# Characterisation of ESBL/AmpC-Producing *Enterobacteriaceae* isolated from three Poultry Farms in West Coast, Peninsular Malaysia

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**ABSTRACT**

Extended-spectrum beta lactamases (ESBLs) and AmpC beta-lactamases (AmpCs)-producing *Enterobacteriaceae* have been increasingly reported and imposing significant threat to public. Livestock production industry might be the important source for clinically important ESBL-producing *Enterobacteriaceae*. This study aims to investigate the resistance profile, phenotypic ESBL production, beta-lactamase genes, virulence factors, and plasmid replicon types among 59 *Enterobacteriaceae* strains isolated from poultry faecal samples in Malaysia's commercial poultry farm. The identity of bacteria was determined by API and 16S rRNA sequencing. Resistance profile was determined by antimicrobial susceptibility test via disk diffusion method. ESBL production was examined via double disk synergy test. Beta-lactamase genes, virulence genes and plasmid replicons were screened using polymerase chain reaction (PCR). There were 38.7% and 32.3% of *E. coli* resistant to cefotaxime and cefoxitin, respectively, while *Klebsiella spp.* demonstrated resistance rate of 52.6% to both mentioned antimicrobials. Majority of the *E. coli* isolates carried $bla_{\text{TEM}}$ and $bla_{\text{CMY-2 group}}$. $bla_{\text{SHV}}$ was the most prevalent genes detected in *Klebsiella spp.*, followed by $bla_{\text{DHA}}$ and $bla_{\text{TEM}}$. Resistance to extended spectrum cephalosporin in our isolates was primarily mediated by plasmid mediated AmpC beta-lactamase such as CMY-2 group and DHA enzyme. The CTX-M genes were found in two ESBL-producing *E. coli*. IncF, IncI1, and IncN plasmids were most frequently detected in *E. coli* and *Klebsiella spp.* The virulence factor, including EAST1 and pAA were identified at low frequency. This study highlights the poultry as a reservoir of resistance and virulence determinants and prevalence of plasmids in *Enterobacteriaceae* might drive their dissemination.

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**TABLE OF CONTENTS**

## ABBREVIATIONS

| | |
|---|---|
| µg | microgram |
| µL | microlitre |
| µM | micromolar |
| β-lactam | beta-lactam |
| β-lactamase | beta-lactamases |
| AMC | amoxicillin/clavulanic acid |
| AmpCs | AmpC beta-lactamases |
| API | Analytical Profile Index |
| ATCC | American Type Culture Collection |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair |
| ℃ | degree Celsius |
| CA | clavulanic acid |
| cAmpCs | chromosomal AmpC β-lactamases |
| CAZ | ceftazidime |
| CLSI | Clinical and Laboratory Standards Institute |
| CTX | cefotaxime |
| DAEC | diffusely adherent *E. coli* |

| | |
|---|---|
| D-Ala | D-alanine |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| DDST | double-disk synergy test |
| D-Glu | γ-D-glutamic acid |
| dGTP | deoxyguanosine triphosphate |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| dTTP | deoxythymidine triphosphate |
| EAEC | enteroaggregative *E. coli* |
| EDTA | ethylenediaminetetraacetic acid |
| EHEC | enterohaemorrhagic *E. coli* |
| EIEC | enteroinvasive *E. coli* |
| EPEC | enteropathogenic *E. coli* |
| ESBLs | extended-spectrum beta lactamases |
| ETEC | enterotoxigenic *E. coli* |
| ExPEC | extraintestinal pathogenic *E. coli* |
| FEP | cefepime |
| FOX | cefoxitin |

| | |
|---|---|
| g | gram |
| $H_2O_2$ | hydrogen peroxide |
| $H_2S$ | hydrogen sulfide |
| kb | kilobase |
| L | litre |
| L-Ala | L-alanine |
| LB | Luria-Bertani |
| L-Lys | L-lysine |
| mA | milliampere |
| MALDI-TOF-MS | matrix-assisted laser desorption ionization-time of flight mass spectrometry |
| $MgCl_2$ | magnesium chloride |
| MGEs | mobile genetic elements |
| mL | millilitre |
| mM | millimolar |
| NaCl | sodium chlorine |
| NAG | N-acetylglucosamine |
| NAM | N-acetylmuramic acid |
| NCBI | National Center for Biotechnology Information |
| $NH_4^+$ | ammonium |

| | |
|---|---|
| OD | optical density |
| *ori* | origin of replication |
| *oriT* | origin of transfer |
| pAmpCs | plasmid-mediated AmpC β-lactamases |
| PBPs | penicillin-binding proteins |
| PCR | polymerase chain reaction |
| PFGE | pulsed-field gel electrophoresis |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| SNP | single nucleotide polymorphism |
| T4CP | type IV coupling protein |
| T4SS | type IV secretion system |
| $T_{anneal}$ | annealing temperature |
| *Taq* | *Thermus aquaticus* |
| $t_{elongate}$ | elongation time |
| $T_m$ | primer melting temperature |
| TZB | tazobactam |
| UTIs | urinary tract infections |

| | |
|---|---|
| V | volt |
| ZOI | zone of inhibition |

## LIST OF FIGURES

**LIST OF TABLES**

# CHAPTER 1 GENERAL INTRODUCTION

## 1.1 Research background

Antimicrobial resistance is global public health concern. Imprudent use of antimicrobials in livestock is one of the driving forces behind the increasing rate of resistance. In addition to therapeutic purpose, antibiotics could be applied as prophylaxis and growth promoter in animals. This may impose the selective pressure on both commensal and pathogenic microorganisms, which can disseminate among humans via direct contact, food chain or environmental pollution of farm effluents (Roca *et al.*, 2015). Beta-lactam (β-lactam) is one of the most important classes of antimicrobial agents for combating bacterial infection. It can be categorised to several classes, including penicillins, cephalosporins, cephamycins, carbapenems, and monobactams. Resistance to β-lactams is predominantly due to the production of beta-lactamase (β-lactamase), which can inactivate the drug by hydrolysing β-lactam ring. Extended-spectrum beta-lactamase (ESBL) and AmpC-beta-lactamase (AmpC) are enzymes capable of conferring resistance to extended-spectrum β-lactams (Smet *et al.*, 2010).

The bacteria's ability to disseminate genetic materials through horizontal gene transfer plays an important role in the development of antimicrobial resistance and virulence (Stokes and Gillings, 2011). Resistance genes such as ESBL and AmpC are associated with mobile genetic elements (MGEs) including insertion sequences (ISs), integrons, transposons, and plasmids, and can be disseminated across different species through MGEs (Smet *et al.*, 2010; Stokes and Gillings, 2011). The horizontal transfer of resistance via MGEs has been investigated between commensals and pathogens in human, animal and environment setting. Among all, *Escherichia coli* may serve as an

important contributor to the spread of antibiotic resistance because it is an important animal commensal that can persist in the environment, with presence of some pathogenic variants (Stokes and Gillings, 2011).

### 1.2 Problem statement

Since the 2000s, there has been a growing trend of detecting ESBL/AmpC-producing *Enterobacteriaceae* in farm animals, particularly in poultry, which was initially described in human medical practice. This leads to the emergence of hypothesis that animals might become sources or reservoir of infection, contributing to the spread of resistant bacteria (Ewers *et al.*, 2012). A review summarising the data from 2006 until 2011 has found that the prevalence of ESBL in *Enterobacteriaceae* in poultry was high (more than 50%) in countries such as Denmark, Finland, Germany, Japan, Netherlands and Spain (Saliu, Vahjen and Zentek, 2017). Furthermore, many studies in Southeast Asia have revealed similar finding with high prevalence of ESBL-producing *E. coli* in poultry, including Vietnam (88.3%), Thailand (79.2%), and Philippines (60.9%) (Le *et al.*, 2015; Gundran *et al.*, 2019; Sornsenee *et al.*, 2022). In addition, several studies have described the detection of AmpC-carrying bacteria in poultry (Trung *et al.*, 2019; Nakayama *et al*., 2022). It should be taken seriously because ESBL/AmpC producing *E. coli* are able to transmit and persist in a broiler flock even without selective pressure from antibiotics, and often exhibit multidrug resistant features which are regarded as a potential health threat to humans and animals (Huijbers *et al.*, 2016; Nguyen *et al.*, 2016). Poultry meat products produced by Malaysia have been reported to carry ESBL producers, with prevalence as high as 53.8% or 67.9% (Aliyu *et al.*, 2016; Guo *et al.*, 2021). However, the phenotypic and molecular

characteristics of resistant isolates from poultry in Malaysia remains unknown. Thus, to address the gap, the present study was conducted to characterise the antimicrobial resistance profile, ESBL production, beta-lactamase genes, virulence genes, and plasmid replicon among *Enterobacteriaceae* isolates collected from poultry in Malaysia.

### 1.3 Aims and Objectives

This study aims to characterise the *Enterobacteriaceae* isolated from commercial poultry farms located in Peninsular Malaysia. Antimicrobial susceptibility, ESBL production, resistance genes, virulence genes, and plasmid replicon types were examined for better understanding resistance and virulence profile, and epidemiology of resistant strains in poultry in Malaysia. The flowchart of this study is displayed in Figure 1.1.

The specific objectives of this study are as follow:

1. To characterise the antimicrobial resistance profile of *Enterobacteriaceae* isolates
2. To investigate ESBL production of isolates using double disk synergy test
3. To examine the beta-lactamase genes and virulence genes in isolates via PCR
4. To determine the plasmid incompatibility group of all isolates through PCR

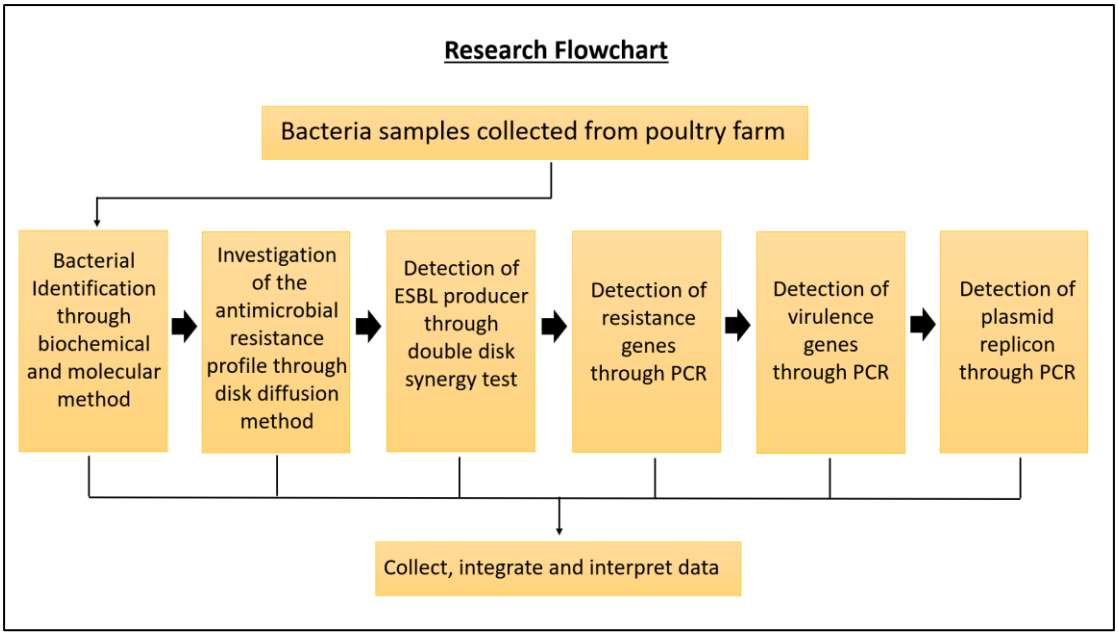


**Figure 1.1**: The flowchart of the research study.

## CHAPTER 2 LITERATURE REVIEW

Antimicrobial resistance poses global public health concern because it increases the challenge of treating bacterial infections (Ventola, 2015). In 2019, it was estimated that there were 1.27 million deaths attributable to bacterial antimicrobial resistance and 4.95 million deaths associated with it. Among 21 assigned regions, Western sub-Saharan Africa had the highest antimicrobial resistance burden, with 27.3 deaths per 100 000 attributable to antimicrobial resistance. Additionally, third-generation cephalosporin-resistant *E. coli* and *K. pneumoniae* were responsible for an estimated 50 000–100 000 deaths in 2019 (Murray *et al.*, 2022). Resistance to extended spectrum cephalosporins is primarily mediated by ESBLs and AmpC (George A Jacoby, 2009; Canton, Gonzalez-Alba and Galán, 2012). Genes encoding ESBLs and AmpC are mainly located on MGEs and horizontal transfer via MGEs accelerates the spread of ESBLs/pAmpCs between commensals and pathogens in intestinal microbiota of

animals and humans (Sukmawinata *et al*., 2020; Ewers *et al*., 2021). Commensals might act as reservoir of resistance genes that poses serious threat to human health (Smet *et al.*, 2010). *E. coli* and *Klebsiella spp.* are gut flora in both human and animal, and resistance genes in these bacteria contribute to dissemination of multidrug-resistant bacteria among animals, humans, and environment through food chain (Subramanya *et al.*, 2021). The excessive use of antimicrobials in agriculture, as well as the horizontal transfer of resistance genes, may be related to the worldwide emergence of ESBL- and AmpC-producing *Enterobacteriaceae* in both humans and animals (Ewers *et al.*, 2012).

### 2.1 Antibiotic resistance

In 1928, the modern era of antibiotics started with the discovery of penicillin (Ventola, 2015). Antibiotics play a vital role in health care systems. Major advances in medicine and surgery namely cancer therapy, organ transplantation, chronic diseases, might not be achieved without antibiotic treatment to control for bacterial infections (Ventola, 2015; Aslam *et al.*, 2018). Antibiotics extend expected life spans and decrease the morbidity and mortality caused by food-borne and other poverty-related infections (Ventola, 2015).

Antibiotic resistance was first identified in 1940s and this prompted the development of new beta-lactam antibiotics (Ventola, 2015). Resistance to an antibiotic is defined as the ability of microorganism to grow or survive in the presence of a concentration of antibiotic that is generally sufficient to inhibit or kill organisms of the same species (Sabtu, Enoch and Brown, 2015). Antimicrobial resistance has become worrying threat to global health because it threatens the ability to treat infectious diseases, leading to prolonged illness, disability, and death (Cepas and Soto, 2020). In

addition to the greater mortality risk, antimicrobial resistance also poses financial burden to healthcare system and society. It is noticeable that resistance among bacteria is increasing in both community and hospital settings, but the development of new and innovative antimicrobials has not kept up (Ventola, 2015; Cepas and Soto, 2020). Thus, patients infected with resistant organisms are associated with long hospitalisation and high medical expenses (Roca *et al.*, 2015).

The aetiology of antibiotic resistance includes excessive use of antibiotics in humans and animals due to inadequate regulations and imprudent usage, and lack of awareness in best practices. Poor sanitation, poor sewerage disposal system, wildlife spread, increased international travel and weak infection control standards are potent drivers of antibiotic resistance (Aslam *et al.*, 2018).

## 2.2 β-lactam and β-lactamase

### 2.2.1 β-lactam and its mechanism of action

Beta-lactams (β-lactams) are one of the most important classes of antimicrobial agents in human and veterinary medicine (Guenther, Ewers and Wieler, 2011). They are used to treat pneumonia, urinary tract and bloodstream infections, and as prophylaxis before surgery (Brolund, 2014). In addition to clinical settings, β-lactams are also widely applied in agriculture for disease treatment, growth promotion and prophylaxis (Manyi-Loh *et al.*, 2018). β-lactams can be categorised to several classes based on structural and functional differences, including monobactams, penams (e.g. penicillin G), penems (e.g. carbapenems), and cephems (e.g. first to fifth generation of cephalosporins) (Turner *et al.*, 2022).

Antibiotics can exert either a cytotoxic or cytostatic effect on micro-organisms via different mechanisms, including interfering bacterial cell wall synthesis, nucleic acid synthesis, protein synthesis, metabolic pathways, and altering structure of bacterial cell membrane. β-lactams are antimicrobial agents that interfere with the synthesis of bacterial cell wall (Levy and Marshall, 2004)(Abushaheen *et al.*, 2020). The bacterial cell wall plays important roles in maintaining cell shape and preventing lysis caused by high intracellular osmotic pressure (Johnson, Fisher and Mobashery, 2013). The major component of bacterial cell wall is peptidoglycan, which provides mechanical support (Vollmer, Blanot and De Pedro, 2008). Thick peptidoglycan layer on the cytoplasmic membrane is found in Gram-positive bacteria while Gram-negative bacteria have thin peptidoglycan between the outer membrane and the cytoplasmic membrane (Smet *et al.*, 2010).

The structure of peptidoglycan is made up of glycan chains of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which are cross-linked with short peptides by penicillin-binding proteins (PBPs) (Sauvage *et al.,* 2008). The peptide chain consists of alternating D- and L-forms of L-alanine, γ-D-glutamic acid, L-lysine, D-alanine, and D-alanine residues linked to NAM. The last D-Ala residue is eliminated by hydrolysis during cross-linking (Sawa, Kooguchi and Moriyama, 2020). PBP mediated cross-linking of the glycan strands occurs between the carboxyl group of the fourth D-alanine and the amino group of the third diaminopimelic acid (a ε-carboxyl derivative of lysine) (Vollmer, Blanot and De Pedro, 2008; Sawa, Kooguchi and Moriyama, 2020). The amount of PBPs differs between bacterial species, and PBPs can be located in membrane and cytoplasm (Smet *et al.,* 2010).

The structure of β-lactams is highly similar to D-Ala-D-Ala, which is a nascent structure of peptidoglycan (Zapun, Contreras-Martel and Vernet, 2008). Covalent

binding of β-lactams to serine active sites of PBPs impairs the cross-linking of peptidoglycan, weakens cell wall structure, and eventually leads to cell lysis (Zapun, Contreras-Martel and Vernet, 2008; Rawat and Nair, 2010). The mechanism action of β-lactams against bacterial cell wall synthesis is showed in Figure 2.1.

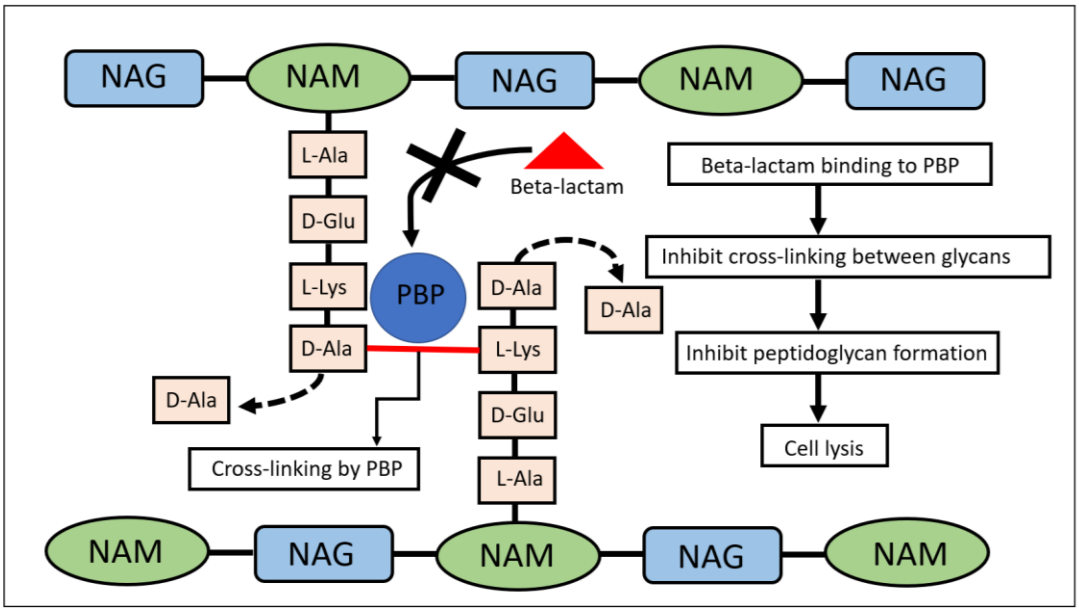


**Figure 2.1**: The mechanism action of β-lactam against bacterial cell wall synthesis. *NAG: N-acetylglucosamine; NAM: N-acetylmuramic acid; PBP: penicillin-binding proteins; L-Ala: L-alanine; D-Glu: γ-D-glutamic acid; L-Lys: L-lysine; D-Ala: D-alanine. Adapted from (Sawa, Kooguchi and Moriyama, 2020).

### 2.2.2 Resistance mechanism against β-lactam

The excellent genetic plasticity of bacteria enables them to withstand the damage brought by antibiotic through two strategies, including mutation in genes altering antibiotic action and acquisition of resistance gene. Genetic mutations that affect the mechanisms of action of antibiotic are complicated and diverse (Hoffman, 2001). The mechanisms of antimicrobial resistance can be categorised into four groups, including drug uptake limitation, drug efflux, drug target modification, and drug inactivation (Uddin *et al.*, 2021).

Drug uptake primarily depends on the permeability of outer membrane, and situations such as porin downregulation and biofilm formation can limit the uptake of antimicrobials into bacteria. Efflux pumps in bacteria are responsible for actively transporting antibiotics out of the cell (Uddin *et al.*, 2021). Overexpression of multidrug efflux pumps can mediate resistance by actively effluxing β-lactams (Li *et al.*, 2007). Drug target modification can mediate resistance through reducing the amount of drug target and altering the structure of the drug target, thus changing the drug-binding site (Uddin *et al.*, 2021). For example, mutations in genes encoding penicillin-binding proteins can mediate resistance if the altered penicillin-binding proteins show reduced affinity for β-lactams but retain cell wall synthesis (Smet *et al.*, 2010). Drug inactivation can occur by altering the drug through the attachment of various chemical groups or by destroying key structures of the drug (Uddin *et al.*, 2021). Drug inactivation by beta-lactamase (β-lactamase) production is the most common mechanism of resistance against β-lactam in Gram-negative bacteria, especially in *Enterobacteriaceae* (Smet *et al.*, 2010).

### 2.2.3 β-lactamase and its classification

β-lactamase is capable of hydrolysing the amide bond of the β-lactam ring, ultimately rendering antibiotics inactive (Zapun, Contreras-Martel and Vernet, 2008). It is speculated that β-lactamases have evolved from penicillin-binding proteins due to some sequence homology (Bradford, 2001). Penicillinase, a β-lactamase was discovered in 1940 after the first use of antibiotics (Blair *et al.*, 2014).

β-lactamases can be classified based on molecular or functional characteristics. The molecular classification is determined by the amino acid sequence homology, and

β-lactamases are categorised into four molecular classes according to Ambler's classification: A, B, C, and D. Classes A, C, and D belong to serine-β-lactamases, which hydrolyse their substrates by forming an acyl enzyme through an active site serine. In contrast, class B β-lactamases are metallo-enzymes that utilise zinc ion for β-lactam hydrolysis (Bush and Jacoby, 2010). The functional grouping, known as the Bush–Jacoby system, is based on substrate hydrolysis profiles and inhibitor profile (Bonomo, 2017).

