs) that leads to a lack of β-lactam binding (23).

In the case of erythromycin, all isolates were shown to be 100% (n=6) resistant. This is expected as it is known that many strains of *E. coli* have intrinsic resistance against erythromycin. According to the previous study by (23), 60% of *E. coli* isolates recovered from cow faeces (n=52), the farm environment (n=43) and 100% (n=6) from beef samples in Malaysia are resistant to erythromycin (24). The potential resistance of Enterobacteriaceae against macrolides may develop through several mechanisms. These mechanisms include the modification of the target site by methylases encoded by *erm*(A), *erm*(B), and *erm*(C); the deactivation of the enzyme by esterases (via the expression of by *ere*(A) or *ere*(B)) or phosphotransferases (encoded by *mph*(A), *mph*(B), and *mph*(D) genes) (25).

## CONCLUSION

In conclusion, the bacteria in the livestock may develop resistance after receiving antibiotics for an extended period of time. These bacteria multiply rapidly within the livestock and can spread to other animals through interactions, leading to the formation of other sub-population or ecological niches of antibiotic-resistant bacteria. Furthermore, *E. coli* serve as represented microorganisms for the reservoir of antibiotic resistance genes observed in a community. Out of 30 isolates, six isolates are identified as *E. coli* based on morphological characteristics of colonies grown on selection and differential media: EMB agar and MacConkey agar. All isolates were also tested using different types of biochemical tests such as catalase, Methyl Red (MR), Triple Sugar Ion (TSI), oxidase, citrate, and Voges-Proskauer (VP) tests and shown to match the biochemical profiles of *E coli*. Based on AST result, all isolates are found to be resistant to erythromycin and Isolate P4 were observed to be resistant against all tested antibiotics and therefore is classified as a multidrug resistant *E. coli*. The information generated from this study showed the presence of resistant and MDR bacteria within this local farm. This baseline data emphasise the importance of a local farm to develop appropriate strategies and mitigation efforts to enhance the safety of farm management and livestock meat sold for public consumption. In the future, it is recommended to screen for antibiotic resistance genes that confer resistance in identified resistant isolates to elucidate their resistance mechanisms.

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