Functional group 1 enzymes, which belong to molecular class C, are active against cephamycins and resistant to inhibition by clavulanic acid. Group 2 β-lactamase, including molecular classes A and D, comprise the largest group of β-lactamases. Enzymes in this group are involved in the hydrolysis of benzylpenicillin, penicillin, and early cephalosporins (subgroup 2a, 2b), oxyimino-cephalosporins (subgroup 2e, 2be, 2ber, 2de), carbenicillin or ticarcillin (subgroup 2c, 2ce), cloxacillin or oxacillin (subgroup 2d, 2de, 2df), and carbapenem (subgroup 2f, 2df). Subgroup 2br and 2ber are β-lactamases that are resistant to clavulanic acid, a β-lactamase inhibitors. Group 3 consists of metallo-β-lactamases that require a zinc ion at the active site. They primarily hydrolyse carbapenems and are inhibited by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) (Bush and Jacoby, 2010). The alignment of structural and functional classifications, along with corresponding substrates, inhibitors, and examples, is presented in Table 2.1.

**Table 2.1**: Classification scheme of β-lactamases. Adapted from (Bush and Jacoby, 2010; Bonomo, 2017).

| Ambler molecular class | Bush Jacoby group | Substrate | Inhibited by | Example |
|---|---|---|---|---|
| C | 1 | Cephalosporins (Cephamycin) | - | ACT-1, CMY-2, FOX-1, MIR-1 |
| A | 2a | Penicillins | CA/TZB | PC1 |
| | 2b | Penicillins, early cephalosporins | CA/TZB | TEM-1, TEM-2, SHV-1 |
| | 2br | Penicillins, cephalosporins | - | TEM-30, SHV-10 |
| | 2be | Extended-spectrum cephalosporins, monobactams | CA/TZB | TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1 |
| | 2ber | Extended-spectrum cephalosporins, monobactams | - | TEM-50 |
| | 2c | Carbenicillin | CA/TZB | PSE-1, CARB-3 |
| | 2ce | Carbenicillin, cephalosporins | CA/TZB | RTG-4 |
| | 2e | Extended-spectrum cephalosporins | CA/TZB | CepA |
| | 2f | Carbapenems | Variable (CA/TZB) | KPC-2, IMI-1, SME-1 |
| D | 2d | Cloxacillin | Variable (CA/TZB) | OXA-1, OXA-10 |
| | 2de | Cloxacillin, extended-spectrum cephalosporins | Variable (CA/TZB) | OXA-11, OXA-15 |
| | 2df | Cloxacillin, Carbapenems | Variable (CA/TZB) | OXA-23, OXA-48 |
| B | 3a | Carbapenems | EDTA | IMP-1, VIM-1, CcrA, IND-1 |
| | 3b | Carbapenems | EDTA | CphA, Sfh-1 |

*CA: clavulanic acid; TZB: tazobactam; EDTA: Ethylenediaminetetraacetic acid

### 2.2.4 Extended-spectrum β-lactamases (ESBLs)

Early β-lactamases against first-generation β-lactams were identified first, followed by the emergence of extended-spectrum β-lactamases (ESBLs) (Blair *et al.*, 2014). ESBLs are a rapidly evolving group of β-lactamases that hydrolyse penicillins, first-, second- and third-generation (oxyimino-) cephalosporins, as well as aztreonam. ESBLs are not active against cephamycins and carbapenems (Paterson and Bonomo, 2005). Inhibition of ESBLs by clavulanic acid is another feature of ESBLs (Rawat and Nair, 2010). The majority of ESBLs belong to Ambler's molecular class A and to functional group 2be (Bradford, 2001). Common enzyme families of ESBLs include TEM (Temoneira) -type β-lactamases, SHV (Sulfhydryl variable) -type β-lactamases, CTX (cefotaximase) -M-type β-lactamases, and OXA (oxacillinase) -type β-lactamases. In addition to these four groups, less common ESBL families include GES, PER, VEB, TLA, BEL, SFO, and OXY β-lactamases (Bradford, 2001; Castanheira, Simner and Bradford, 2021).

ESBLs have been found worldwide in many different genera of *Enterobacteriaceae* (Bradford, 2001). Until the end of the 1990s, the majority of detected ESBLs belonged to SHV and TEM types, and *Klebsiella pneumoniae* was the dominant carrier of ESBLs. Isolates carrying TEM-ESBLs and SHV-ESBLs were usually associated with nosocomial outbreaks. However, this situation has changed dramatically since 2000, when CTX-M enzymes were predominantly investigated. *E. coli* expressing CTX-M β-lactamases has emerged worldwide and is associated with community infections, particularly urinary tract infections (Cantón *et al.*, 2008). The detection of CTX-M enzymes has increased overtime in most countries (Bevan, Jones and Hawkey, 2017). ESBLs in Gram-negative bacteria such as *E. coli* and *K. pneumoniae* have serious implications in the treatment of severe infections (Blair *et al.*,

2014). ESBL infections have led to the use of last-line drugs, such as carbapenems, resulting in the emergence and dissemination of strains producing carbapenemases (Bevan, Jones and Hawkey, 2017).

#### 2.2.4.1 TEM-type β-lactamases

In 1965, TEM-1 enzyme was initially identified in an *E. coli* strain isolated from a patient named Temoniera in Greece, leading to its designation as TEM (DATTA and KONTOMICHALOU, 1965). TEM-1 was the first plasmid-mediated β-lactamase described in gram-negative bacteria. It can hydrolyse penicillins and early cephalosporins, including cephalothin and cephaloridine (Bradford, 2001). TEM-1 is inhibited by clavulanic acid. TEM-2 is a less common β-lactamase that shares the same hydrolytic profile as TEM-1 (Paterson and Bonomo, 2005). It is the first derivative of TEM-1 with a single amino acid substitution (Bradford, 2001). Numerous point mutations in the parental penicillinases result in amino acid substitutions that affect the enzyme's structure and activity in different ways (Gniadkowski, 2001). These amino acid substitutions in TEM enzyme can lead to ESBL or inhibitor-resistant phenotypes (Bradford, 2001).

In 1987, TEM-3, a TEM-type ESBL, was detected in *K. pneumoniae* in France (Sirot *et al.*, 1987). TEM-52 is found to be the predominant TEM-type ESBLs in *E. coli* and *Salmonella spp.* isolated from poultry faecal sample (Smet *et al.*, 2008; Leverstein-van Hall *et al.*, 2011; Geser, Stephan and Hächler, 2012; Laube *et al.*, 2013; de Jong *et al.*, 2014). In addition, TEM-215 and TEM-219 are other TEM-type ESBLs found in chicken origin (Trung *et al.*, 2019).

### 2.2.4.2 SHV-type β-lactamases

Similar to TEM enzyme, SHV β-lactamases can be categorised into broad-spectrum β-lactamases, inhibitor-resistant β-lactamases, and ESBLs (Liakopoulos, Mevius and Ceccarelli, 2016). However, unlike the TEM-type β-lactamases, there are relatively few derivatives of SHV-1, and most of SHV variants exhibit the ESBL phenotype. SHV-type ESBLs are commonly found in strains of *K. pneumoniae* (Bradford, 2001).

In 1983, the first ESBLs, SHV-2, was detected in a *Klebsiella ozaenae* isolate from Germany. SHV-2 effectively hydrolysed cefotaxime and differs from SHV-1 by a single amino acid substitution (Paterson and Bonomo, 2005). SHV-2 and SHV-12 are SHV-ESBLs normally detected in faecal samples from poultry (Blanc *et al.*, 2006; Geser, Stephan and Hächler, 2012; de Jong *et al.*, 2014).

### 2.2.4.3 CTX-M-type β-lactamases

CTX-M enzymes can be subdivided based on amino acid sequence similarities. They can be classified into five groups through phylogenetic studies: CTX-M-1 group, CTX-M-2 group, CTX-M-8 group, CTX-M-9 group, and CTX-M-25 group. The members of each group exhibit >94% identity, and ≤90% amino acid identity is investigated between members of different groups (Bonnet, 2004).

CTX-M ESBL genes are more frequently detected in *Enterobacteriaceae*, particularly in *E. coli* and *K. pneumoniae* (Bonnet, 2004; Cantón and Coque, 2006). Chromosomal β-lactamase genes in *Kluyvera spp.* are speculated to be the progenitors of the CTX-M family (Humeniuk *et al.*, 2002). For example, $bla_{\text{CTX-M-2}}$ is likely to have

originated from *bla*$_{KLUA}$ in *Kluyvera ascorbate*, and *bla*$_{CTX-M-14}$ from *bla*$_{KLUY}$ in *Kluyvera georgiana* (Di Conza *et al.*, 2002; Olson *et al.*, 2005).

CTX-M group 1 (including CTX-M-55, CTX-M-15) and CTX-M group 9 (including CTX-M-14, CTX-M-65) are the predominant CTX-M groups detected in *E. coli* from poultry faecal samples in several Asian countries, including China, Japan, Korea, Vietnam, Thailand, and Nepal (Zheng *et al*., 2012; Kameyama *et al*., 2013; Wu *et al*., 2018; Tansawai, Walsh and Niumsup, 2019; Trung *et al*., 2019; Subramanya *et al.*, 2020; Kim *et al.*, 2021; Seo and Lee, 2021).

Among European countries, CTX-M-1 enzyme has been more prevalent in investigations involving *E. coli* from poultry faeces. For example, it has been studied in Switzerland, Belgium, the Netherlands, and England (Smet *et al*., 2008; Leverstein-van Hall *et al.*, 2011; Randall *et al*., 2011; Geser, Stephan and Hächler, 2012; Toszeghy *et al*., 2012; Huijbers *et al*., 2014; Blaak *et al.*, 2015).

### 2.2.5 AmpC β-lactamases (AmpCs)

AmpC β-lactamases are a class of enzymes that can confer resistance to penicillins, narrow-spectrum cephalosporins (such as cefazolin and cephalothin), oxyimino-cephalosporins (such as cefotaxime, cefpodoxime, and ceftazidime), and cephamycins (such as cefoxitin and cefotetan). However, their hydrolysis rate for fourth-generation cephalosporins (such as cefepime) is very low and negligible (Meini *et al.*, 2019). Moreover, AmpC β-lactamases are usually resistant to β-lactam-based inhibitors such as clavulanic acid, sulbactam, and tazobactam, but they can be inhibited by cloxacillin and oxacillin (Jacoby, 2009).

The gene encoding AmpC β-lactamases can be found on either the chromosome or a plasmid in Gram-negative bacteria. The expression of chromosomal AmpC β-lactamases (cAmpCs) can be constitutive or inducible and is often found in *Enterobacter spp.*, *Serratia marcescens*, *Citrobacter freundii*, *Providencia stuartii*, and *Morganella morganii*. The expression of cAmpCs can be induced by β-lactams. In *E. coli*, chromosomal AmpC is normally expressed at a low level and is non-inducible due to the lack of transcriptional regulator *ampR* (Philippon, Arlet and Jacoby, 2002).

Plasmid-mediated AmpC β-lactamases (pAmpCs) usually originate from cAmpCs in some Gram-negative bacteria (Meini *et al.*, 2019). *K. pneumoniae*, *P. mirabilis*, and *Salmonella spp.* lack a chromosomal AmpC enzyme (Jacoby, 2009). *Enterobacterales* that harbour pAmpCs include *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *Klebsiella oxytoca*, *Salmonella enterica*, and *Shigella spp.* (Meini *et al.*, 2019). pAmpC enzymes have been named according to i) substrate profile, including CMY (cephamycins), FOX (cefoxitin), MOX (moxalactam), LAT (latamoxef); ii) type of β-lactamase, including ACT (AmpC type), ACC (Ambler class C); iii) site of discovery, including MIR-1 (Miriam Hospital), DHA (Dhahran hospital); iv) patient's name, including BIL-1 (Bilal) (Philippon, Arlet and Jacoby, 2002).

Contamination of chicken meat by CMY-2-producing *E. coli* has been reported in many countries, including Columbia, Portugal, Brazil, Finland, USA, Canada, and the Netherlands (Park *et al.*, 2012; Sheikh *et al.*, 2012; Voets *et al.*, 2013; Castellanos *et al.*, 2017; Koga *et al.*, 2019; Päivärinta *et al.*, 2020; Clemente *et al.*, 2021). In contrast, DHA-1 is less commonly reported in livestock compared to clinical cases. DHA-1-producing *K. pneumoniae* isolates have been reported in clinical setting (Diestra *et al.*, 2011; Hennequin *et al.*, 2012; Compain *et al.*, 2014; Hennequin *et al.*, 2018). *K. pneumoniae* producing DHA from a clinical origin has demonstrated multidrug

resistance and virulence attributes (Hennequin *et al.*, 2012; Kis *et al.*, 2016; Hennequin *et al.*, 2018). Plasmid-borne AmpC-producing *K. pneumoniae* is associated with a clinical outbreak with high treatment failure and mortality (Pai *et al.*, 2004). The production of cAmpC is associated with nosocomial infections, while pAmpC-producing *Enterobacterales* are mainly observed in community-acquired infections (Meini *et al.*, 2019).

### 2.3 Virulence factors

Pathogenicity refers to the ability of a bacterium to cause disease. Pathogens are known to cause infection with their ability to colonise, invade, and harm host tissues (Aslam *et al.*, 2018). Bacterial pathogenicity is a complex and multifactorial phenomenon that takes into consideration the host's immune status and bacterial virulence (Cepas and Soto, 2020). The coevolution of the host's immune system and bacteria may last millions of years (Aslam *et al.*, 2018).

*E. coli* is also the common pathogen that causes human infections in both industrialised and developing countries (Kuhnert, Boerlin and Frey, 2000). The mechanism of pathogenicity in *E. coli* involves numerous virulence factors that are responsible for colonisation, adhesion, invasion, and survival against host defenses (Paixão *et al.*, 2016). Virulence is mediated by adhesins, invasins, toxins, host cell surface modifying enzymes, capsules, outer membrane proteins, siderophores (iron acquisition systems), biofilm, and secretion systems (Schroeder, Brooks and Brooks, 2017; Cepas and Soto, 2020).

Some strains of *E. coli* that acquire specific virulence attributes become highly adapted to new niches, leading to the development of various diseases (Kaper, Nataro

and Mobley, 2004). *E. coli* strains that causes enteric or diarrhoeal diseases can be further categorised into six groups, including 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). *E. coli* pathotypes that are involved in extraintestinal infections such as urinary tract infections (UTIs), meningitis and septicaemia are referred to as extraintestinal pathogenic *E. coli* (ExPEC) (Kaper, Nataro and Mobley, 2004).

EAST1 is heat-stable toxin associated with diarrheal disease in humans, cattle, and swine (Veilleux and Dubreuil, 2006; Dubreuil, 2019). pAA (porcine attaching- and effacing-associated factor), originally identified in a porcine enteropathogenic strain, is a type of adhesins found in EPEC, EHEC, and ETEC (Kaper, Nataro and Mobley, 2004; Leclerc *et al.*, 2007). F18 is a common fimbriae associated with ETEC in swine (Lee *et al.*, 2008). Enterotoxins like STa (human) and LT (human and swine) have been identified among ETEC strains from humans or animals (Gomes *et al.*, 2016). A gene *eae* encodes an outer membrane protein called intimin, which is responsible for the tight association of both pathogens with the host cell. The *eae* gene has been cloned from different EPEC and EHEC strains isolated from humans and animals (Oswald *et al.*, 2000). A gene *est*, which encodes heat-stable enterotoxins, has been described in humans and pigs with diarrhoeal symptoms (Hornes, Wasteson and Olsvik, 1991). AIDA (adhesin involved in diffuse adherence) is an adhesin found in *E. coli* strains isolated from human and swine with diarrhoea (Niewerth *et al.*, 2001). A gene *aspU*, which encodes a cryptic secreted protein with unknown role, has been identified in EAEC (Czeczulin *et al.*, 1999; Toma *et al.*, 2003).

The virulence attributes can be mobilised into different strains to create novel combinations of virulence factors, and the clone containing the most successful

combinations of virulence factors become pathotype (Kaper, Nataro and Mobley, 2004). The mobile genetic elements play important role in the distribution and co-selection of resistance and virulence genes (Aslam *et al.*, 2018).

### 2.4 Strategy of disseminating resistance genes

The rapid dissemination of resistance can be attributed to the propagation of resistant strains and mobile genetic elements that harbour resistance genes (Blair *et al.*, 2014). Horizontal gene transfer is one of the most important mechanisms for the dissemination of antimicrobial resistance (Schroeder, Brooks and Brooks, 2017). It is defined as a lateral exchange of genetic material between organisms, where the genetic material from one cell can be incorporated into the genome of recipient, allowing for stable inheritance. This is different from vertical DNA transfer, which occurs from parent to offspring (Stokes and Gillings, 2011; San Millan, 2018). The units of horizontal gene transfer, including integrons, transposons, integrative conjugative elements, and plasmids, play a crucial role in the physical mobilisation of resistance genes (Stokes and Gillings, 2011).

The resistance can be propagated between bacteria of the same or different species or genera through three types of horizontal gene transfer: transformation, transduction, and conjugation (Schroeder, Brooks and Brooks, 2017). Transformation, which involves incorporation of naked DNA into chromosome, is rarely observed. Transduction, on the other hand, is mediated by bacteriophages. During conjugation, MGEs such as plasmids and transposons, which carry valuable genetic information, are transferred between bacterial cells via cell-to-cell contact (Hoffman, 2001). Furthermore, insertion sequences are also present in natural plasmids (Mahillon,

Léonard and Chandler, 1999). These insertion sequences and transposons have the ability to move between the chromosome and a plasmid, or between different plasmids, or back to the chromosome within a bacterial cell. However, this process requires the presence of a conjugative element, such as a plasmid or conjugative transposon (Smet *et al.*, 2010). Among these mechanisms, conjugation is believed to be the primary means of spreading resistance genes (Peterson and Kaur, 2018).

The use of antimicrobial agents can alter bacterial evolution by accelerating the mutation rate and promoting horizontal gene transfer. Exposure to antimicrobial agents induces a stress response in bacterial cells, which includes facilitating the lateral transfer of resistance genes and integron combination events. Horizontal gene transfer can also contribute to the evolution of multidrug-resistant pathogens with enhanced virulence by accumulating different virulence factors and plasmids (Stokes and Gillings, 2011).

## 2.5 Plasmid

A plasmid is a double-stranded, circular deoxyribonucleic acid (DNA) molecule that serves as an extrachromosomal genetic element with self-replicating ability in bacteria (Carattoli, 2009). Apart from self-replicating ability, plasmids can carry dispensable genes that are involved in plasmid transmission among bacteria, as well as gene conferring resistance to antibiotics and heavy metals (del Solar *et al.*, 1998). Therefore, it plays an essential role in the communication of genetic information among bacteria, promoting bacterial evolution and adaption (Shintani, Sanchez and Kimbara, 2015).

### 2.5.1 Plasmid DNA replication and replicon

In general, circular plasmids can replicate through three main mechanisms: theta type, strand displacement, and rolling circle. Theta-type replication is the most well-studied mechanism among circular plasmids in gram-negative bacteria. This type of replication involves the melting of the parental strands, synthesis of a primer RNA, and initiation of DNA synthesis by extending the primer RNA.

A conserved region within plasmid contains genes or loci responsible for replication and its control, collectively known as the replicon. The origin of replication (referred to as *ori*), the gene encoding Rep protein (*repA*) involved in the initiation of replication, and genes involved in the replication control are essential and conserved regions within the plasmid. Iterons, which are repeated sequences in the *ori*, serve as binding sites for the plasmid-encoded Rep proteins and play an important role in plasmid replication and its control. Rep proteins binding to specific sequences at the *ori* generate a nucleoprotein initiation complex where essential macromolecular interactions occur. On the other hand, replication control can also be mediated by antisense RNA (del Solar *et al.*, 1998).

### 2.5.2 Plasmids incompatibility (Inc) grouping

Plasmids are miscellaneous because they exhibit variation in size, G+C content, copy number, replication mechanism, and genetic cargo (Norman, Hansen and Sørensen, 2009). Hedges and Datta established a plasmid classification scheme plasmids known as incompatibility (Inc) grouping, which categorises plasmids into different groups. This grouping system involves introducing a plasmid of unknown Inc group into a strain containing a known Inc group of plasmids. If the resident plasmid is

removed, the incoming plasmid is assigned to the same Inc group as resident plasmid (Datta and Hedges, 1971). This phenomenon, called incompatibility, occurs when two plasmids with common replication controls are incompatible and cannot be stably propagated within the same cell line. Conversely, plasmids with different replication controls are considered compatible and can be co-resident in transconjugants (Datta and Hughes, 1983; Couturier *et al.*, 1988).

In *Enterobacteriaceae,* there are 27 recognised Inc groups, including IncA/C, IncD, IncJ, IncFI, IncFII, IncFIV, IncFV, IncFVI, IncI1, IncI2, IncIγ, IncHI1, IncHI2, IncHI3, IncHII, IncK, IncL/M, IncN, IncP, IncT, IncU, IncV, IncW, IncX, IncY, IncB/O, and com9 (Couturier *et al.*, 1988). Carattoli *et al.* developed a PCR-based replicon typing method that targets different replicon regions of plasmids, including *rep* genes, iterons, and RNAI (counter-transcript RNA). This method allows the screening 18 types of Inc groups, including FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA (Carattoli *et al.*, 2005).

### 2.5.3 Dissemination of plasmids via conjugation

Conjugation is considered as one of the most effective mechanisms for the dissemination of genetic elements among different bacteria (Guglielmini *et al.*, 2011). During conjugation, relaxase enzyme covalently binds to an origin of transfer (*oriT)* and mediates the cleavage of the transferring DNA at the *oriT* site. The resulting DNA-protein complex is then transported to the recipient cell through a protein export mechanism called the type IV secretion system (T4SS). This process is facilitated by the type IV coupling protein (T4CP), which actively pumps the single-stranded DNA into the recipient cell (Garcillán-Barcia, Francia and de La Cruz, 2009). Interestingly,

plasmids possessing conjugative transfer systems, such as IncF, IncI1, IncN, and IncHI1, display highly conserved genes that exhibit homology to a subset of the chromosome-located type IV secretion system. This unique characteristic enables these plasmids to participate in the uptake and export of DNA in various bacterial species (Carattoli, 2009; Wallden, Rivera-Calzada and Waksman, 2010).

Self-transmissible or conjugative plasmids are characterised by carrying a complete protein machinery for conjugal transfer, as mentioned earlier. In contrast, mobilisable plasmids possess a minimal gene set and rely on a helper conjugative plasmid to facilitate their conjugative transmission. It is important to note that all transmissible plasmids contain relaxase, while self-transmissible plasmids also carry T4SS. Conjugative plasmids typically exhibit a large size (>30 kb) and have a low copy number, whereas mobilisable plasmids tend to be smaller in size (<15 kb) and have a high copy number (Garcillán-Barcia, Francia and de La Cruz, 2009).

Apart from plasmid incompatibility grouping, another plasmid classification method has been established to categorise all transmissible plasmids whether conjugative or mobilizable, into six mobility (MOB) types: $MOB_C$, $MOB_F$, $MOB_H$, $MOB_P$, $MOB_Q$, and $MOB_V$. This classification is based on the amino acid sequences of the relaxase proteins (Garcillán-Barcia, Francia and de La Cruz, 2009; Smillie *et al.*, 2010; Garcillán-Barcia, Alvarado and de la Cruz, 2011). It is important this classification method specifically applies to transmissible plasmids (Shintani, Sanchez and Kimbara, 2015).

Plasmid classification based on replicon typing has two limitations: (i) it can be challenging to assign a plasmid into a single replicon group due to its multi-replicon status, and (ii) identifying replication regions for other types of plasmids is difficult due

to limited detailed information about Inc groups or Rep types across various microbial taxonomies. The MOB classification overcomes these limitations because plasmids rarely carry more than one relaxase gene, and this classification system can encompass the entirety of microbial plasmids (Shintani, Sanchez and Kimbara, 2015).

### 2.5.4 The association between resistance genes and plasmids

Carattoli conducted a review on the emergence and spread of antibiotic resistance mediated by plasmids in *Enterobacteriaceae*. Plasmid carrying resistance genes against β-lactam, aminoglycoside, and quinolone were identified in *Enterobacteriaceae* isolated from human and animal sources. This plasmids belonged to various incompatibility groups, including IncF, IncA/C, IncL/M, IncI1, IncHI2, and IncN (Carattoli, 2009). Plasmids carrying antimicrobial resistance genes such as ESBLs and AmpC , as well as virulence genes, have been documented (Carattoli, 2011). Plasmids carrying antimicrobial resistance can be horizontally transferred among *Enterobacteriaceae* via conjugation in the human gut, animals, and the environment (Bevan, Jones and Hawkey, 2017). The dissemination of ESBLs-carrying plasmids presents a significant challenge in the treatment of *Enterobacteriaceae* infections (Paterson, 2006).

#### 2.5.4.1 IncF and IncN plasmids

IncF plasmids have been associated with resistance genes for β-lactams (ESBLs and AmpC), quinolone, aminoglycosides, and virulence genes (Villa *et al.*, 2010). TEM-1, an ancestor of TEM-type ESBL, is predominantly carried by IncF plasmids, which are well adapted to *E. coli* and exhibit high conjugative ability (Marcadé *et al.*,

2009). The close relationship between IncF plasmids and the widespread dissemination of *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$, has been observed (Zhao and Hu, 2012). Furthermore, IncF plasmids have been implicated in the dissemination of SHV-type ESBLs in poultry (Pouget *et al.*, 2013).

IncN plasmids, known for their broad host-range, plays a significant role in the global dissemination of various genes in *Enterobacteriaceae* from animals to humans (Carattoli *et al.*, 2010). Multiple studies have investigated the association between IncN plasmids and the spread of various resistance genes, including ESBL (CTX-M-1), carbapenemase (VIM, KPC), and plasmid-mediated quinolone resistance gene (*qnr*) (Carattoli *et al.*, 2010; Karah *et al.*, 2010; Zhao and Hu, 2012; Dolejska *et al.*, 2013). IncN plasmids have implicated in the dissemination of SHV-type ESBLs in poultry (C. Dierikx *et al.*, 2013). In addition, genes conferring resistance to sulphonamides, aminoglycosides, tetracyclines, and streptomycin have been identified in IncN plasmids (Rozwandowicz *et al.*, 2018).

#### 2.5.4.2 IncI1 plasmids

The IncI plasmids encompass a group of low-copy-number plasmids with a narrow host range, including variants such as I1 (also named IncIα), I-γ, and I2 (also named IncIδ). Distinguishing between IncI-γ and IncI1 using PCR-based replicon typing can be challenging due to their high similarity. IncI plasmids have been identified as carriers of ESBL and pAmpC genes, such as CTX-M-1, TEM-52, and CMY-2 (Rozwandowicz *et al.*, 2018). The dissemination of *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-1}}$, and *bla*$_{\text{CTX-M-3}}$ is predominantly mediated by the IncI1 group (Zhao and Hu, 2012). IncI1 plasmids also plays a role in the dissemination of SHV-type ESBLs in poultry

(Bortolaia *et al.*, 2011; Accogli *et al.*, 2013; Pouget *et al.*, 2013). Furthermore, IncI plasmids carrying genes encoding resistance to aminoglycosides, tetracyclines, and quinolones are commonly detected in *S. enterica* (Rozwandowicz *et al.*, 2018).

#### 2.5.4.3 IncK and IncB/O plasmids

The similarity between RNAI sequences of IncK and IncB/O poses challenges in the PCR-based replicon typing scheme (Rozwandowicz *et al.*, 2018). It has been reported that non-specific amplification can occur when using the IncK and IncB/O primer pairs in PCR-based replicon typing (Williams *et al.*, 2013). IncK plasmids are commonly associated with the spread of β-lactam resistance genes, such as CMY-2 and CTX-M-14, while IncB/O plasmids are less prevalent and carry resistance genes against β-lactam, sulphonamide, streptomycin, and aminoglycoside (Zhao and Hu, 2012; Rozwandowicz *et al.*, 2018).

#### 2.5.4.4 IncHI1 and IncHI2 plasmids

IncHI1 and IncHI2 are known to commonly carry genes that confer resistance to various antibiotics, including β-lactams (ESBLs), sulphonamides, aminoglycosides, tetracyclines, streptomycin, chloramphenicols, and fluoroquinolones (Rozwandowicz *et al.*, 2018; McMillan, Jackson and Frye, 2020). IncHI1 plasmids are particularly associated with driving multiple resistance in *Salmonella* Typhi. On the other hand, IncHI2 plasmid have been implicated in colistin resistance gene, carrying genes such as *mcr-1* and *mcr-3* (Rozwandowicz *et al.*, 2018).

#### 2.5.4.5 IncA/C, IncX and IncY plasmids

IncA/C replicon has been further subclassified into two variants, IncA/C1 and IncA/C2, through molecular analysis (Hancock *et al.*, 2017). IncA/C plasmids are associated with multidrug resistance, including resistance to ESBLs, AmpC, carbapenemases, sulphonamides, aminoglycosides, tetracyclines, chloramphenicol, and trimethoprim, particularly in IncA/C2 (Rozwandowicz *et al.*, 2018). In humans, TEM-type ESBLs were found to be more prevalent in IncA/C plasmids in *E. coli* (Marcadé *et al.*, 2009).

IncX plasmid group can be further categorised into six subtypes (X1-X6) (Rozwandowicz *et al.*, 2018). The primers designed by Carattoli *et al.* (Carattoli *et al.*, 2005) primarily target the IncX2 subgroup (Johnson *et al.*, 2012). IncX plasmids are commonly isolated from *E. coli* and *Salmonella* originating from poultry sources. Among poultry samples, IncX1 is mainly associated with ESBLs such as TEM-52, while IncX2 frequently carries genes encoding plasmid-mediated quinolone resistance, such as *qnrS1* (Bielak *et al.*, 2011; Fortini *et al.*, 2011; Johnson *et al.*, 2012). IncX3, X4, X5, and X6 have been reported to carry genes encoding carbapenemases, particularly KPC and NDM, originating from human sources. TEM-1 is one of the resistance-associated genes found in IncX1 and IncX2 plasmids derived from poultry (Fortini *et al.*, 2011; Johnson *et al.*, 2012). IncY plasmids have been described as conferring resistance to ampicillin (Rozwandowicz *et al.*, 2018). IncY carrying SHV-2 has been detected (Billard-Pomares *et al.*, 2014).

### 2.5.5 The association between virulence genes and plasmids

In addition, a large number of virulence plasmids have been identified in *Enterobacterales*, including *Enterobacter*, *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia* (Shintani, Sanchez and Kimbara, 2015). These virulence-associated plasmids carry genes encoding colonisation factors, adherence factors, and toxins, which are often found in human intestinal pathogenic *E. coli* strains such as ETEC, EAEC, EIEC, EHEC, and EPEC (Kaper, Nataro and Mobley, 2004; Johnson and Nolan, 2009). Notably, plasmids encoding virulence-associated traits in *E. coli* are exclusively found within IncF family (Johnson and Nolan, 2009).

In human cell line models, *spv* genes found in the *Salmonella* virulence plasmids have been reported to associate with the intracellular proliferation of *Salmonella* in macrophages, apoptosis in intestinal epithelial cells, and intestinal barrier dysfunction (Libby *et al.*, 2000; Paesold *et al.*, 2002; Sun *et al.*, 2020). In the case of *Shigella*, the virulence plasmid carries genes involved in epithelial cells invasion, intracellular survival, the secretion and translocation of other effector proteins into eukaryotic host cells, and macrophage killing (Schroeder and Hilbi, 2008). It is important to note that plasmids play a significant role in the dissemination of virulence determinants among bacterial population.

## 2.6 Insertion sequence and its association with β-lactam resistance gene

Insertion sequences are the small DNA elements capable of independent transposition, leading to insertion mutations and rearrangements in the genome (Zhao and Hu, 2012). These sequences consist of a transposase gene flanked by terminal inverted repeats and generally do not encode any functions other than those involved in their mobility (Mahillon and Chandler, 1998). Insertion sequences are more commonly found on plasmids than on chromosomes (Siguier, Gourbeyre and Chandler, 2014). Transposons and insertion sequences can cause genome alterations, including deletions, duplications, and inversions within a single cell (Williams, 2016).

One specific insertion sequence, *IS26*, has been associated with the mobilisation of $bla_{SHV}$ (Ford and Avison, 2004). The gene encoding SHV-type ESBLs is commonly associated with the *IS26* element, as observed through sequence analysis of the regions surrounding these β-lactamase genes in *Enterobacteriaceae*. *IS26* is known to preferentially transpose within plasmids rather than into the chromosome, providing an explanation for the association between SHV-type ESBLs and specific plasmids through the linkage of *IS26* (Liakopoulos, Mevius and Ceccarelli, 2016).

It is commonly believed that CTX-M genes originate from the chromosome of *Kluyvera* species in soil (Blair *et al.*, 2014). These genes are then transferred to conjugative plasmids after escaping from the chromosome, aided by insertion sequences, particularly *ISEcp1* (Poirel, Naas and Nordmann, 2008; Blair *et al.*, 2014). In fact, several insertion sequence, such as *ISEcp1*, *ISCR1*, and various plasmids, have been associated with the mobilisation and dissemination $bla_{CTX\text{-}M}$ genes (Zhao and Hu, 2012). The roles of *ISEcp1* include encoding the transposase, which allows the mobilisation of $bla_{CTX\text{-}M}$ onto plasmids and acting as a strong promoter for the

expression of *bla*$_{\text{CTX-M}}$ (Bevan, Jones and Hawkey, 2017). It is interesting to note that CTX-M genes can be mobilised by *ISEcp1* onto non-IncF replicons, such as IncL/M, IncN, and IncI1. Additionally, transposition of CTX-M genes from these replicons to IncF plasmids can occur because they are not hindered by Tn3 mediated-transposition immunity (Marcadé *et al.*, 2009). This may explain the common co-carriage of TEM-1 and CTX-M-3/CTX-M-15 (Smith *et al.*, 2015).

Similarly, CMY-2 β-lactamase is also associated with the insertion sequence *ISEcp1* (Pietsch *et al.*, 2018), which has the ability to mobilise plasmid-borne AmpC gene onto plasmids of different replicon types and contains the promoter for high level expression of *bla*$_{\text{CMY-2}}$ (Jacoby, 2009). The presence of *ISEcp1* upstream of the CMY-2 gene has been observed in IncK, IncI1, and InA/C plasmids (Pietsch *et al.*, 2018).

*ISCR1*, *IS26*, and class 1 integrons are commonly described as mobile genetic elements in DHA-1 carrying plasmids. DHA-1 plasmids have been found to carry several resistance genes, such as those conferring resistance to β-lactam (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{KPC}}$), aminoglycosides, fluoroquinolones, phenicols, rifampicine, sulfonamides, trimethoprim, tetracyclines, and macrolides. It is concerning to observe that DHA-1 plasmids can accommodate many resistance genes, especially the carbapenemase gene (Hennequin, Ravet and Robin, 2018).

### 2.7 Transposon and its association with β-lactam resistance gene

Transposons are DNA sequence that can jump into different locations in the genome. They are categorised into two types: retrotransposons, commonly found in eukaryotes, and DNA transposons, present in both eukaryotes and prokaryotes. In bacteria, transposons belong to the DNA transposons and the Tn family, often carrying

additional antibiotic resistance genes. (Babakhani and Oloomi, 2018). While transposons share similarities with insertion sequences, they differ in that they encode additional factors that are independent of their mobility functions, such as antibiotic resistance or virulence genes (Williams, 2016). The mobilisation of DNA transposons is mediated by a cut-and-paste mechanism in which transposon is excised from one location and reintegrated into another site via recognition of terminal inverted repeats by transposase (Muñoz-López and García-Pérez, 2010). This ability of transposons to transfer between plasmids or between a chromosome and a plasmid, and vice versa, contribute to the transmission of antibiotic resistance genes in bacteria (Babakhani and Oloomi, 2018).

In a study by *Cloeckaert et al*., an IncI1 plasmid carrying TEM-52 (TEM-type ESBLs) on the Tn3 transposon was detected from *Salmonella* of poultry origin (Cloeckaert *et al.*, 2007). All TEM genes, including TEM-1 or TEM-2 and their derivatives, are carried by three of the earliest described bacterial transposons, namely Tn1, Tn2, and Tn3, which encode the transposase (*tnpA*) and resolvase genes (*tnpR*) (Poirel, Naas and Nordmann, 2008). However, a plasmid containing a copy of Tn3 is resistant to further insertions of Tn3, known as transposition immunity. Therefore, the transposition of TEM-type ESBLs from other replicons to IncF plasmids carrying TEM-1 is unlikely due to transposition immunity, which may explain why two TEM genes cannot be located within the same replicon (Marcadé *et al.*, 2009).

### 2.8 Dissemination of ESBL/AmpC-producing isolates between poultry and human

It has been hypothesised that food-producing animals might become a potential source of antimicrobial-resistant bacteria in humans (Lazarus *et al.*, 2015). There are

two mechanisms through which transmission may occur from animals to humans. The first mechanism is known as whole bacterium transmission, where bacteria carrying resistance may be directly transmitted from animals to humans. Animals-derived bacterial clone can propagate during the food production process and may cause extraintestinal infection in humans after ingestion. The second mechanism is MGE-mediated transmission, in which resistance genes can be spread from bacteria of animal origin to human bacteria through mobile genetic elements such as plasmids. This mode of transmission can occur at any stage of the transmission paradigm, including within the human gastrointestinal tract (Lazarus *et al.*, 2015).

Several studies have been conducted to compare resistance genes, plasmid types, and bacterial strain types between *E. coli* strains collected from poultry and humans. These studies have provided evidence supporting the concept of whole bacterium transmission between these two sources, as indistinguishable sequence types of *E. coli*, ESBL genes (such as CTX-M-1 and TEM-52), and plasmids were identified in both poultry and human isolates (Leverstein-van Hall *et al.*, 2011; Overdevest *et al.*, 2011; Kluytmans *et al.*, 2013; Huijbers *et al.*, 2014). Furthermore, Berg *et al.* conducted a study that supported the hypothesis of clonal transmission of AmpC-producing *E. coli* from poultry to humans. This study revealed the presence of isolates from poultry and humans that shared an identical sequence type (ST38) and exhibited less than 15 single nucleotide polymorphism (SNP) differences in 2.5 megabases of shared genome sequences (Berg *et al.*, 2017).

There are several studies that provide support for the hypothesis of MGE-mediated transmission through plasmids. For instance, IncI1 plasmids carrying $bla_{\text{CTX-M-1}}$ were found in heterogenous *E. coli* lineages from both humans and poultry, suggesting that these plasmids may play an important role in the effective dissemination

of ESBL β-lactamases among *E. coli* populations in different reservoirs (de Been *et al.*, 2014; Huijbers *et al.*, 2014; Börjesson *et al.*, 2016). Additionally, *E. coli* lineages carrying *bla*$_{\text{CMY-2}}$ on IncK or IncI1 plasmids showed high variability among isolates from human and poultry, further supporting the involvement of plasmid-mediated horizontal transfer in the transmission of CMY-2-mediated cephalosporin resistance between humans and animals (Voets *et al.*, 2013; de Been *et al.*, 2014; Huijbers *et al.*, 2014; Hansen *et al.*, 2016; Pietsch *et al.*, 2018).

Highly similar IncK plasmid carrying *bla*$_{\text{CMY-2}}$ have been reported in both clinical and poultry isolates (Berg *et al.*, 2017). Whole genome sequencing has revealed the presence of two plasmid addiction systems in poultry derived-IncK/*bla*$_{\text{CMY-2}}$ plasmids (Mo *et al.*, 2016). Plasmid addiction systems enhance plasmid stabilisation by eliminating plasmid-free daughter cells after cell division (Unterholzner, Poppenberger and Rozhon, 2013). This mechanism may contribute to the persistent maintenance and dissemination of pAmpC-producing *E. coli* in the broiler production, even in the absence of selection pressure from antimicrobial use (Mo *et al.*, 2016).

It has been suggested that the ingestion of contaminated meat might be responsible for the widespread dissemination of antimicrobial-resistant bacteria between animals and humans (Leverstein-van Hall *et al.*, 2011). Expanded-spectrum cephalosporin-resistant *Enterobacteriaceae* present in the guts of animals can directly contaminate meat during slaughter or be released into the soil and water, leading to indirect contamination (Lazarus *et al.*, 2015). For example, the occurrence of resistant bacteria in meat is suspected to result from faecal contamination, as identical PFGE (pulsed-field gel electrophoresis) patterns were observed in *E. coli* carrying *bla*$_{\text{CMY-2}}$ on IncK plasmid from Swedish broiler cecum and Swedish meat (Börjesson *et al.*, 2013). After ingestion, resistant bacteria may colonize the intestinal tract and increase the risk

of extraintestinal infection, such as urinary tract infections (Lazarus *et al.*, 2015). Extensive gene transfer occurring in human intestine microbiome may accelerate the spread of resistance genes in gut commensals, posing a potential threat when treating infections (Salyers, Gupta and Wang, 2004).

### 2.9 Use of antibiotics in poultry farming

Poultry is a globally prevalent food industries globally due to its relatively low production costs and lack of restrictions on consumption from cultural and religious perspectives (Nhung, Chansiripornchai and Carrique-Mas, 2017). Bacterial and parasitic infections are common in intensive animal production industries (Hao *et al.*, 2014). Antibiotics are frequently used in food animals for disease treatment. In the case of poultry, treating entire groups by medicating feed or water is a feasible method instead of treating individual animals (McEwen and Fedorka-Cray, 2002). Subtherapeutic doses of antibiotics are routinely administered to promote animal growth and improve feed conversion (Hao *et al.*, 2014). In addition, antimicrobials can prevent disease, even at subtherapeutic doses (McEwen and Fedorka-Cray, 2002). The use of subtherapeutic antibiotic doses can alter the immune status of broiler chickens and modify the gastrointestinal microbiota (Torok *et al*., 2011; Lee *et al*., 2012; Singh *et al*., 2013).

There are variations among countries regarding the antimicrobial agents used for animal treatment (Blanc *et al.*, 2006). Antimicrobials such as tetracyclines, aminoglycosides, sulfonamides, and penicillins are approved for use in poultry in countries like Brazil, the US, the United Kingdom, Germany, France, Poland, Spain, and China (Roth *et al.*, 2019). It is noteworthy that many of the antimicrobials used in

livestock production are also important medicines for humans (Nhung, Chansiripornchai and Carrique-Mas, 2017). In several countries, ampicillin and amoxicillin are commonly used to treat avian infections (Smet *et al.*, 2010). These antibiotics are administered to combat bacterial diseases such as collibacilosis (caused by pathogenic *E. coli*), *Ornithobacterium rhinotracheale* infection, *Riemerella anatipestifer* infections, and fowl cholera (caused by *Pasteurella multocida*). Benzylpenicillins are employed to address dysbacteriosis. The use of third-generation cephalosporins in poultry is less frequent (Smet *et al.*, 2010). Ceftiofur, a third-generation cephalosporin, is not authorised for use in poultry within the European Union. However, the United States permits its use in 1-day-old chickens to prevent early mortality (Heinrich *et al.*, 2013). The use of extended-spectrum cephalosporins and cephamycins for treatment purposes is very uncommon. The detection of ESBLs/AmpC β-lactamase in bacteria originating from livestock may be associated with co-selection due to the administration of other non-beta-lactam antibiotics (Blanc *et al.*, 2006).

### 2.10 Alternatives to antibiotics with less health impact on human and environment

The use of sub-lethal concentrations of antibiotics promotes the selection, mobilisation, and the transfer of resistance determinants (Gullberg *et al.*, 2011; Bengtsson-Palme, Kristiansson and Larsson, 2018). Antibiotics used in livestock increase selective pressure on both commensal and pathogenic bacteria, which can potentially spread to humans through direct contact and food chain, or indirectly through environmental pollution from farm effluents (Roca *et al.*, 2015).

Due to the harmful effects of antibiotic use on the environment and consumer health, many studies have been conducted to search for natural alternatives to antibiotics. These alternatives must fulfil specific requirements, such as maintaining a low mortality rate, promoting animal yield, and preserving the environment and consumer health. Several alternatives are available, including phytogenic feed additives, essential oils, probiotics, prebiotics, enzymes, organic acids, and others (Mehdi *et al.*, 2018).

Phytogenic feed additives are derived from plants, herbs, and spices (Mehdi *et al.*, 2018). Several studies have suggested that phytogenic feed additives serve as viable alternatives to antibiotics, as they have demonstrated positive effects on broiler chicken growth, improved immune system function, and antimicrobial activity (Ghasemi, Kasani and Taherpour, 2014; Jarriyawattanachaikul, Chaveerach and Chokesajjawatee, 2016).

Essential oils are aromatic oily liquids extracted from plants, known for their odoriferous and volatile properties (Mehdi *et al.*, 2018). Essential oil and organic acids have shown promising effects as growth promoters, improving chicken production (Adil *et al.*, 2010; Khattak *et al.*, 2014).

In addition, probiotics are live microorganisms intended to provide health benefits to the host when administered in adequate amounts (Mehdi *et al.*, 2018). Several studies have explored the roles of probiotics in terms of their antibacterial and anticoccidial effects, as well as their potential to improve poultry meat quality (Giannenas *et al.*, 2012; Levkut *et al.*, 2012; Popova, 2017).

Prebiotics are non-digestible food ingredients that can stimulate the growth and/or activity of beneficial microorganisms in the colon, thereby providing beneficial

functions to host health (Gibson and Roberfroid, 1995). Prebiotics have been found to enhance chicken intestinal health by modifying the morphological development of the intestine and promoting colonization by beneficial bacteria (Baurhoo, Ferket and Zhao, 2009).

# CHAPTER 3 MATERIAL and METHODS

## 3.1 Medium preparation

### 3.1.1 LB (Luria-Bertani) agar

LB agar was prepared by dissolving 15g of Agar Bacteriological (Oxoid, UK), 10g of tryptone, 10g of sodium chloride (NaCl), and 5g of yeast extract (Oxoid, UK) in 1L of purified water in a bottle, followed by autoclaving at 121℃ for 15 minutes. After autoclaving, the tightly closed bottle was gently rolled on the table to ensure homogenisation. In the biosafety cabinet, the cap of bottle was opened, and the agar medium was poured gently onto a sterile petri dish when the temperature of the agar medium reached 50-60℃. The filled petri dish was swirled gently to cover the entire surface and prevent the formation of bubbles. The lid of the petri dish was left slightly open in the biosafety cabinet for 5 to 15 minutes to allow agar to solidify. The lid was closed once the agar plate was completely solidified. The solidified agar plate was labelled with the agar type and date of preparation, stacked, packed, and sealed in a sterile plastic bag. The agar plates were stored upside down in a refrigerator at 4℃.

### 3.1.2 LB (Luria-Bertani) broth

LB broth was prepared by adding 10g of tryptone, 10g of NaCl, and 5g of yeast extract (Oxoid, UK) to 1L of purified water in a beaker. The mixture was thoroughly stirred and then dispensed evenly into small bottles, which were subsequently autoclaved at 121℃ for 15 minutes to ensure sterility. After autoclaving, the bottles containing the broth were stored in a refrigerator at 4℃.

### 3.1.3 Mueller Hinton agar

Mueller Hinton agar was prepared by adding 38g of Mueller Hinton Agar (Oxoid, UK) to 1L of purified water in a bottle, followed by autoclaving at 121℃ for 15 minutes. The preparation procedure for Mueller Hinton agar is similar to that of LB agar, as mentioned above.

## 3.2 Sample collection

Fifty-nine bacterial strains isolated from poultry faecal samples in Malaysian commercial poultry farm were provided by Dr. Nabin Rayamajhi (Warrenton Regional Animal Health Laboratory, 272 Academy Hill Rd,Warrenton, VA 20186 , USA). The faecal samples were collected from three commercial poultry farms in West coast, Peninsular Malaysia. Farm 1 and Farm 2 have three to five sheds, while Farm 3 has two big sheds with a temperature control system. Diseased broiler chicken samples (under enrofloxacin treatment) were collected from farms 1 and 2, while the samples from healthy broiler chickens were collected from farm 3. Farm 1 and 2 raised broiler chickens aged 3-7 weeks, while farm 3 raised younger broiler chickens aged 10-12 days.

A total of 100 faecal samples were collected from randomly selected chickens, and 30-40 samples were obtained from each farm. The faecal swabs were stored at 4°C and subjected to microbiological analysis within 24 hours after sample collection. The faecal swabs were pooled in 20 mL of Luria-Bertani (LB) broth, and each enriched sample was streaked onto MacConkey agar plate to select *Enterobacteriaceae*. All samples yielded positive results for *Enterobacteriaceae* isolates, and one enteric isolate were isolated from each sample. In this study, a total of 59 stock cultures of *Enterobacteriaceae* isolated from poultry faecal samples were used. All isolates were archived in glycerol stocks and stored at -80℃ for further use.

### 3.3 Revival of bacterial glycerol stock

The glycerol stocks of bacteria obtained from poultry faecal samples were revived by culturing them on LB agar. The glycerol stocks, which were stored at -80℃, were kept in an ice box during the entire procedure. Inside the biosafety cabinet, the inoculation loop was inserted into Inotech Steri 350 (Simon Keller AG, Switzerland) containing glass beads. The Inotech Steri 350 maintained an inner temperature of 250℃, and the loop was kept for 30 seconds, followed by a few seconds of cooling. The loop was sterilised before being used to scrape the top of the frozen glycerol stock of bacteria. The scraped inoculation loop was then used to streak the LB agar using the four-way streaking method. The loop was sterilised before each streaking. The streaked agar plates were then placed in an incubator set at 37℃ for 18-20 hours to allow for bacterial growth.

### 3.4 Bacterial DNA extraction

The bacterial DNA was extracted using the boiling method. (Briñas *et al.*, 2002). Single colony bacteria were picked and suspended in 100µl of sterile water. The bacterial suspension was then boiled at 99℃ for 10 minutes using an Eppendorf ThermoMixer (Eppendorf, Germany). After boiling, the suspension was centrifuged at 13,000 rpm (15,871 × *g*) for 2 minutes to spin down the cell debris. The supernatant, containing the crude DNA, was carefully transferred to a new sterile tube, which was labelled accordingly, and stored at -20℃ for further use. The obtained supernatant served as the template for PCR analysis.

### 3.5 Polymerase chain reaction (PCR)

The PCR reaction mixture was prepared by adding the reagents in the following order: sterile water, 10x $NH_4^+$ Reaction Buffer, dNTPs (deoxynucleoside triphosphate), $MgCl_2$ (magnesium chloride), primers, *Taq* DNA polymerase, and template (refer to Table 3.1) (Lorenz, 2012). The volume of sterile water varied depending on the concentration of primers, aiming for a final volume of 20µL per reaction. Calculation was necessary to determine the amount of each component to be pipetted when preparing a master mix for multiple PCR tests. The volume of each component was multiplied by the number of PCR tests. An example of how to calculate the volume of components required when preparing a 10x master mix with a primer concentration of 0.5 µM is provided in Table 3.2. The master mix was prepared by adding the corresponding volume of reagents, excluding the DNA template. 19µL of the master mix was aliquoted into sterile PCR tubes. Each PCR reaction included negative and positive controls. The PCR tubes were then placed into a Bio-Rad T100$^{TM}$ thermal

cycler (Bio-Rad, USA). The PCR condition was set as follows: initial denaturation at 95℃ for 5 minutes, followed by 30 cycles of denaturation at 95℃ for 30 seconds, annealing at 52-63℃ for 30 seconds, elongation at 72℃ for 30-60 seconds, and a final extension at 72℃ for 5 minutes.

**Table 3.1**: Descriptive information of volume and concentration of various reagents in each PCR reaction mixture.

| **PCR reagent** | **Volume (µL)** | **Final Concentration** |
|---|---|---|
| Sterile distilled water | Variable | - |
| 10X $NH_4^+$ Reaction Buffer (Bioline, UK) | 2 | 1X |
| dNTP Mix (Total 10mM, 2.5mM of dATP, dTTP, dGTP, and dCTP) (Promega, USA) | 1.6 | 800 µM (200 µM each dNTP) |
| $MgCl_2$ (50 mM) (Bioline, UK) | 0.4 | 1 mM |
| 10µM Forward Primer (Integrated DNA Technologies, USA) | 0.3-1.2 | 0.15-0.6 µM |
| 10µM Reverse Primer (Integrated DNA Technologies, USA) | 0.3-1.2 | 0.15-0.6 µM |
| BIOTAQ DNA Polymerase (5 unit/µL) (Bioline, UK) | 0.4 | 0.1 unit/µL |
| DNA Template | 1 | - |
| Total volume | 20 | |

*$NH_4^+$: ammonium; dNTP: deoxynucleoside triphosphate; dATP: deoxyadenosine triphosphate; dTTP: deoxythymidine triphosphate; dGTP: deoxyguanosine triphosphate; dCTP: deoxycytidine triphosphate; $MgCl_2$: magnesium chloride

**Table 3.2**: An example of how to calculate the volume of components required when preparing a PCR master mix with a 0.5 μM primer concentration.

| PCR reagent | Final Concentration | Volume (μL) per reaction | Volume (μL) in master mix (10 reactions) |
|---|---|---|---|
| Sterile distilled water | - | 12.6 | 126 |
| 10X $NH_4^+$ Reaction Buffer | 1X | 2 | 20 |
| dNTP Mix | 800 μM | 1.6 | 16 |
| $MgCl_2$ (50 mM) | 1 mM | 0.4 | 4 |
| 10μM Forward Primer | 0.5 μM | 1 | 10 |
| 10μM Reverse Primer | 0.5 μM | 1 | 10 |
| BIOTAQ DNA Polymerase (5 unit/μL) | 0.1 unit/μL | 0.4 | 4 |
| Total volume | | 19 | 190 |

*$NH_4^+$: ammonium; dNTP: deoxynucleoside triphosphate; $MgCl_2$: magnesium chloride

### 3.6 Agarose gel electrophoresis and gel imaging

To prepare 2% agarose gel, approximately 1.2g of agarose powder was added into 60mL of 1X TBE buffer (89mM Tris, 89mM Boric acid, 2mM EDTA). The mixture was heated in microwave until fully dissolved. Once dissolved, the gel was pre-stained by adding 3μL of FloroSafe DNA Stain (First base, Singapore) to the solution. The solution was then poured into a gel tray with a comb when the temperature cooled down to 50-60℃. After solidification, the agarose gel was placed in a tank containing 1X TBE buffer.

For gel loading, approximately 10μL of PCR product was gently mixed with 2μL of Tri-Color 6X DNA Loading Dye (First base, Singapore). 10μL of mixture and

6μL of GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific, USA) were loaded into separate wells in the pre-stained 2% TBE-agarose gel. The gel electrophoresis was conducted at 70V, 400mA for 50 minutes.

To visualise the gel, a ChemiDoc™MP Gel Imaging System (Bio-rad, USA) was used. The size reference of the GeneRuler™ 1 kb Plus DNA Ladder can be found in Appendix 1.

### 3.7 Bacterial identification by biochemical tests

#### 3.7.1 Gram stain

All isolates were selected to perform Gram staining. The inoculation loop sterilised by a Bunsen burner was used to add a few loopfuls of sterile water onto a new microscope slide. A single colony on LB agar, cultured for 18-24 hours, was scraped and transferred to the slide. It was evenly spread on the slide (15mm x 30mm) using the sterilised loop. The slide was then dried by moving it in a circular motion over the flame of the Bunsen burner.

Next, a few drops of crystal violet, the primary stain, were added to cover the fixed culture on the slide for 60 seconds, followed by rinsing with water. The slide was then covered with iodine solution for 60 seconds and rinsed again with water. Subsequently, a few drops of 95% ethyl alcohol (ethanol solvent) were added to the slide for 15 seconds, followed by immediate rinsing with water. The smear on the slide was counterstained with 0.1% basic fuchsin solution for 60 seconds, followed by rinsing and air-drying. The dried slide was examined under a microscope. Gram-positive bacteria retained a purple colour, while gram-negative bacteria were stained pink.

### 3.7.2 Catalase test

To perform the catalase test, a single colony from LB agar (cultured for 18-24 hours) was picked using a sterile plastic inoculating loop or toothpick and placed onto a sterile petri dish. One drop of 3% hydrogen peroxide ($H_2O_2$) was added onto the smear, and the immediate formation of bubbles was observed in catalase-positive bacteria.

### 3.7.3 Oxidase test

For oxidase test, a piece of filter paper was placed in sterile petri dish and soaked with 2-3 drops of an oxidase test kit, which contains N,N-dimethyl-1,4-phenylene diamine and alpha-naphtol. A single colony from LB agar (cultured for 18-24 hours) was picked and scraped onto the soaked filter paper using a sterile plastic inoculating loop or toothpick. The colour change to purple within 5-90 seconds was interpreted as oxidase positive, while no colour change or a change that takes longer than 2 minutes was interpreted as oxidase negative.

### 3.7.4 API 20E test

All Gram-negative, catalase-positive, and oxidase-negative isolates were further identified using API® 20E test kit (BioMérieux, France) according to the manufacturer's instruction. The API 20E strip consists of 20 microtubes, each containing different tests, including ONPG, ADH, LDC, ODC, CIT, $H_2S$, URE, TDA, IND, VP, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY, and ARA. Each microtube contains specific active ingredients that indicate the presence of certain reactions or enzymes (refer to Table 3.3).

A single colony from LB agar plate (cultured for 18-24 hours) was selected and emulsified in 5mL of sterile distilled water. The resulting bacterial suspension was transferred into the tubes of all tests, excluding the cupules, using a sterile pipette. For the CIT, VP, and GEL tests, the tubes and cupules were completely filled with the suspension. The cupules for the ADH, LDC, ODC, $H_2S$, and URE tests were overlaid with sterile mineral oil. The API 20E strip was placed in an incubation box containing a small amount of distilled water and incubated at 37℃ for 18 hours. After the incubation periods, specific reagents were added to three tests: TDA, IND, and VP. One drop of TDA reagent and JAMES reagent was added to the tubes of the TDA and IND test, respectively. The tube of the VP test was added with one drop of VP 1 and VP 2 reagents, and the result was observed after 10 minutes. The strip was read and interpreted according to Table 3.3. The 20 tests and the oxidase test (total of 21 tests) were divided into groups of 3, and a value 1, 2 or 4 was assigned for each test (total of 7 groups). After interpreting the result, the sums of the values for positive reaction were calculated within each group to obtain a seven-digit numerical profile, which was then input into the API 20E software to identify the bacterial genus.

**Table 3.3**: The information of 20 microtubes in API 20E strip, including active ingredients, reactions/enzymes, colour interpretation result, and numerical profile.

| Tests | Ingredients | Reactions/Enzyme | Result | | Numerical profile | |
|---|---|---|---|---|---|---|
| | | | Negative | Positive | Mark | Group |
| ONPG | 2-nitrophenyl-ßD-galactopyranoside | ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase) | colorless | yellow | 1 | 1 |
| ADH† | L-arginine | Arginine Dihydrolase | yellow | red / orange | 2 | |
| LDC† | L-lysine | Lysine Decarboxylase | yellow | red / orange | 4 | |
| ODC† | L-ornithine | Ornithine Decarboxylase | yellow | red / orange | 1 | 2 |
| CIT* | trisodium citrate | Citrate utilization | pale green / yellow | blue-green / blue | 2 | |
| $H_2S$† | sodium thiosulfate | $H_2S$ production | colorless / greyish | black deposit / thin line | 4 | |
| URE† | urea | Urease | yellow | red / orange | 1 | 3 |
| TDA | L-tryptophane | Tryptophane Deaminase | yellow | reddish brown | 2 | |
| IND | L-tryptophane | Indole production | Colorless pale green / yellow | pink | 4 | |
| VP* | sodium pyruvate | acetoin production (Voges Proskauer) | colorless | pink / red | 1 | 4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| GEL* | Gelatin (bovine origin) | Gelatinase | no diffusion | diffusion of black pigment | 2 | |
| GLU | D-glucose | fermentation / oxidation of glucose | blue / blue-green | yellow / greyish yellow | 4 | |
| MAN | D-mannitol | fermentation / oxidation of mannitol | blue / blue-green | yellow | 1 | 5 |
| INO | inositol | fermentation / oxidation of inositol | blue / blue-green | yellow | 2 | |
| SOR | D-sorbitol | fermentation / oxidation of sorbitol | blue / blue-green | yellow | 4 | |
| RHA | L-rhamnose | fermentation / oxidation of rhamnose | blue / blue-green | yellow | 1 | 6 |
| SAC | D- sucrose | fermentation / oxidation of sucrose | blue / blue-green | yellow | 2 | |
| MEL | D-melibiose | fermentation / oxidation of melibiose | blue / blue-green | yellow | 4 | |
| AMY | amygdalin | fermentation / oxidation of amygdalin | blue / blue-green | yellow | 1 | 7 |
| ARA | L-arabinose | fermentation / oxidation of L-arabinose | blue / blue-green | yellow | 2 | |
| | | Oxidase test (not in API test kit) | | | 4 | |

*For the CIT, VP and GEL tests: both the tube and cupule were filled with the bacterial suspension

†For the ADH, LDC, ODC, $H_2S$ and URE tests: the tube was filled with the bacterial suspension, and the cupule was filled with mineral oil

### 3.8 16S rRNA sequencing

A few strains from each genus groups identified by the API test were randomly selected for 16S rRNA sequencing to further confirm the identity of the bacteria. DNA extraction was performed using the method described in Method 3.4. The specific primers used for 16S rRNA amplification are displayed in Table 3.4, and their primer melting temperature ($T_m$) and GC content were determined using OligoAnalyzer Tool (Integrated DNA Technologies, USA). The PCR reaction mixture was prepared according to Method 3.5, with different primer concentration/volume and water volume as specified in Table 3.5. The thermal cycler was set with condition similar to those mentioned in Method 3.5, except for the annealing temperature and duration of elongation, as indicated in Table 3.5. A negative control was prepared by substituting water for the DNA template while DNA template of *E. coli* ATCC® 25922 was used as positive control. The PCR products were subjected to agarose gel electrophoresis (refer to Method 3.6) for verification. The successful amplification of the 16S rRNA gene was confirmed by the presence of a 444bp band, similar to the positive control, and the absence of band in the negative control. The PCR amplicons were then sent for sequencing at Apical Scientific in Singapore, after verification of PCR quality by gel electrophoresis. The obtained sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis, which generates a similarity score by comparing the resulting sequence with reference sequence of known species (specifically, Accession number KY962911.1, OM816745.1, OL958647.1, OM666543.1, and MT525340.1). A similarity score of $\geqslant$99% allows identification at the species level, while the score of <99% and $\geqslant$95% is sufficient for assigning the identification to the genus level (Bosshard *et al.*, 2003).

### 3.9 Differentiation between *E. coli* from *Shigella spp.* by screening *lacY*

Two *Shigella spp.* isolates, previously identified through API test, underwent further confirmation of their identity by screening for the presence of a lactose permease gene (*lacY*). This gene is typically found in *E. coli* and can be used to differentiate it from *Shigella spp.* The characteristics of the primers, including their sequence, melting temperature ($T_m$), GC content, and the size of amplified fragment, are listed in Table 3.4. The PCR reaction mixture for screening *lacY* was prepared following the instructions in Method 3.5, with different primer concentration/volume and water volume as specified in Table 3.5. *E. coli* ATCC® 25922 was used as a positive control, and sterile water served as a negative control. The thermal cycler was set with condition similar to those mentioned in Method 3.5, except for the annealing temperature and duration of elongation, as indicated in Table 3.5. After amplification, the PCR products were analysed by gel electrophoresis, and the result were visualized using a gel imaging system (refer to Method 3.6).

**Table 3.4**: Primers used for 16S rRNA sequencing and screening of *lacY*.

| Target of PCR | Primers' feature | | | | | Reference |
|---|---|---|---|---|---|---|
| | Primer | Sequence (5'-3') | $T_m$ (℃) | GC content (%) | Size (bp) | |
| 16S rRNA | 341-F | CCTACGGGAGGCAGCAG | 58.2 | 70.6 | 444 | (Klindworth *et al.*, 2013) |
| | 785-R | GACTACCAGGGTATCTAATCC | 51.2 | 47.6 | | |
| *lacY* | lacY-F | ACCAGACCCAGCACCAGATAAG | 58.7 | 54.5 | 463 | (Horakova, Mlejnkova and Mlejnek, 2008) |
| | lacY-R | GCACCTACGATGTTTTTGACCA | 55.7 | 45.5 | | |

* $T_m$: Primer melting temperature.

**Table 3.5**: PCR reaction mixture and PCR conditions for 16S rRNA sequencing and screening of *lacY*.

| Target of PCR | Primer | PCR reaction mixture | | | PCR condition | | Positive control |
|---|---|---|---|---|---|---|---|
| | | Final primer concentration (µM) | Volume of 10µM primer in 20µL PCR reaction mixture (µL) | Volume of distilled water in 20µL PCR reaction mixture (µL) | $T_{anneal}$ (℃) | $t_{elongate}$ (second) | |
| 16S rRNA | 341-F | 0.4 | 0.8 | 13 | 58 | 45 | *E. coli* ATCC® 25922 |
| | 785-R | | 0.8 | | | | |
| *lacY* | lacY-F | 0.5 | 1 | 12.6 | 58 | 45 | *E. coli* ATCC® 25922 |
| | lacY-R | | 1 | | | | |

*$T_{anneal}$: annealing temperature; $t_{elongate}$: elongation time; Positive control: bacterial strain used as positive control for gene of interest.

### 3.10 Antimicrobial susceptibility test via disk diffusion

The antimicrobial susceptibility test was conducted using the disk diffusion method following the procedure outlined by Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2015). All isolates were tested for susceptibility to four cephalosporins: cefepime (FEP, 4$^{th}$ generation cephalosporin, 30µg), cefotaxime (CTX, 3$^{rd}$, 30µg), ceftazidime (CAZ, 3$^{rd}$, 30µg), and cefoxitin (FOX, 2$^{nd}$ generation cephalosporin/cephamycin, 30µg) (Oxoid, UK). The results of the antimicrobial susceptibility test were analysed according to the criteria provided by CLSI (CLSI, 2021). *E. coli* ATCC® 25922 was used as a quality control (QC) strain for the test.

All strains were streaked on LB agars and incubated at 37℃ overnight. For each strain, a single colony was transferred into a 5mL LB broth and incubated in a shaker incubator at 37℃ and 180rpm for 2-4 hours. The turbidity of the broth was adjusted with sterile LB broth to achieve a 0.5 McFarland standard, which corresponds to an optical density (OD) of 0.08-0.1 at 625nm (Balouiri, Sadiki and Ibnsouda, 2016). The OD was measured using a Biochrom Libra S12 UV-Vis Spectrophotometer (Biochrom, UK).

To sterilise the Cell Spreader Stainless Steel and forceps, they were inserted into the glass beads of the Inotech Steri 350 and sterilised for 30 seconds. After sterilisation, they were allowed to cool down for a few seconds. Approximately 100µL of the adjusted culture was pipetted onto the Mueller Hinton agar and spread evenly using the sterilised Cell Spreader Stainless Steel. The horizontal part of the Cell spreader was brought into contact with the agar, and the petri dish was rotated in a clockwise or anticlockwise direction to ensure even spreading. After spreading, the lid of petri dish was left partially open for 3-5 minutes until the suspension was fully absorbed. The

inoculum suspension was used within 15 minutes after adjusting the turbidity to match that of the 0.5 McFarland standard.

The sealed packages containing antimicrobial disk cartridges were taken out from the refrigerator 1-2 hours prior to use to allow them to equilibrate to room temperature. The packages were then opened. The antimicrobial disks were placed on inoculated Mueller Hinton agar plates using sterilised forceps. Two disks were distributed evenly and pressed down on each plate, ensuring a distance of no less than 24mm from the centre of one disk to the centre of the other. The plates were inverted and incubated at 37℃.

After 16-18 hours of incubation, the results were examined by measuring the diameters of the zone of inhibition (ZOI) using a ruler held against the back of the inverted plate. The ZOI measurements were recorded and interpreted according to Table 3.6, only when the ZOI of the QC strain fell within the standard range specified in Table 3.7. The QC strain was tested with every batch, and each strain was tested in triplicate.

**Table 3.6**: Zone Diameter Breakpoints of antimicrobial agents for *Enterobacterales*. Adapted from (CLSI, 2021).

| Group | Antimicrobial Agent | Disk Content | Interpretive Categories and Zone Diameter Breakpoints (mm) | | | |
|---|---|---|---|---|---|---|
| | | | S | SDD | I | R |
| 4th generation Cephalosporin | Cefepime | 30µg | ≥ 25 | 19-24 | - | ≤18 |
| 3rd generation Cephalosporin | Cefotaxime | 30µg | ≥ 26 | - | 23-25 | ≤ 22 |
| 3rd generation Cephalosporin | Ceftazidime | 30µg | ≥ 21 | - | 18-20 | ≤17 |
| 2nd generation Cephalosporin/ Cephamycin | Cefoxitin | 30µg | ≥ 18 | - | 15-17 | ≤14 |

*S: Susceptible; SDD: Susceptible-dose dependent; I: Intermediate; R: Resistant

**Table 3.7**: Disk Diffusion QC Ranges of *E. coli* ATCC® 25922 for four antimicrobial agents. Adapted from (CLSI, 2021).

| Antimicrobial Agent | Disk Content | Disk Diffusion QC Ranges, mm |
|---|---|---|
| | | ***E. coli* ATCC® 25922** |
| Cefepime | 30µg | 31-37 |
| Cefotaxime | 30µg | 29-35 |
| Ceftazidime | 30µg | 25-32 |
| Cefoxitin | 30µg | 23-29 |

### 3.11 Phenotypic ESBL detection by double disk synergy test

All CTX-resistant isolates were tested for phenotypic ESBL production by the double-disk synergy test (DDST) (Drieux *et al.*, 2008). The bacterial suspension with a turbidity of 0.5 McFarland standard (equivalent to OD 0.08-0.1 at 625nm) was prepared for the inoculum using a spectrophotometer.

The Cell Spreader Stainless Steel and forceps were sterilised using the Inotech Steri 350 for 30 seconds, followed by a brief cooling period. Approximately 100µL of the adjusted inoculum suspension was pipetted onto the Mueller Hinton agar and spread evenly using Cell Spreader Stainless Steel. The plate was then left undisturbed for 3-5 minutes to allow suspension to fully absorb.

The antimicrobial disk cartridges were removed from the refrigerator 1-2 hours before use. The sterilised forceps were used to place a disk of amoxicillin/clavulanic acid (AMC, 20/10 µg) and a 30-µg cephalosporin disk (CTX, cefotaxime; CAZ, ceftazidime) at the distance of 20mm and 30 mm (centre to centre) from each other, as illustrated in Figure 3.1. The disks were gently pressed down onto the agar, and the plate was incubated at 37℃ for 18 hours.

The test was interpreted as positive when there was enhancement of thr inhibition zones around the cephalosporin disk, extending toward the direction of the amoxicillin/clavulanic acid disk, often resulting in a shape resembling a ‘keyhole’ zone. *E. coli* ATCC® 25922 was used as the quality control strain.

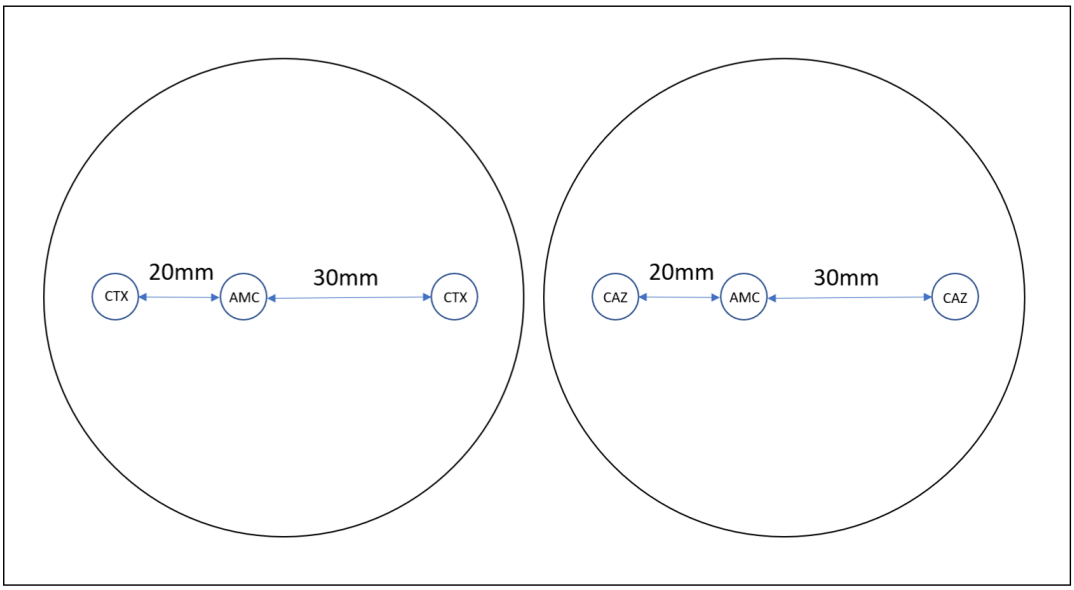


**Figure 3.1**: Placement of antibiotic disks on agar for double disk synergy test. The augmentin and cephalosporin were placed at the distance of 20mm and 30mm on agar plate. * AMC: Amoxicillin/clavulanic acid; CTX: Cefotaxime; CAZ: Ceftazidime.

### 3.12 Molecular detection of β-lactamase genes

PCR was conducted to investigate the presence of genes encoding β-lactamases, specifically TEM, SHV, CTX-M, CMY-2 group, DHA, and MIR/ACT types, in all bacterial isolates. The DNA of all bacteria was isolated using boiling method described in Method 3.4. The descriptive information of the primers, including sequence, primer melting temperature ($T_m$), GC content, and fragment size, is presented in Table 3.8. The PCR reaction mixture was prepared following the protocol described in Method 3.5, with the primer concentration/volume and water volume varying according to Table 3.9. Negative controls were prepared by replacing DNA template with water. The thermal cycling conditions were set according to the parameters outlined in Table 3.9 and Method 3.5. After amplification, the PCR products were analysed by gel electrophoresis, and the result were visualized using gel imaging (refer to Method 3.6).

**Table 3.8**: Primers used for screening β-lactamase genes.

| Target of PCR | Primers' features | | | | | Reference |
|---|---|---|---|---|---|---|
| | **Primer** | **Sequence (5'-3')** | **$T_m$ (℃ )** | **GC content (%)** | **Size (bp)** | |
| *bla*$_{TEM}$ | TEM-F | CATTTCCGTGTCGCCCTTATTC | 56.3 | 50.0 | 800 | (Dallenne *et al.*, 2010) |
| | TEM-R | CGTTCATCCATAGTTGCCTGAC | 55.8 | 50.0 | | |
| *bla*$_{SHV}$ | SHV-F | AGCCGCTTGAGCAAATTAAAC | 54.4 | 42.9 | 713 | (Dallenne *et al.*, 2010) |
| | SHV-R | ATCCCGCAGATAAATCACCAC | 55.0 | 47.6 | | |
| *bla*$_{CTX-M}$ | CTX-M-F | ATGTGCAGTACCAGTAAGGTGATGGC | 60.7 | 50.0 | 593 | (Hasman *et al.*, 2005) |
| | CTX-M-R | TGGGTAAAGTAGGTCACCAGAACCAGCGG | 64.6 | 55.2 | | |
| *bla*$_{CMY-2\ group}$ | CMY-2-F | GCACTTAGCCACCTATACGGCAG | 59.6 | 56.5 | 758 | (Hasman *et al.*, 2005) |
| | CMY-2-R | GCTTTTCAAGAATGCGCCAGG | 57.5 | 52.4 | | |
| *bla*$_{DHA}$ | DHA-F | AACTTTCACAGGTGTGCTGGGT | 59.3 | 50.0 | 405 | (Pérez-Pérez and Hanson, 2002) |
| | DHA-R | CCGTACGCATACTGGCTTTGC | 58.9 | 57.1 | | |
| *bla*$_{MIR/ACT}$ | MIR/ACT-F | TCGGTAAAGCCGATGTTGCGG | 60.3 | 57.1 | 302 | (Pérez-Pérez and Hanson, 2002) |
| | MIR/ACT-R | CTTCCACTGCGGCTGCCAGTT | 62.9 | 61.9 | | |

*$T_m$: Primer melting temperature

**Table 3.9**: PCR reaction mixture and PCR conditions for screening β-lactamase genes (Pérez-Pérez and Hanson, 2002; Hasman et al., 2005; Dallenne et al., 2010).

| Target of PCR | Primer | PCR reaction mixture | | | PCR condition | |
|---|---|---|---|---|---|---|
| | | Final primer concentration (µM) | Volume of 10µM primer in 20µL PCR reaction mixture (µL) | Volume of distilled water in 20µL PCR reaction mixture (µL) | $T_{anneal}$ (℃) | $t_{elongate}$ (second) |
| *bla*$_{TEM}$ | TEM-F | 0.4 | 0.8 | 13 | 60 | 60 |
| | TEM-R | | 0.8 | | | |
| *bla*$_{SHV}$ | SHV-F | 0.4 | 0.8 | 13 | 60 | 60 |
| | SHV-R | | 0.8 | | | |
| *bla*$_{CTX-M}$ | CTX-M-F | 0.4 | 0.8 | 13 | 63 | 40 |
| | CTX-M-R | | 0.8 | | | |
| *bla*$_{CMY-2\ group}$ | CMY-2-F | 0.5 | 1 | 12.6 | 62 | 60 |
| | CMY-2-R | | 1 | | | |
| *bla*$_{DHA}$ | DHA-F | 0.6 | 1.2 | 12.2 | 63 | 30 |
| | DHA-R | | 1.2 | | | |
| *bla*$_{MIR/ACT}$ | MIR/ACT-F | 0.5 | 1 | 12.6 | 63 | 30 |
| | MIR/ACT-R | | 1 | | | |

$T_{anneal}$: annealing temperature; $t_{elongate}$: elongation time; Positive control: bacterial strain used as positive control for gene of interest.

### 3.13 Molecular detection of virulence genes

The genes encoding toxin and virulence factors were detected using PCR. The features of the primers, including sequence, primer melting temperature ($T_m$), GC content, and fragment size, are shown in Table 3.10. The PCR reaction mixture was prepared by adding water, 10X NH4+ Reaction Buffer, dNTPs, $MgCl_2$, primers, *Taq* DNA polymerase, and DNA template as described in Method 3.5. However, the primer concentration and volume, and water volume were calculated differently and are specified in Table 3.11. The thermal cycle was set according to the PCR conditions outlined Table 3.11 and Method 3.5. After PCR amplification, gel electrophoresis and gel imaging were conducted to analyse the results (refer to Method 3.6).

**Table 3.10**: Primers used for screening virulence genes.

| Target of PCR | Primers' features | | | | | Reference |
|---|---|---|---|---|---|---|
| | Primer | Sequence (5'-3') | $T_m$ (℃) | GC content (%) | Size (bp) | |
| EAST1 | EAST1-F | CCATCAACACAGTATATCCGA | 51.7 | 42.9 | 111 | (Yamamoto and Nakazawa, 1997) |
| | EAST1-R | GGTCGCGAGTGACGGCTTTGT | 62.5 | 61.9 | | |
| pAA | pAA-F | CCATAAAGACAGCTTCAGTGAAAA | 53.6 | 37.5 | 162 | (Zhang *et al.*, 2007) |
| | pAA-R | GTATTACTGGTACCACCACCATCA | 56.1 | 45.8 | | |
| F18 | F18-F | CTTTCACATTGCGTGTGGAG | 54.4 | 50 | 441 | (Lee *et al.*, 2008) |
| | F18-R | ATTCGACGCCTTAACCTCCT | 56 | 50 | | |
| STa | Sta-F | GAAACAACATGACGGGAGGT | 55.2 | 50 | 229 | (Lee *et al.*, 2008) |
| | Sta-R | GCACAGGCAGGATTACAACA | 55.5 | 50 | | |
| LT | LT-F | GGTTTCTGCGTTAGGTGGAA | 55.2 | 50 | 605 | (Lee *et al.*, 2008) |
| | LT-R | GGGACTTCGACCTGAAATGT | 54.7 | 50 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *eae* | *eae*-F | CCCGAATTCGGCACAAGCATAAGC | 60.9 | 54.2 | 881 | (Oswald *et al.*, 2000) |
| | *eae*-R | CCCGGATCCGTCTCGCCAGTATTCG | 64.5 | 64 | | |
| *est* | *est*-F | TTAATAGCACCCGGTACAAGCAGG | 58.9 | 50 | 147 | (Hornes, Wasteson and Olsvik, 1991) |
| | *est*-R | CCTGACTCTTCAAAAGAGAAAATTAC | 52.3 | 34.6 | | |
| AIDA | AIDA-F | TGGTGGGAAAACCACTGCTA | 56.5 | 50 | 771 | (Lee *et al.*, 2008) |
| | AIDA-R | TAGCCGCCATCACTAACCAG | 56.9 | 55 | | |
| *aspU* | *aspU*-F | GCCTTTGCGGGTGGTAGCGG | 64 | 70 | 282 | (Toma *et al.*, 2003) |
| | *AspU*-R | AACCCATTCGGTTAGAGCAC | 55 | 50 | | |

* $T_m$: Primer melting temperature

**Table 3.11**: PCR reaction mixture and PCR conditions for screening virulence genes.

| Target of PCR | Primer | PCR reaction mixture | | | PCR condition | |
|---|---|---|---|---|---|---|
| | | Final primer concentration (µM) | Volume of 10µM primer in 20µL PCR reaction mixture (µL) | Volume of distilled water in 20µL PCR reaction mixture (µL) | $T_{anneal}$ (℃) | $t_{elongate}$ (second) |
| *EAST1* | EAST1-F | 0.25 | 0.5 | 13.6 | 58 | 60 |
| | EAST1-R | | 0.5 | | | |
| *pAA* | pAA-F | 0.45 | 0.9 | 12.8 | 58 | 60 |
| | pAA-R | | 0.9 | | | |
| *F18* | F18-F | 0.25 | 0.5 | 13.6 | 55 | 60 |
| | F18-R | | 0.5 | | | |
| *STa* | Sta-F | 0.25 | 0.5 | 13.6 | 55 | 60 |
| | Sta-R | | 0.5 | | | |
| *LT* | LT-F | 0.25 | 0.5 | 13.6 | 55 | 60 |
| | LT-R | | 0.5 | | | |
| *eae* | *eae*-F | 0.25 | 0.5 | 13.6 | 60 | 60 |
| | *eae*-R | | 0.5 | | | |
| *est* | *est*-F | 0.25 | 0.5 | 13.6 | 55 | 60 |
| | *est*-R | | 0.5 | | | |
| *AIDA* | AIDA-F | 0.25 | 0.5 | 13.6 | 55 | 60 |
| | AIDA-R | | 0.5 | | | |
| *aspU* | *aspU*-F | 0.25 | 0.5 | 13.6 | 60 | 60 |
| | *AspU*-R | | 0.5 | | | |

* $T_{anneal}$: annealing temperature; $t_{elongate}$: elongation time; Positive control: bacterial strain used as positive control for gene of interest

### 3.14 Plasmid replicon typing

To determine the plasmid incompatibility (Inc) groups, PCR-based replicon typing was performed on all isolates using 13 pairs of primers targeting replicon I1, FIC, FIA, FIB, N, X, K, A/C, HI1, HI2, Y, FrepB, and B/O (Carattoli *et al.*, 2005). The primer sequence, primer melting temperature ($T_m$), GC content, and fragment size are displayed in Table 3.12. As shown in Appendix 2, the primers of PCR-based replicon typing were designed based on the specific replicon regions for each plasmid group, including *rep* genes, iterons, and RNAI (counter-transcript RNA) (Carattoli *et al.*, 2005). The replicons were amplified in seven panels, including one multiplex (three primer pairs), four duplex (two primer pairs), and two simplex (one primer pair) PCR. For duplex or multiplex PCR, the DNA template of the positive control strain was prepared by mixing the corresponding isolate's DNA in a 1:1 or 1:1:1 ratio. The PCR reaction mixture was prepared as described in Method 3.5, with the number of primer pair, primer concentration/volume and water volume adjusted according to Table 3.13. The PCR conditions in thermal cycle were adjusted according to Table 3.13 and Method 3.5. After amplification, the PCR product was analysed by gel electrophoresis and gel imaging, following the protocol described in Method 3.6.

**Table 3.12**: Primers used for plasmid replicon typing (Carattoli *et al.*, 2005).

| Target of PCR | Primers' features | | | | |
|---|---|---|---|---|---|
| | **Primer** | **Sequence of primer (5'-3')** | **$T_m$ (℃ )** | **GC content (%)** | **Size (bp)** |
| IncI1 | I1-F | CGAAAGCCGGACGGCAGAA | 60.8 | 63.2 | 139 |
| | I1-R | TCGTCGTTCCGCCAAGTTCGT | 61.5 | 57.1 | |
| IncFIC | FIC-F | GTGAACTGGCAGATGAGGAAGG | 57.7 | 54.5 | 262 |
| | FIC-R | TTCTCCTCGTCGCCAAACTAGAT | 57.7 | 47.8 | |
| IncFIA | FIA-F | CCATGCTGGTTCTAGAGAAGGTG | 57.2 | 52.2 | 462 |
| | FIA-R | GTATATCCTTACTGGCTTCCGCAG | 57 | 50 | |
| IncFIB | FIB-F | GGAGTTCTGACACACGATTTTCTG | 55.9 | 45.8 | 702 |
| | FIB-R | CTCCCGTCGCTTCAGGGCATT | 62.2 | 61.9 | |
| IncN | N-F | GTCTAACGAGCTTACCGAAG | 52.6 | 50 | 559 |
| | N-R | GTTTCAACTCTGCCAAGTTC | 51.9 | 45 | |
| IncX | X-F | AACCTTAGAGGCTATTTAAGTTGCTGAT | 56.2 | 35.7 | 376 |
| | X-R | TGAGAGTCAATTTTTATCTCATGTTTTAGC | 54.3 | 30 | |

| | | | | | |
|---|---|---|---|---|---|
| IncK | K-F | GCGGTCCGGAAAGCCAGAAAAC | 61.2 | 59.1 | 160 |
| | K-R | TCTTTCACGAGCCCGCCAAA | 59.8 | 55 | |
| IncA/C | A/C-F | GAGAACCAAAGACAAAGACCTGGA | 56.8 | 45.8 | 465 |
| | A/C-R | ACGACAAACCTGAATTGCCTCCTT | 59.1 | 45.8 | |
| IncHI1 | HI1-F | GGAGCGATGGATTACTTCAGTAC | 55 | 47.8 | 471 |
| | HI1-R | TGCCGTTTCACCTCGTGAGTA | 58.5 | 52.4 | |
| IncHI2 | HI2-F | TTTCTCCTGAGTCACCTGTTAACAC | 56.6 | 44 | 644 |
| | HI2-R | GGCTCACTACCGTTGTCATCCT | 58.8 | 54.5 | |
| IncY | Y-F | AATTCAAACAACACTGTGCAGCCTG | 58.7 | 44 | 765 |
| | Y-R | GCGAGAATGGACGATTACAAAACTTT | 56.1 | 38.5 | |
| IncFrepB | $F_{repB}$-F | TGATCGTTTAAGGAATTTTG | 46.1 | 30 | 270 |
| | $F_{repB}$-R | GAAGATCAGTCACACCATCC | 52.7 | 50 | |
| IncB/O | B/O-F | GCGGTCCGGAAAGCCAGAAAAC | 61.2 | 59.1 | 159 |
| | B/O-R | TCTGCGTTCCGCCAAGTTCGA | 61.6 | 57.1 | |

* $T_m$: Primer melting temperature

**Table 3.13**: Primers' features and PCR conditions for plasmid replicon typing (Carattoli *et al.*, 2005).

| Panel | Primer | Final primer concentration (µM) | Volume of 10µM primer in 20µL PCR reaction mixture (µL) | Volume of water in 20µL PCR reaction mixture (µL) | $T_{anneal}$ (℃) | $t_{elongate}$ (second) |
|---|---|---|---|---|---|---|
| Panel 1 (Duplex) | I1-F | 0.35 | 0.7 | 12.2 | 63 | 60 |
| | I1-R | | 0.7 | | | |
| | FIC-F | 0.25 | 0.5 | | | |
| | FIC-R | | 0.5 | | | |
| Panel 2 (Duplex) | FIA-F | 0.25 | 0.5 | 12.6 | 63 | 60 |
| | FIA-R | | 0.5 | | | |
| | FIB-F | 0.25 | 0.5 | | | |
| | FIB-R | | 0.5 | | | |
| Panel 3 (Duplex) | N-F | 0.35 | 0.7 | 12.2 | 60 | 60 |
| | N-R | | 0.7 | | | |
| | X-F | 0.25 | 0.5 | | | |
| | X-R | | 0.5 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Panel 4 (Duplex) | K-F | 0.35 | 0.7 | 11.8 | 63 | 60 |
| | K-R | | 0.7 | | | |
| | A/C-F | 0.35 | 0.7 | | | |
| | A/C-R | | 0.7 | | | |
| Panel 5 (Multiplex) | HI1-F | 0.25 | 0.5 | 11.6 | 63 | 45 |
| | HI1-R | | 0.5 | | | |
| | HI2-F | 0.35 | 0.7 | | | |
| | HI2-R | | 0.7 | | | |
| | Y-F | 0.15 | 0.3 | | | |
| | Y-R | | 0.3 | | | |
| Panel 6 (Simplex) | $F_{repB}$-F | 0.25 | 0.5 | 13.6 | 52 | 60 |
| | $F_{repB}$-R | | 0.5 | | | |
| Panel 7 (Simplex) | B/O-F | 0.45 | 0.9 | 12.8 | 63 | 45 |
| | B/O-R | | 0.9 | | | |

*$T_{anneal}$: annealing temperature; $t_{elongate}$: elongation time; Positive control: bacterial strain used as positive control for gene of interest

# CHAPTER 4 RESULT

## 4.1 Bacteria identification

### 4.1.1 Bacterial identification by biochemical tests

A total of 59 *Enterobacteriaceae* isolates from poultry origin were examined in this study. The identity of the isolates was determined by the API test until genus or species level. Based on biochemical identification, *E. coli* was found to be the most common type of *Enterobacteriaceae* strains isolated from the poultry faecal samples, followed by *Klebsiella spp.*, *Proteus spp.*, *Salmonella spp.*, and *Shigella spp.* The code of isolates and bacterial identity determined by API test were demonstrated in Table 4.1.

**Table 4.1**: Isolate's code and API-determined identity of 59 isolates collected from poultry in Malaysia.

| Isolate's Code | Identity suspected by API | | Isolate's Code | Identity suspected by API |
|---|---|---|---|---|
| A6 | *E. coli* | | A5 | *Klebsiella spp.* |
| A7 | *E. coli* | | A13 | *Klebsiella spp.* |
| A17 | *E. coli* | | A15 | *Klebsiella spp.* |
| B3 | *E. coli* | | A16 | *Klebsiella spp.* |
| B5 | *E. coli* | | A18 | *Klebsiella spp.* |
| B6 | *E. coli* | | B4 | *Klebsiella spp.* |
| B15 | *E. coli* | | B7 | *Klebsiella spp.* |
| B17 | *E. coli* | | B10 | *Klebsiella spp.* |
| B18 | *E. coli* | | B13 | *Klebsiella spp.* |
| B20 | *E. coli* | | B14 | *Klebsiella spp.* |

| | | | | |
|---|---|---|---|---|
| B21 | *E. coli* | | B19 | *Klebsiella spp.* |
| B24 | *E. coli* | | B22 | *Klebsiella spp.* |
| B26 | *E. coli* | | B27 | *Klebsiella spp.* |
| B28 | *E. coli* | | 4-1 | *Klebsiella spp.* |
| B29 | *E. coli* | | 8-1 | *Klebsiella spp.* |
| B31 | *E. coli* | | 10-1 | *Klebsiella spp.* |
| B32 | *E. coli* | | 36-1 | *Klebsiella spp.* |
| B33 | *E. coli* | | 43-1 | *Klebsiella spp.* |
| B34 | *E. coli* | | 62-1 | *Klebsiella spp.* |
| 16-1 | *E. coli* | | A12 | *Proteus spp.* |
| 18-1 | *E. coli* | | 7-2 | *Proteus spp.* |
| 19-1 | *E. coli* | | 9-1 | *Proteus spp.* |
| 33-1 | *E. coli* | | 14-1 | *Proteus spp.* |
| 34-1 | *E. coli* | | B8 | *Salmonella spp.* |
| 47-1 | *E. coli* | | 2-2B | *Salmonella spp.* |
| 48-1 | *E. coli* | | 15-1B | *Salmonella spp.* |
| 49-1 | *E. coli* | | B25 | *Shigella spp.* |
| 50-1 | *E. coli* | | 32-2B | *Shigella spp.* |
| 53-1 | *E. coli* | | | |
| 55-1 | *E. coli* | | | |
| 59-1 | *E. coli* | | | |

### 4.1.2 16S rRNA sequencing

To verify the biochemical identification and allow further confirmation of bacterial identity, a total of six isolates from five different genus groups were selected for 16S rRNA sequencing in this study. 16S ribosomal RNA (rRNA) gene is a highly conserved gene that is important component of cell function. The length of the 16S rRNA gene sequence is about 1,550 bp, consisting both variable and conserved regions (Clarridge and III, 2004). Sequence in hypervariable regions is diverse among different bacteria while the surrounding regions are conserved regions in most bacteria, making it feasible to amplify target sequences by using primers that can be complementary to the consensus sequence. Bacterial 16S rRNA genes contain nine hypervariable regions (Chakravorty *et al.*, 2007). Comparing the 16S rRNA gene sequences allows for differentiation between organisms at the genus, species, and subspecies level (Clarridge and III, 2004).

The results of the partial sequence of the 16 rRNA gene sequence were recorded in Appendix 3. The similarity score was obtained by comparing the resulting sequences with corresponding references (Accession number KY962911.1, OM816745.1, OL958647.1, OM666543.1, MT525340.1) through BLAST. The results of the biochemical and molecular identification were demonstrated in Table 4.2. Based on the result shown in Table 4.2, the genus of the isolates (33-1, B7, A12, B8) can be determined because the result of API system match the results generated by 16S rRNA sequencing. However, there was a discrepancy in the identity of isolate B25 when using different identification methods. According to the result of 16S rRNA sequencing, the identity of isolate B25 was assumed to be *Enterobacter spp.* even though its identity was regarded as *Shigella spp.* via API system. Isolate 32-2B cannot be identified by this method since it showed high similarity (>99%) to both *E. coli* and *Shigella flexneri.*

**Table 4.2**: Molecular identification of six *Enterobacteriaceae* isolates via 16S rRNA sequencing.

| Isolate | Conventional identification (API) | Molecular identification (16s rRNA sequencing) | | |
|---|---|---|---|---|
| | **Result** | **Similarity (%)** | **Reference sequence** | **Accession number** |
| 33-1 | *E. coli* | 100% [431/431] | *E. coli* | KY962911.1 (partial rRNA sequence) |
| | | 99.77% [431/432] | *E. coli* | CP091925.1 (complete genome) |
| | | 99.77% [431/432] | *S. flexneri* | OM909218.1 (partial rRNA sequence) |
| | | 99.77% [431/432] | *S. flexneri* | CP058796.1 (complete genome) |
| B7 | *Klebsiella spp.* | 99.54% [429/431] | *K. pneumoniae* | OM816745.1 (partial rRNA sequence) |
| | | 99.54% [429/431] | *K. pneumoniae* | CP092901.1 (complete genome) |

| | | | | |
|---|---|---|---|---|
| A12 | *Proteus spp.* | 99.77% [429/430] | *P. mirabilis* | OL958647.1 (partial rRNA sequence) |
| | | 99.77% [429/430] | *P. mirabilis* | CP092652.1 (complete genome) |
| B8 | *Salmonella spp.* | 99.77% [432/433] | *S. enterica* | OM666543.1 (partial rRNA sequence) |
| | | 99.77% [433/434] | *S. enterica* | CP037893.1 (complete genome) |
| B25 | *Shigella spp.* | 99.77% [431/432] | *Enterobacter cloacae* | MT525340.1 (partial rRNA sequence) |
| | | 99.54% [430/432] | *Enterobacter cloacae* | AP024913.1 (complete genome) |
| | | 99.77% [431/432] | *Enterobacter cancerogenus* | MN620423.1 (partial rRNA sequence) |
| | | 99.77% [431/432] | *Enterobacter hormaechei* | MN006223.1 (partial rRNA sequence) |
| | | 99.77% [431/432] | *Enterobacter asburiae* | MK522141.1 (partial rRNA sequence) |

| | | | | |
|---|---|---|---|---|
| | | 99.54% [430/432] | *Enterobacter asburiae* | CP074177.1 (complete genome) |
| | | 99.54% [430/432] | *Enterobacter mori* | CP084692.1 (complete genome) |
| | | 99.54% [430/432] | *Enterobacter bugandensis* | OM910728.1 (partial rRNA sequence) |
| 32-2B | *Shigella spp.* | 99.53% [426/428] | *E. coli* | MN416978.1 (partial rRNA sequence) |
| | | 99.30% [426/429] | *E. coli* | CP091925.1 (complete genome) |
| | | 99.30% [426/429] | *S. flexneri* | OM909218.1 (partial rRNA sequence) |
| | | 99.30% [426/429] | *S. flexneri* | CP058796.1 (complete genome) |

### 4.1.3 Differentiation between *E. coli* and *Shigella spp.*

16S rDNA sequencing is unable to distinguish between two closely related bacterial species, *E. coli* and *Shigella spp*., because they share more than 99% sequence similarity (Woo *et al.*, 2008; Jenkins *et al.*, 2012). In such case, screening of other housekeeping gene targets is required for identification (Woo *et al.*, 2008). The gene that codes for lactose permease, *lacY*, has been established as a hallmark of *E. coli*. Lactose permease plays important role in lactose fermentation by transporting lactose across the cytoplasmatic membrane. *lacY* can be applied to distinguish *E. coli* from *Shigella spp.* because *Shigella spp.* lacks this gene (Horakova, Mlejnkova and Mlejnek, 2008). According to Figure 4.1, *lacY* was not detected in isolates 32-2B. Thus, isolate 32-2B was tentatively identified as *Shigella spp. lacY* was also not detected in isolate B25, which was identified as *Enterobacter spp.* through 16S rRNA sequencing.

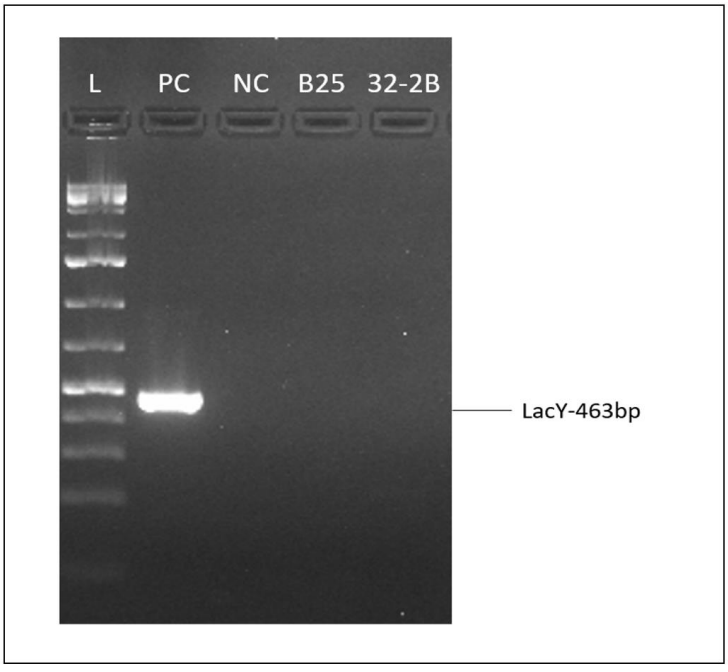


**Figure 4.1**: Screening of *lacY* in suspected *Shigella spp.*via PCR. *L: GeneRuler™ 1 kb Plus DNA Ladder; PC (positive control): *E. coli* ATCC® 25922; NC (negative control): sterile water; B25 & 32-2B: isolates that were presumptively identified as *Shigella spp.* via API test.

After bacterial identification by biochemical and molecular techniques, it was determined that the *Enterobacteriaceae* strains isolated from the poultry faecal samples included *E. coli* (n=31), *Klebsiella spp.* (n=19), *Proteus spp.* (n=4), *Salmonella spp.* (n=3), *Shigella spp.* (n=1), and *Enterobacter spp.* (n=1). The percentages of these various bacterial types were displayed in Figure 4.2.

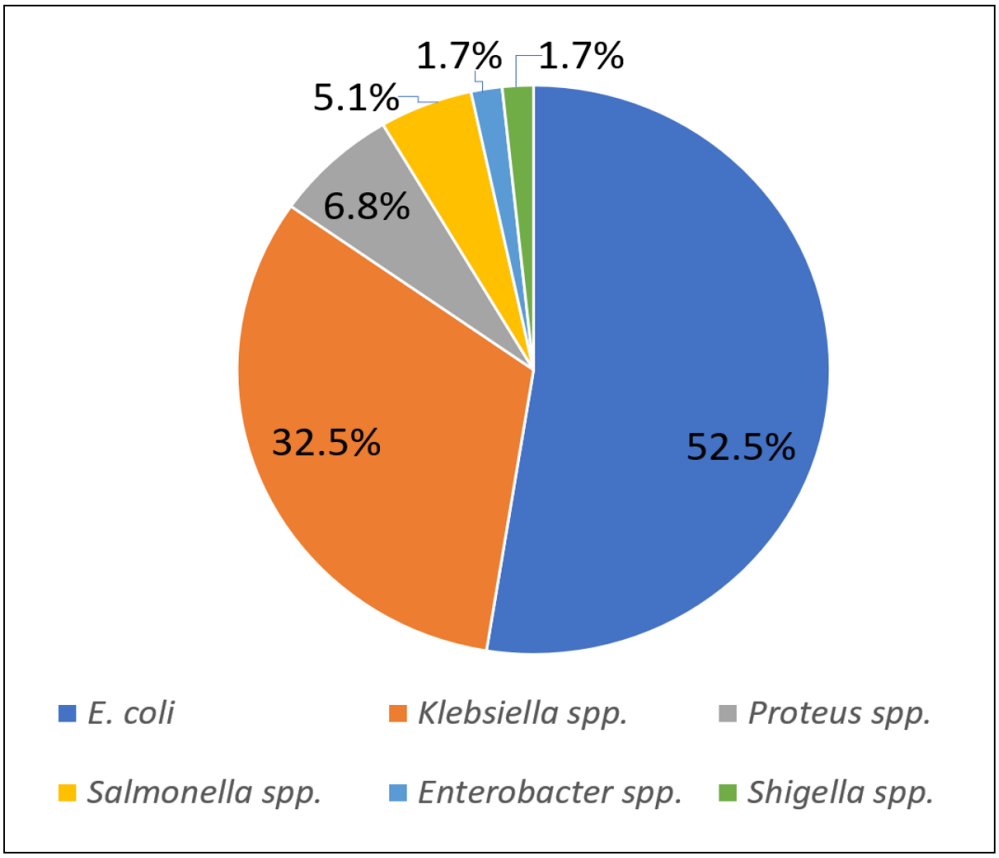


**Figure 4.2**: Percentage of various *Enterobacteriaceae* isolates obtained from poultry faecal samples.

## 4.2 Antimicrobial susceptibility test

The antimicrobial susceptibility test enables the investigation of the resistance profile among bacteria isolated from poultry. The representative results of the antimicrobial susceptibility test were displayed in Figure 4.3. The full data regarding antimicrobial susceptibility test were recorded in Appendix 4 and 5, and the susceptibility of all isolates to four antimicrobials was summarised in Table 4.3. Among *E. coli*, the highest percentage of resistance was observed in cefotaxime (38.7%), followed by cefoxitin (32.3%) and ceftazidime (22.6%). For *Klebsiella spp*., a high resistance rate was reported in cefotaxime (52.6%), cefoxitin (52.6%), and ceftazidime (47.4%). About 25% of *Proteus spp.* was resistant to cefotaxime and cefoxitin. Cefepime-resistant isolates were found at the lowest frequency, with only two *E. coli* strains being resistant. One *Enterobacter spp.* exhibits resistance to cefoxitin. None of *Salmonella spp.* and *Shigella spp.* were resistant to all four antimicrobials.

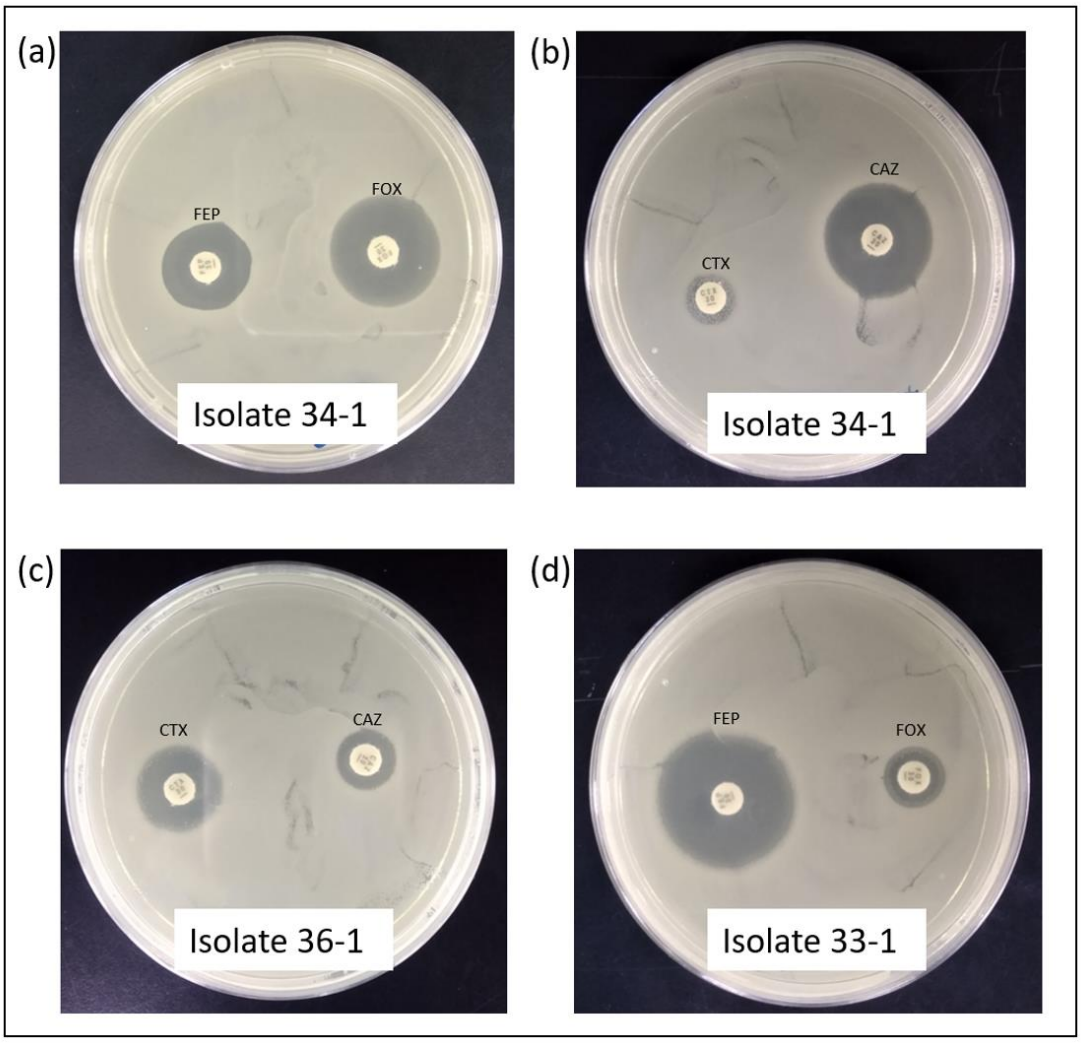


**Figure 4.3**: Representative results from the antimicrobial susceptibility. (a) *E. coli* (Isolate 34-1) resistant to cefepime (ZOI = 17mm). (b) *E. coli* (Isolate 34-1) resistant to cefotaxime (ZOI = 10mm) (c) *Klebsiella spp.* (Isolate 36-1) resistant to cefotaxime (ZOI = 16mm) and ceftazidime (ZOI = 11mm). (d) *E. coli* (Isolate 33-1) resistant to cefoxitin (ZOI = 11mm). Zone Diameter Breakpoints for *Enterobacterales* to interpret as resistant phenotype in cefepime (ZOI ≤ 18mm), cefotaxime (ZOI ≤ 22mm), ceftazidime (ZOI ≤ 17mm) and cefoxitin (ZOI ≤ 14mm) (CLSI, 2021). * ZOI: Zone of inhibition; FEP: Cefepime; CTX: Cefotaxime; CAZ: Ceftazidime; FOX: Cefoxitin.

**Table 4.3**: Summary description of antibiotics resistance profile of 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm (summarised from Appendix 4 and Appendix 5).

| | Total No. | No. isolate (%) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cefepime-30µg (FEP 30) | | | Cefotaxime-30µg (CTX 30) | | | Ceftazidme-30µg (CAZ 30) | | | Cefoxitin-30µg (FOX 30) | | |
| | | S | SDD | R | S | I | R | S | I | R | S | I | R |
| *E. coli* | 31 | 27 (87.1) | 2 (6.5) | 2 (6.5) | 13 (41.9) | 6 (19.4) | 12 (38.7) | 20 (64.5) | 4 (12.9) | 7 (22.6) | 21 (67.7) | 0 (0) | 10 (32.3) |
| *Klebsiella spp.* | 19 | 16 (84.5) | 3 (15.8) | 0 (0) | 4 (21.1) | 5 (26.3) | 10 (52.6) | 9 (47.4) | 1 (5.3) | 9 (47.4) | 9 (47.4) | 0 (0) | 10 (52.6) |
| *Proteus spp.* | 4 | 4 (100.0) | 0 (0) | 0 (0) | 3 (75.0) | 0 (0) | 1 (25.0) | 3 (75.0) | 1 (25.0) | 0 (0) | 3 (75.0) | 0 (0) | 1 (25.0) |
| *Salmonella spp.* | 3 | 3 (100.0) | 0 (0) | 0 (0) | 2 (66.7) | 1 (33.3) | 0 (0) | 2 (66.7) | 1 (33.3) | 0 (0) | 3 (100.0) | 0 (0) | 0 (0) |
| *Enterobacter spp.* | 1 | 1 (100.0) | 0 (0) | 0 (0) | 0 (0) | 1 (100.0) | 0 (0) | 1 (100.0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (100.0) |
| *Shigella spp.* | 1 | 1 (100.0) | 0 (0) | 0 (0) | 0 (0) | 1 (100.0) | 0 (0) | 1 (100.0) | 0 (0) | 0 (0) | 1 (100.0) | 0 (0) | 0 (0) |
| Total | 59 | 52 (88.1) | 5 (8.5) | 2 (3.4) | 22 (37.3) | 14 (23.7) | 23 (39.0) | 36 (61.0) | 7 (11.9) | 16 (27.1) | 37 (62.7) | 0 (0) | 22 (37.3) |

*S: Susceptible; SDD: Susceptible-dose dependent; I: Intermediate; R: Resistant

* The value in brackets indicates the percentage (%) of individuals belonging to categories of susceptible, susceptible-dose dependent, intermediate, or resistant within the same genus group.

### 4.3 Phenotypic detection of ESBL production

Isolates that exhibited resistance to cefotaxime were subjected to further testing for ESBL production using the double-disk synergy test. This included 12 *E. coli* isolates, 10 *Klebsiella spp.* isolates, and 1 *Proteus spp.* isolate. An isolate was considered an ESBL producer if there was an enhanced inhibition zone around any of the cephalosporin disks towards the clavulanate-containing disks. Figure 4.4 showed the representative results, including ESBL producers (Isolates 34-1 and 49-1) and non-ESBL producers (Isolates B28, B13 and A12). The complete data of the double disk synergy test was recorded in Appendix 6. The prevalence of ESBL producers among all *Enterobacteriaceae* isolates and resistant isolates was displayed in Table 4.4. Among 23 cefotaxime-resistant isolates, only two isolates (8.7%) were found to be ESBL producer by DDST. Among 12 cefotaxime resistant *E. coli*, 16.7% of them were found to be carrying ESBL (Table 4.4).

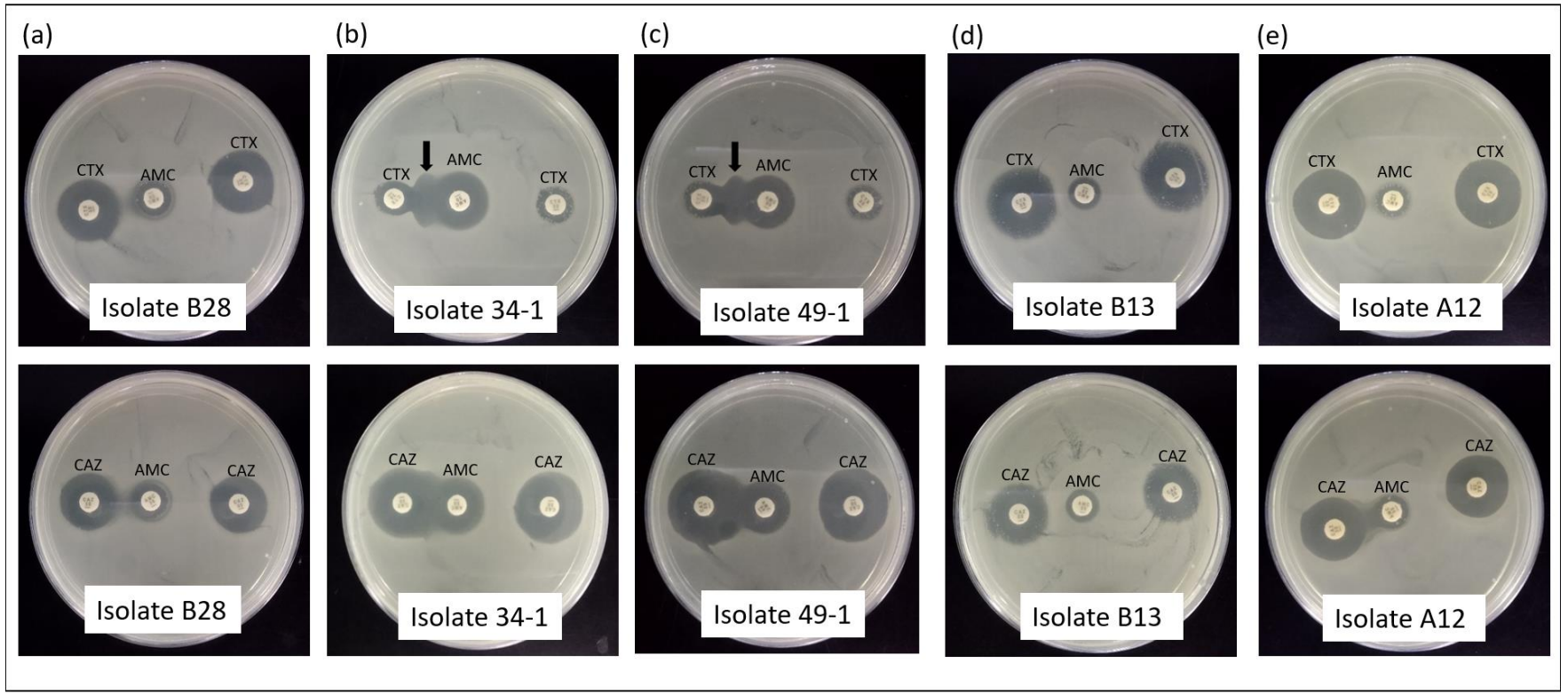


**Figure 4.4**: Representative results from double-disk synergy tests. The disks of CTX/CAZ were placed at the distance of 20/30 mm from AMC disks. The enhancement of inhibition zone of two disks is described as positive for ESBL production (synergy is indicated by arrow). (a) *E. coli* (Isolate B28) carrying $bla_{\text{TEM}}$ and $bla_{\text{CMY-2 group}}$ (No synergy). (b) *E. coli* (Isolate 34-1) carrying $bla_{\text{TEM}}$ and $bla_{\text{CTX-M}}$ (Synergy at distance of 20mm-Positive result). (c) *E. coli* (Isolate 49-1) carrying $bla_{\text{TEM}}$ and $bla_{\text{CTX-M}}$ (Synergy at distance of 20mm-Positive result). (d) *Klebsiella spp.* (Isolate B13) carrying $bla_{\text{SHV}}$ and $bla_{\text{DHA}}$ (No synergy). (e) *Proteus spp.* (Isolate A12) carrying $bla_{\text{CMY-2 group}}$ (No synergy). * AMC: Amoxicillin/clavulanic acid; CTX: Cefotaxime; CAZ: Ceftazidime.

**Table 4.4**: The prevalence of ESBL-producing isolates among 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm (summarised from Appendix 6)

| Bacterial type | Total No. | No. of ESBL producer among all strains (%) | No. of cefotaxime resistant isolates | No. of ESBL producer among cefotaxime resistant strains (%) |
|---|---|---|---|---|
| *E. coli* | 31 | 2 (6.5) | 12 | 2 (16.7) |
| *Klebsiella spp.* | 19 | 0 (0) | 10 | 0 (0) |
| *Proteus spp.* | 4 | 0 (0) | 1 | 0 (0) |
| *Salmonella spp.* | 3 | 0 (0) | 0 | 0 (0) |
| *Enterobacter spp.* | 1 | 0 (0) | 0 | 0 (0) |
| *Shigella spp.* | 1 | 0 (0) | 0 | 0 (0) |
| Total | 59 | 2 (3.4) | 23 | 2 (8.7) |

## 4.4 Molecular detection of β-lactamase genes

PCR was conducted to evaluate the presence of β-lactamase genes, namely *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY-2 group}}$, *bla*$_{\text{DHA}}$, and *bla*$_{\text{MIR/ACT}}$, in all bacterial isolates. The representative PCR results for β-lactamase genes were demonstrated in Figure 4.5, and all gel images for β-lactamase gene screening were provided in Appendix 8. The distribution of resistance genes among different genera was displayed in Table 4.5, and the complete data for screening β-lactamase genes was recorded in Appendix 7.

In this study, *bla*$_{TEM}$ was identified as the most prevalent β-lactamase gene and detected in a wide range of bacterial types, particularly in *E. coli* (87.1%) (Table 4.5). Meanwhile, *bla*$_{SHV}$ was predominantly detected in *Klebsiella spp.* and *bla*$_{CTX-M}$ was detected in two ESBL producers previously identified by DDST. One the other hand, *bla*$_{CMY-2\ group}$ was the most common pAmpC β-lactamase gene, detected in *E. coli* (29%), *Proteus spp.* (25%), and *Klebsiella spp.* (15.8%). Furthermore, *bla*$_{DHA}$ was predominantly found in *Klebsiella spp.* (36.8%), and *bla*$_{MIR/ACT}$ was detected in *Enterobacter spp.*

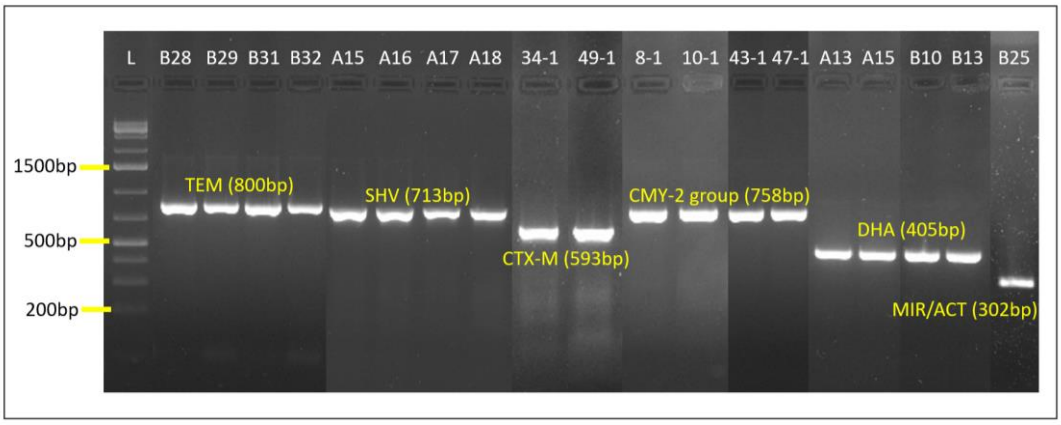


**Figure 4.5**: Representative result of detection of β-lactamase genes. The figure was generated by combining different gel images. All PCR products were resolved on 2% TBE agarose gels. The top labels indicate the isolate reference. L: GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific, USA).

**Table 4.5**: Summary of molecular detection of β-lactamase gene screening in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm (full data was shown in Appendix 7)

| | Total No. | No. Positive (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | ***bla*TEM** | ***bla*SHV** | ***bla*CTX-M** | ***bla*CMY-2 group** | ***bla*DHA** | ***bla*MIR/ACT** |
| *E. coli* | 31 | 27 (87.1) | 1 (3.2) | 2 (6.5) | 9 (29.0) | 1 (3.2) | 0 (0.0) |
| *Klebsiella spp.* | 19 | 7 (36.8) | 15 (78.9) | 0 (0.0) | 3 (15.8) | 7 (36.8) | 0 (0.0) |
| *Proteus spp.* | 4 | 3 (75.0) | 0 (0.0) | 0 (0.0) | 1 (25.0) | 0 (0.0) | 0 (0.0) |
| *Salmonella spp.* | 3 | 1 (33.3) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| *Enterobacter spp.* | 1 | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (100.0) |
| *Shigella spp.* | 1 | 1 (100.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Total | 59 | 39 (66.1) | 16 (27.1) | 2 (3.4) | 13 (22.0) | 8 (13.6) | 1 (1.7) |

## 4.5 Molecular detection of virulence genes

The prevalence of virulence genes, namely *EAST*, *pAA*, *F18*, *STa*, *LT*, *eae*, *est*, *AIDA*, and *aspU*, in all isolates was assessed using PCR screening. The representative PCR profile was displayed in Figure 4.6 and the prevalence rates of each specific virulence genes were summarised in Table 4.6. The results of the virulence genes screening and gel images were presented in Appendices 9 and 10, respectively. None of the isolates in this study were found to carry genes encoding F18 (fimbriae), STa (Heat stable enterotoxin a), LT (Heat labile enterotoxin), eae (intimin), est (Heat stable

enterotoxin-ST I), AIDA-I (adhesin involved in diffuse adherence) or aspU (Cryptic secreted protein / EAEC-secreted protein U). However, the gene encoding EAST1 (Heat stable enterotoxin) was found in 16.1% (5/31) of *E. coli* and 5.3% (1/19) of *Klebsiella spp.*, but it was not detected in *Proteus spp.*, *Salmonella spp.*, *Enterobacter spp.*, and *Shigella spp.* (Table 4.6). In addition, two (6.5%, 2/31) *E. coli* isolates were found to carry gene encoding pAA (porcine attaching and effacing-associated factor).

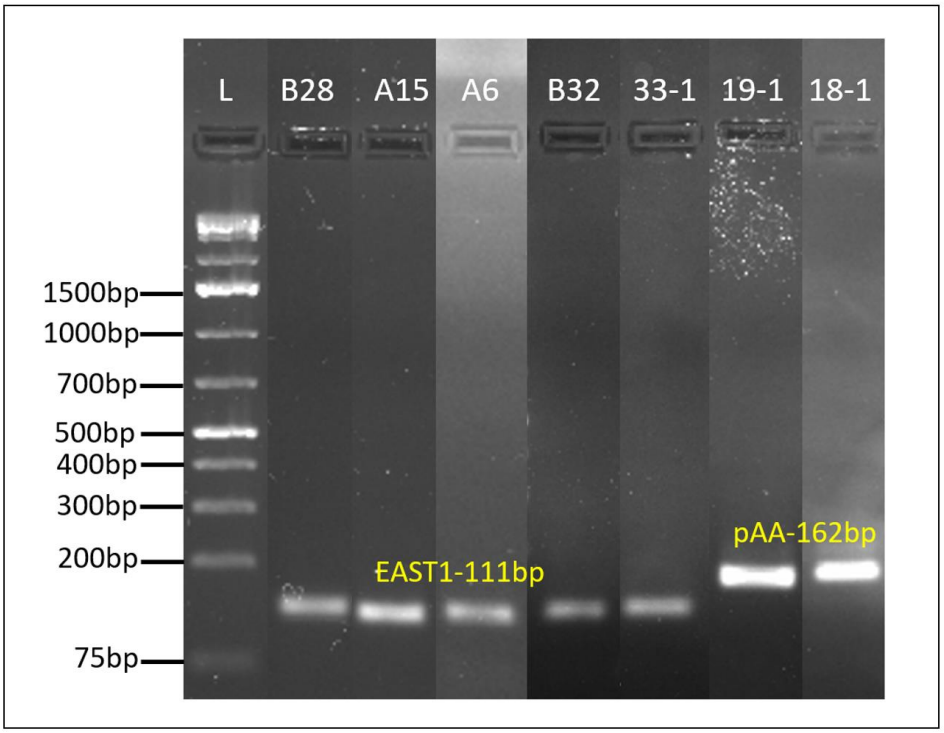


**Figure 4.6**: Representative result of detection of virulence genes. The figure was generated by combining different gel images. All PCR products were resolved on 2% TBE agarose gels. The top labels indicate the isolate reference. L: GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific, USA).

**Table 4.6**: Summary of molecular detection of virulence genes screening in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm (full data was shown in Appendix 9)

| | **Total No.** | **No. Positive (%)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ***EAST1*** | ***pAA*** | ***F18*** | ***STa*** | ***LT*** | ***eae*** | ***est*** | ***AIDA*** | ***aspU*** |
| *E. coli* | 31 | 5 (16.1) | 2 (6.5) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Klebsiella spp.* | 19 | 1(5.3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Proteus spp.* | 4 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Salmonella spp.* | 3 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Enterobacter spp.* | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Shigella spp.* | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Total | 59 | 6 (10.2) | 2 (3.4) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

## 4.6 Plasmid replicon typing

This study employed single, duplex, or multiplex PCR to determine plasmid incompatibility groups of all 59 bacterial isolates. Thirteen type of plasmid replicons were successfully amplified, with the representative PCR results for plasmid replicon typing are shown in Figure 4.7. All gel images of plasmid replicon typing were provided in Appendix 12. The distribution of plasmid replicons among different genera was presented in Table 4.7, and the complete data of plasmid replicon typing was recorded in Appendix 11.

The most frequent types of plasmid replicons in *E. coli* were IncFIB (71%) and FrepB (64.5%), followed by IncI1 (29%) and IncN (29%) (Table 4.7). Meanwhile, IncN (47.4%) was the dominant replicon types in *Klebsiella spp.*, followed by IncI1 (26.3%)

and IncFIB (21.1%). Thirteen types of replicons were all identified in *E. coli*. However, fewer replicon types were detected in *Salmonella spp.* (2 types), *Shigella spp.* (2 types), and *Klebsiella spp.* (5 types). No replicons were detected in *Proteus spp.* and *Enterobacter spp.*

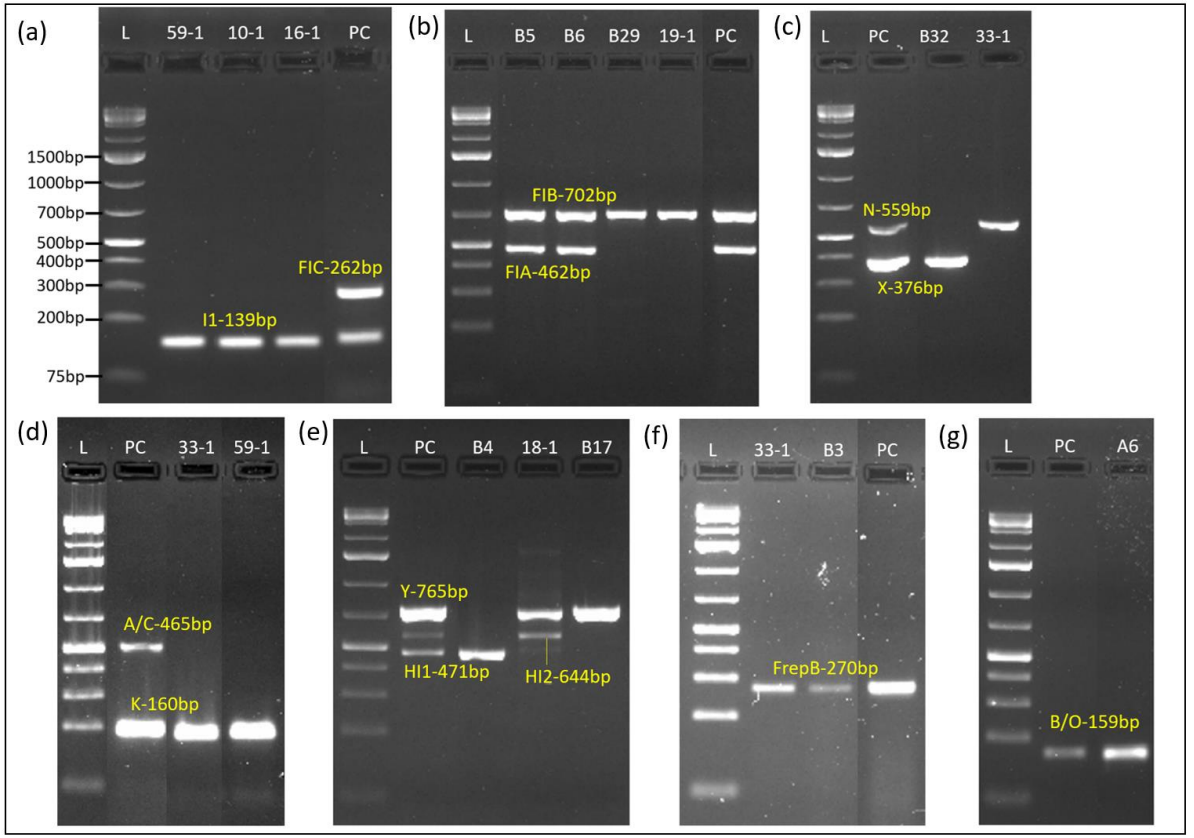


**Figure 4.7**: Representative results of plasmid replicon typing. Plasmid replicon typing were obtained using different panels. (a) Panel 1 (Duplex PCR-I1 & FIC). (b) Panel 2 (Duplex PCR-FIA & FIB). (c) Panel 3 (Duplex PCR-N & X). (d) Panel 4 (Duplex PCR-K & A/C). (e) Panel 5 (Multiplex-PCR-HI1, HI2 & Y). (f) Panel 6 (Simplex PCR-FrepB). (g) Panel 7 (Simplex PCR-B/O). All PCR products were run on 2% TBE agarose gels. The top labels indicate the isolate code. The gel images (a), (b), (c), (d), (e), (f), and (g) were edited and derived from an individual gel images, as displayed in Appendix 12. L: GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific, USA), PC: Positive control.

**Table 4.7**: Summary of molecular detection of plasmid replicon in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm (full data was shown in from Appendix 11)

| | **Total No.** | **No. of Positive Strains (%)** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **I1** | **FIC** | **FIA** | **FIB** | **N** | **X** | **K** | **A/C** | **HI1** | **HI2** | **Y** | **FrepB** | **B/O** |
| *E. coli* | 31 | 9 (29.0) | 1 (3.2) | 4 (12.9) | 22 (71.0) | 9 (29.0) | 1 (3.2) | 5 (16.1) | 1 (3.2) | 2 (6.5) | 3 (9.7) | 1 (3.2) | 20 (64.5) | 5 (16.1) |
| *Klebsiella spp.* | 19 | 5 (26.3) | 0 (0) | 0 (0) | 4 (21.1) | 9 (47.4) | 0 (0) | 1 (5.3) | 0 (0) | 2 (10.5) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Proteus spp.* | 4 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Salmonella spp.* | 3 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (33.3) | 0 (0) | 0 (0) | 0 (0) | 1 (33.3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Enterobacter spp.* | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Shigella spp.* | 1 | 0 (0) | 0 (0) | 0 (0) | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Total | 59 | 14 (23.7) | 1 (1.7) | 4 (6.8) | 27 (45.8) | 19 (32.2) | 1 (1.7) | 6 (10.2) | 1 (1.7) | 6 (10.2) | 3 (5.1) | 1 (1.7) | 20 (33.9) | 5 (8.5) |

### 4.7 Combined molecular characteristics of *Enterobacteriaceae* isolates

The characteristics of all *Enterobacteriaceae* isolates obtained from the poultry are presented in Table 4.8 by combining data on resistance profiles, ESBL production, β-lactamase genes, virulence genes and plasmid replicon types.

**Table 4.8**: Summary of characteristics of 59 *Enterobacteriaceae* isolates from poultry in Malaysia, including resistance profile, ESBL production, β-lactamase genes, virulence genes, and plasmid replicons.

| Isolate | Species | Resistance phenotype to β-lactam* | Phenotypic ESBL production† | β-lactamase genes‡ | Virulence genes§ | Plasmid replicons (Inc)¶ |
|---|---|---|---|---|---|---|
| A6 | *E. coli* | FOX, CTX, CAZ | - | TEM, CMY-2 group | EAST1 | FIB, K, B/O |
| A7 | *E. coli* | - | ND | - | - | FIB, HI1, FrepB |
| A17 | *E. coli* | FOX, CTX | - | SHV, DHA | - | - |
| B3 | *E. coli* | - | ND | TEM | - | FIA, FIB, FrepB |
| B5 | *E. coli* | - | ND | TEM | - | FIA, FIB, FrepB |
| B6 | *E. coli* | - | ND | TEM | - | FIA, FIB, FrepB |
| B15 | *E. coli* | - | ND | TEM | - | FrepB |
| B17 | *E. coli* | - | ND | TEM | - | Y, FrepB |
| B18 | *E. coli* | - | ND | TEM | - | FIB, N, FrepB |
| B20 | *E. coli* | - | ND | TEM | - | FIB, N, FrepB |
| B21 | *E. coli* | - | ND | TEM | - | N |
| B24 | *E. coli* | FOX, CTX, CAZ | - | TEM, CMY-2 group | - | I1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| B26 | *E. coli* | - | ND | TEM | - | FIB, N, FrepB |
| B28 | *E. coli* | FOX, CTX, CAZ | - | TEM, CMY-2 group | EAST1 | FIB, K, HI1, B/O |
| B29 | *E. coli* | - | ND | TEM | - | FIB, FrepB |
| B31 | *E. coli* | - | ND | TEM | - | FIB, N, FrepB |
| B32 | *E. coli* | - | ND | TEM | EAST1 | X |
| B33 | *E. coli* | - | ND | TEM | - | I1, FIB, N, FrepB |
| B34 | *E. coli* | - | ND | TEM | - | N |
| 16-1 | *E. coli* | FOX, CTX, CAZ | - | TEM, CMY-2 group | - | I1, FIB, FrepB |
| 18-1 | *E. coli* | - | ND | TEM | pAA | HI2, N |
| 19-1 | *E. coli* | - | ND | - | pAA | FIC, FIB, FrepB |
| 33-1 | *E. coli* | FOX, CTX | - | TEM, CMY-2 group | EAST1 | FIA, FIB, K, B/O, N, FrepB |
| 34-1 | *E. coli* | FEP, CTX | + | TEM, CTX-M | - | I1, FIB, FrepB |
| 47-1 | *E. coli* | FOX, CTX, CAZ | - | TEM, CMY-2 group | - | I1, FIB |
| 48-1 | *E. coli* | FOX, CTX | - | TEM, CMY-2 group | EAST1 | I1, FIB, FrepB |
| 49-1 | *E. coli* | FEP, CTX | + | TEM, CTX-M | - | I1, FrepB |
| 50-1 | *E. coli* | - | ND | TEM | - | FIB, HI2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 53-1 | *E. coli* | FOX, CTX, CAZ | - | CMY-2 group | - | FIB, K, A/C, B/O, FrepB |
| 55-1 | *E. coli* | - | ND | TEM | - | I1, FIB, FrepB |
| 59-1 | *E. coli* | FOX, CTX, CAZ | - | TEM, CMY-2 group | - | I1, FIB, K, HI2, B/O |
| A5 | *Klebsiella spp.* | - | ND | TEM, SHV | - | - |
| A13 | *Klebsiella spp.* | FOX, CTX, CAZ | - | SHV, DHA | - | - |
| A15 | *Klebsiella spp.* | FOX, CTX, CAZ | - | SHV, DHA | EAST1 | - |
| A16 | *Klebsiella spp.* | - | ND | SHV | - | I1, N |
| A18 | *Klebsiella spp.* | FOX, CTX, CAZ | - | SHV, DHA | - | K, N |
| B4 | *Klebsiella spp.* | - | ND | TEM | - | HI1, N |
| B7 | *Klebsiella spp.* | - | ND | - | - | FIB, N |
| B10 | *Klebsiella spp.* | FOX, CTX, CAZ | - | SHV, DHA | - | - |
| B13 | *Klebsiella spp.* | FOX, CTX, CAZ | - | SHV, DHA | - | I1 |
| B14 | *Klebsiella spp.* | - | ND | SHV | - | FIB, N |
| B19 | *Klebsiella spp.* | - | ND | TEM, SHV | - | N |
| B22 | *Klebsiella spp.* | - | ND | TEM | - | - |
| B27 | *Klebsiella spp.* | - | ND | SHV | - | FIB, N |

| | | | | | | |
|---|---|---|---|---|---|---|
| 4-1 | *Klebsiella spp.* | - | ND | SHV | - | HI1 |
| 8-1 | *Klebsiella spp.* | FOX, CTX, CAZ | - | SHV, CMY-2 group | - | I1 |
| 10-1 | *Klebsiella spp.* | FOX, CTX | - | SHV, CMY-2 group | - | I1 |
| 36-1 | *Klebsiella spp.* | FOX, CTX, CAZ | - | TEM, SHV, DHA | - | N |
| 43-1 | *Klebsiella spp.* | FOX, CTX, CAZ | - | TEM, CMY-2 group | - | I1, FIB |
| 62-1 | *Klebsiella spp.* | FOX, CTX, CAZ | - | TEM, SHV, DHA | - | N |
| A12 | *Proteus spp.* | FOX, CTX | - | CMY-2 group | - | - |
| 7-2 | *Proteus spp.* | - | ND | TEM | - | - |
| 9-1 | *Proteus spp.* | - | ND | TEM | - | - |
| 14-1 | *Proteus spp.* | - | ND | TEM | - | - |
| B8 | *Salmonella spp.* | - | ND | TEM | - | HI1, N |
| 2-2B | *Salmonella spp.* | - | ND | - | - | - |
| 15-1B | *Salmonella spp.* | - | ND | - | - | - |
| B25 | *Enterobacter spp.* | FOX | ND | MIR/ACT | - | - |
| 32-2B | *Shigella spp.* | - | ND | TEM | - | FIB, HI1 |

[*]Antimicrobial susceptibility test. FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; "-" indicate not resistant to all four β-lactams

[†]ESBL screening by double disk synergy test. "-" indicate negative result (no synergy observed); "+" indicate positive result (synergy between cephalosporin and amoxicillin/clavulanic acid observed); ND: Not determined (Only the isolates resistant to cefotaxime subjected for screening of ESBL production by double disk synergy test.)

[‡]Molecular detection of β-lactamase genes. "-" indicate absence of any β-lactamase genes

[§]Molecular detection of virulence genes. "-" indicate absence of any virulence factors

[¶]Plasmid replicon typing. Incompatibility group (Inc); "-" indicate absence of any Inc replicons

## CHAPTER 5 DISCUSSION & CONCLUSION

In this study, the bacterial identification of fifty-nine *Enterobacteriaceae* isolates collected from the Malaysia's poultry farm was determined using the biochemical method (API system). The API 20E (analytical profile index 20E) testing apparatus, which consists of 20 tubes containing pH-based substrates, allows the identification of almost 100 taxa (Franco-Duarte *et al.*, 2019). Until 1992, this method was considered the "gold standard", especially in clinical microbiology (Janda and Abbott, 2002; Franco-Duarte *et al.*, 2019). Smith *et al.* reported an overall accuracy of 96.4% for API system when evaluating the identification of 336 *Enterobacteriaceae* isolates (Smith *et al.*, 1972). The advantage of the API 20E system is the availability of an extensive database (Franco-Duarte *et al.*, 2019). Based on Table 4.1 and Table 4.2, the results of biochemical identification (API system) are consistent with those of 16S rRNA sequencing for isolates 33-1, B7, A12, and B8. However, isolate B25, identified as *Enterobacter spp.*, was misidentified as *Shigella spp.* by the API system.

Misidentification of *Enterobacter spp.* as *Shigella spp.* can occur when using the API identification system, particularly in the API 20E, which is commonly used for *Enterobacteriaceae* identification. One reason for this misidentification is the similarity in biochemical characteristics between *Enterobacter spp.* and *Shigella spp.* For example, both *Enterobacter spp.* and *Shigella spp.* can ferment mannitol and do not produce $H_2S$ (Wang *et al.*, 2010; Kus, 2014; Dekker and Frank, 2015). Another possible reason for misidentification is human errors, such as contamination during pipetting or subjective interpretation of colour change. One limitation of phenotypic identification is that biochemical properties might not precisely reflect the genomic complexity of a given species due to potential influence from environmental factors such as pH, temperature, and concentration of growth substances. Relying solely on a single

identification system, whether phenotypic or genotypic, increases the risk of misidentifying bacterial species. Neither system, phenotypic nor genotypic, is 100% accurate for bacterial identification. Ideally, employing both methods would be optimal, even if it is not technically and financially feasible (Janda and Abbott, 2002). It is also important to carefully interpret the results obtained from the API identification system and be aware of its limitations.

To further confirm the bacterial identity, six isolates were randomly selected for 16S rRNA sequencing. The 16S rRNA gene is universal in bacteria and is commonly used for bacterial identification and phylogenetic studies (Clarridge and III, 2004). The standard method of molecular identification involves PCR amplification and sequencing of the 16S rRNA gene, followed by comparison to known databases (Franco-Duarte *et al.*, 2019). 16S rRNA gene sequence analysis has been shown to achieve a genus identification rate of up to 97.2% and a species identification rate of up to 89.2% among Gram negative bacteria (Tang *et al.*, 1998). In general, sequencing the entire 1,500-bp region of the gene is required for accurate identification (Clarridge and III, 2004). However, for specific applications, such as identifiying *Mycobacterium* and *coryneform* bacteria, MicroSeq 500 system is commonly used, which analyse the first 527-bp fragment of the 16s rRNA gene (Woo *et al.*, 2003). Some researchers have also utilised a shorter 400-bp sequence for identification purpose (Bosshard *et al.*, 2003). When it comes to clinical bacterial isolates, identification using 500-bp sequence is typically sufficient and may provide a greater ability to detect differences between samples due to the slightly higher level of diversity per kilobase sequenced (Clarridge and III, 2004; Fida *et al.*, 2021). Additionally, Tang *et al.* found that 527bp fragment was effective in providing genus information, similar to that of full-length 16S rRNA sequences (Tang *et al.*, 1998).

In this study, the primer pair 341F/785R was utilised to amplify a 430bp fragment of the 16S rRNA gene, which proved to be sufficient to provide bacterial identification up to the genus level (as shown in Table 4.2). The selection of this primer set was based on its *in silico* evaluation, which indicated high coverage of the hypervariable region 3 – 4 of *Gammaproteobacteria*, including *Enterobacteriaceae* (Klindworth *et al.*, 2013). Additionally, the 341F/785R primer set demonstrates high PCR-amplification efficiency and low non-specificity, making it a suitable for studying bacteria derived from soil and plant (Thijs *et al.*, 2017). PCR-based identification offers a faster alternative to conventional culture-based methods and can identify laboratory unculturable bacteria. In addition, this method requires relatively lower starting material, allowing for the detection of bacteria that may be presence in low abundance (Franco-Duarte *et al.*, 2019).

One drawback of using 16S rRNA gene sequencing for bacterial identification is the lack of a universal definition for species identification. Acceptable criteria for establishing a "species" match varies among researchers, making it challenging to define bacterial genus and species with a universal cut-off. This is due to different evolution rates among different bacteria (Janda and Abbott, 2007; Woo *et al*., 2008). As a result, the cut-off values for species identification can vary depending on the genus being investigated (Drancourt *et al.*, 2000). The interpretation of sequence data also requires consideration of factors such as sequence length and sequence quality, the program used for analysis, and the similarity threshold used to assign bacterial species (Woo *et al.*, 2008). Bosshard *et al.* applied a <99% and ⩾95% similarity score to define identification up to the genus level and ⩾99% for species level (Bosshard *et al.*, 2003). Based on Table 4.2, the partial 16S rRNA sequence of the six isolates showed a ⩾99% similarity score when compared to the reference sequence, which might allow

identification up to the species level according to Bosshard *et al.*'s criteria. However, in this study, the aim of 16S rRNA sequencing was to verify the effectiveness of biochemical identification methods, and presumption of identification up to the genus level was deemed sufficient and acceptable. This decision was made due to the lack of well-established criteria for species identification.

*Enterobacter spp.* are commonly found in the environment and in the gastrointestinal tracts of humans and animals as commensal microbiota. The genus *Enterobacter* comprises 22 species. Among these species, *E. cloacae, E. asburiae, E. hormaechei, E. kobei*, *E. ludwigii*, *E. mori*, and *E. nimipressuralis* are grouped together and referred to as *Enterobacter cloacae* complex group. In clinical setting, *E. cloacae* and *E. hormaechei* are the most commonly detected species. However, differentiating the *Enterobacter cloacae* complex group using 16S rRNA sequencing is not suitable due to their genetic similarity (Davin-Regli, Lavigne and Pagès, 2019). Our result of 16S rRNA sequencing indicated there were many possibilities of *Enterobacter* species for isolate B25, as shown in Table 4.2. Species identification within the *Enterobacter cloacae* complex group requires other techniques such as matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS) (Pavlovic *et al.*, 2012; Godmer *et al.*, 2021).

The effectiveness of 16S rRNA gene sequencing is limited when identifying species with highly similar sequences (Clarridge and III, 2004). *Shigella* species and *E. coli* are very closely related Gram-negative bacteria within the family *Enterobacteriaceae*. For example, EIEC has very similar biochemical properties to *Shigella*, and shares high genotypic similarity (Brenner *et al.*, 1972; Lan and Reeves, 2002). Initially, *Shigella* strains were considered clones of *E. coli* and were later classified as separate species based on their distinct biochemical characteristics and

clinical relevance (Lan and Reeves, 2002; Devanga Ragupathi *et al.*, 2018). It is challenging to differentiate *Shigella* from *E. coli* due to their close relatedness (Khot and Fisher, 2013). Jenkins *et al.* revealed that *E. coli* and *Shigella spp.* share >99% sequence identity in their 16S rRNA gene sequences (Jenkins *et al.*, 2012). Similarly, a comparison of 16S rRNA sequences from the NCBI database showed a narrow (<1%) divergence between EHEC/EIEC and *Shigella spp.* (Devanga Ragupathi *et al.*, 2018). Consequently, 16S rRNA gene sequencing is not ideal for differentiating between *Shigella* species and *E. coli* (Khot and Fisher, 2013). In Table 4.2, the amplified 16S rRNA sequence from isolate 32-2B showed a high similarity score to both *E. coli* and *S. flexneri*, suggesting the need for other identification methods to differentiate between these two closely related bacterial species.

Triple Sugar Iron test serves as a biochemical test to assess a bacterium's capacity to ferment carbohydrate (lactose, glucose, and sucrose) and produce hydrogen sulfide ($H_2S$) via a pH-indicator-containing growth medium (Lehman, 2005). In general, *Shigella* typically lacks lactose fermentation whereas *E. coli* is known to ferment lactose (Lehman, 2005; Devanga Ragupathi *et al.*, 2018). However, the presence of "inactive" *E. coli*, charaterised by exhibiting two or more of the following traits: absence of lactose fermentation, lack of motility, and lack of gas production, has introduced a challenge in distinguishing between *Shigella spp.* and inactive *E. coli* (Khot and Fisher, 2013). Due to the limited specificity of the Triple Sugar Iron test, the molecular differentiation method was subsequently implemented in this study.

Subsequently, the *lacY*, which encodes lactose permease, was screened to differentiate between *E. coli* and *Shigella spp.* (Horakova, Mlejnkova and Mlejnek, 2008). Although isolate 32-2B showed high similarity with *E. coli* based on 16S rRNA sequencing (Table 4.2), the absence of *lacY* (Figure 4.1) suggested its identity as

*Shigella spp*. *Shigella* is a Gram-negative bacterium known to cause dysentery, an acute form of diarrhoea characterised by the presence of blood or mucus in the stool (Hilbi *et al.*, 1998). *Shigella* species, including *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* are capable of causing the disease by invading the colonic mucosa. They can multiply within colonic epithelial cells, leading to cell death, mucosal ulceration, inflammation, and bleeding. Transmission of *Shigella* infection occurs through contaminated food and water or person-to-person contact (Niyogi, 2005). Shigellosis is a major cause of morbidity and mortality, particularly among children in developing countries (Løbersli *et al.*, 2016). Another worrying fact is that *Shigella* infection can be spread via faecal–oral route, with a low infectious dose of organisms (10-100 organisms) (Ojha *et al.*, 2013).

*E. coli* is the most abundant facultative anaerobe found in the intestinal microflora of humans and animals (Kaper, Nataro and Mobley, 2004). A wide range of commensal *E. coli* strains are non-pathogenic and can be detected in faeces of healthy individuals (Kuhnert, Boerlin and Frey, 2000). Due to its prevalence in the environment and its presence in various food and water sources, *E. coli* is often used as an indicator for the dissemination of the antibiotic resistance (Kuhnert, Boerlin and Frey, 2000; Caruso, 2018). As shown in Figure 4.2, *E. coli* was the most frequently detected *Enterobacteriaceae* in our poultry samples.

*K. pneumoniae* is a natural inhabitant of the gastrointestinal tract in both healthy humans and animals. However, it is also a common opportunistic pathogen in hospitals, causing extra-intestinal infections that can result in high morbidity and mortality rates due to limited treatment options. *K. pneumoniae* is a major contributor to antibiotic resistance. Data from the European Antimicrobial Resistance Surveillance Network have showed a steady increase in resistance rates of *K. pneumoniae* against the third-

generation cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems in several countries, especially in endemic countries, compared to non-susceptible *E. coli* (European Antimicrobial Resistance Surveillance Network, 2022). The continuous accumulation of antibiotic resistance genes in *K. pneumoniae*, driven by de novo mutations and the acquisition of transferable genetic elements under antibiotic selective pressure, has led to the emergence of extremely drug resistant strains (Navon-Venezia, Kondratyeva and Carattoli, 2017). In Figure 4.2, our results showed that *Klebsiella spp.* was the second common *Enterobacteriaceae* isolates in poultry faecal samples, accounting for 32.5% (19/59) of the isolates.

*E. coli* was found to be the predominant species (81.6-90.5%) identified in poultry faeces, while *K. pneumoniae* (1.5-13.2%) and *E. cloacae* (2.6-6.9%) were less frequently detected (Ferreira *et al.*, 2018; Moawad *et al.*, 2018; Subramanya *et al.*, 2020). Our study showed similar bacterial composition as shown in Figure 4.2. *E. coli*, *K. pneumoniae*, *E. cloacae*, and *P. mirabilis* are known to cause bloodstream and urinary tract infections, whereas *E. coli*, *Salmonella spp.*, and *Shigella spp.* are common gastrointestinal pathogens (Fletcher, McLaws and Ellis, 2013; Diekema *et al*., 2019; Medina and Castillo-Pino, 2019). However, it is important to note that the low percentage of *Salmonella spp.*, *Proteus spp.*, *Enterobacter spp.*, and *Shigella spp.* investigated in this study (Figure 4.2) may not be the representative of the prevalence of ESBL producers, β-lactamase genes, virulence genes, and plasmid replicons in the overall population, as our sample size was relatively small. Other studies have reported the presence of *Salmonella*, *P. mirabilis* and *E. cloacae* in broiler faeces (Marin *et al.*, 2011; Moawad *et al.*, 2018; Chinnam *et al.*, 2021).

The emergence of antimicrobial resistance in food products is a global health concern. In developed and developing countries, animal and food animal products were

reported to carry multidrug-resistant strains and ESBL-producing *Enterobacteriaceae* (Carattoli, 2008; Founou, Founou and Essack, 2016). ESBLs are β-lactamases that can hydrolyse penicillins, first-, second-, and third-generation cephalosporins (Paterson and Bonomo, 2005). In this study, 59 *Enterobacteriaceae* isolates were analysed for susceptibility to the second (cefoxitin), third (cefotaxime & ceftazidime), and fourth (cefepime) generation cephalosporins. The resistance rates of *E. coli* against cefotaxime, ceftazidime, and cefoxitin were found to be 38.7%, 22.6%, and 32.3%, respectively (Table 4.3). Similar studies conducted in Spain and China reported resistance rates of 34% and 21% for cefotaxime, and 31% and 10% for ceftazidime, respectively, with lower rates for cefoxitin (Solà-Ginés *et al.*, 2015; Dou *et al*., 2016). Our study's results showed comparable findings, as all studies used similar methods that included all isolates from samples without selection through antimicrobial enrichment, which is same method employed in our study. In contrast, ESBL-producing *E. coli* isolated from poultry in Vietnam demonstrated high resistance rates to cefotaxime (100%) and ceftazidime (67%), possibly due to the initial isolation using 2 µg/ml of cefotaxime, and the evaluation of resistance profiles among resistant isolates, particularly ESBL-producing *E. coli* (Nakayama *et al.*, 2022).

ESBLs have been increasingly reported in food-producing animals, including cattle, poultry, and pig since the 2000s (Carattoli, 2008; Li *et al.*, 2007). Several methods are available for detecting ESBLs, each with different sensitivities, including DDST, CLSI combination disks, ESBL Etests, Vitek ESBL test, and three-dimensional test (Drieux *et al.*, 2008; Rawat and Nair, 2010; Hélène *et al.*, 2011). In our study, we selected a total of 23 cefotaxime-resistant isolates for phenotypic ESBL detection using the DDST method. The DDST is typically performed by placing the clavulanate-containing disk and the third-generation cephalosporin disk at a distance of 30 mm

(centre to centre). Reducing the distance between these two types of disk to 20mm can significantly improves the test sensitivity (Drieux *et al.*, 2008). For example, the DDS20 method, where AMC disk was placed at 20 mm, center to center, to CTX and CAZ on Mueller-Hinton agar, has demonstrated a sensitivity of 87% and specificity of 98% in detecting ESBL when screening 107 strains of *Enterobacteriaceae* that are not susceptible to extended-spectrum cephalosporin (Hélène *et al.*, 2011). In our study, we employed both the 20mm and 30 mm distance in the DDST method to increase sensitivity.

The present study detected ESBL-producers in 6.5% of the total *E. coli* isolates (n=31) (Table 4.4). The prevalence rate is lower than that reported in similar studies conducted in Thailand and Belgium, where the ESBL prevalence among *E. coli* isolated from faecal samples was reported to be 24.2% (32/132) and 45% (133/295), respectively (Smet *et al.*, 2008; Boonyasiri *et al.*, 2014). These studies used selective methods (1 μg/ml ceftriaxone in Thailand and 8 μg/ml ceftiofur in Belgium) to determine the ESBL prevalence by using antibiotic to select resistant isolates. Pre-enrichment with cephalosporin can increase the detection efficiency of ESBL-producers (Schauss *et al.*, 2015). However, it may not be appropriate to make direct comparison with our ESBL prevalence rate, as all isolates in our study were randomly selected without using antimicrobial enrichment. This approach may underestimate the prevalence of ESBL/AmpC producers in poultry samples.

Antimicrobial resistance is linked to the imprudent and excessive use of antimicrobials in both agriculture and human medicine (Velazquez-Meza *et al.*, 2022). The growing demand for protein and extensive farming practices has led to an increase in antimicrobial use, with global consumption expected to rise by 17% between 2017 to 2030 (Tiseo *et al.*, 2020). Animal farms are known to be a vast reservoir of antibiotic

resistance genes and bacteria, and human exposure can occur through direct contact with animals or animal excretion, consumption of animal products, or indirect contact with animal manure. β-lactamases, encoded by the *bla* genes, confer resistance to β-lactam antibiotics, a major class of antibiotic (Xu *et al.*, 2022). In this study, we employed the PCR method to screen six β-lactamase genes, namely $bla_{\text{TEM}}$, $bla_{\text{SHV}}$, $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2 group}}$, $bla_{\text{DHA}}$, and $bla_{\text{MIR/ACT}}$, in 59 *Enterobacteriaceae* isolates.

This study revealed that $bla_{\text{TEM}}$ was the most commonly detected β-lactamase gene in *Enterobacteriaceae*, particularly in *E. coli* (87.1%, 27/31) (Table 4.5). This observation may be due to the fact that TEM-1 is the most prevalent β-lactamase in Gram-negative bacteria in general (Bradford, 2001). Similar findings have been reported in other studies where $bla_{\text{TEM-1}}$ was detected in poultry faecal samples, often in combinations with other β-lactamase genes such as $bla_{\text{CMY}}$, $bla_{\text{CTX-M}}$, and $bla_{\text{SHV}}$ (Laube *et al.*, 2013; Trung *et al.*, 2019). Our study showed comparable results, showing the coexistence of $bla_{\text{TEM}}$ with the $bla_{\text{CMY-2 group}}$ and $bla_{\text{CTX-M}}$, as indicated in Table 4.8. In addition, three out of four *Proteus* spp. (3/4, 75%) in this study carried $bla_{\text{TEM}}$ (Table 4.5). It is worth noting that *P. mirabilis* is inherently susceptible to β-lactam and has negligible expression of chromosomal β-lactamase. Therefore, resistance in *P. mirabilis* is generally mediated by TEM-type β-lactamase (Livermore, 1995).

The SHV-1 gene was first identified in *E. coli* during the 1970s. The SHV-1 enzyme exhibited activity against penicillins and first generation cephalosporins (Liakopoulos, Mevius and Ceccarelli, 2016). SHV-1 is a common plasmid-mediated β-lactamase found in *K. pneumoniae* and *E. coli* (Bradford, 2001). In *K. pneumoniae*, the SHV-1 enzyme is encoded in the chromosome, while in *E. coli*, it is commonly plasmid-mediated (Bradford, 2001). Our results showed a low prevalence rate of $bla_{\text{SHV}}$

in *E. coli* (3.2%, 1/31), but a high prevalence in *Klebsiella spp.* (78.9%, 15/19), as shown in Table 4.5.

ESBLs are enzymes that are derived from TEM-1, TEM-2, and SHV-1, but these parent enzymes themselves are not considered ESBLs as they lack the ability to hydrolyse third generation cephalosporins (Paterson, 2000; Paterson and Bonomo, 2005). In Table 4.8, the detection of TEM and SHV genes in isolates that are susceptible to third/fourth generation cephalosporins indicates that they are more likely to be broad-spectrum type or inhibitor-resistant type rather than ESBL types. To accurately identify the specific type of β-lactamase present in isolates that are resistant to third/fourth generation cephalosporins and cephamycin, and exhibit negative synergy results, sequencing is necessary. This is because the presence of AmpC enzyme can mask the detection of ESBLs when using the DDST method for ESBL screening (Drieux *et al.*, 2008; Kaur *et al.*, 2013). The primers developed by Dallenne *et al.*, targeting $bla_{TEM}$ and $bla_{SHV}$, can amplify both ESBLs and broad-spectrum β-lactamases of TEM and SHV types, but direct sequencing of PCR products can help to discriminate between them (Dallenne *et al.*, 2010).

Based on Table 4.5, CTX-M enzyme was the only confirmed ESBL detected in this study. The CTX-M β-lactamase was firstly described in Germany in 1989 and is known to confer resistance to cefotaxime while remaining susceptible to ceftazidime (Bauernfeind, Schweighart and Grimm, 1990; Paterson and Bonomo, 2005). This is attributed to the unique geometry of the β-lactam binding site in CTX-M enzyme, which allows efficient binding of cefotaxime but not the bulkier molecule of ceftazidime (Rossolini, D'Andrea and Mugnaioli, 2008). However, certain variants of CTX-M have demonstrated enhanced catalytic activity against ceftazidime (Zhao and Hu, 2012). CTX-M has been shown to efficiently hydrolyse cefepime but not cephamycins such as

cefoxitin, which can be used to differentiate it from AmpC β-lactamase (Paterson and Bonomo, 2005; Olsen *et al.*, 2014). As what shown in Table 4.8, isolate 34-1 and 49-1, which only harboured $bla_{\text{CTX-M}}$, exhibited resistance to cefepime and cefotaxime while remaining susceptible to ceftazidime and cefoxitin.

It is worth noting that the prevalence of CTX-M-producing *E. coli* can vary significantly across different regions and populations due to various factors, including antibiotic usage, farming practices, and surveillance methods. In this study, only two (6.5%) *E. coli* isolates tested positive for $bla_{\text{CTX-M}}$ (Table 4.5). The prevalence of CTX-M-producing *E. coli* in poultry faecal samples observed in this study was lower as compared to the prevalence in Thailand (26%), Korea (41%), Japan (40%), China (87%), and England (54.5%) (Randall *et al.*, 2011; Zheng *et al*., 2012; Kameyama *et al*., 2013; Tansawai, Walsh and Niumsup, 2019; Seo and Lee, 2021). These studies reported high CTX-M rates because they specifically screened for the prevalence of CTX-M in cefotaxime-resistant or ESBL-producing strains. In contrast, in this study, $bla_{\text{CTX-M}}$ was directly screened from isolates derived from poultry faecal samples without antibiotic enrichment (Olsen *et al.*, 2014). This might be a contributing factor to the low CTX-M rates observed in this study.

The pAmpC gene families comprise various types, such as CMY-2-like, LAT, DHA, MIR, ACT, ACC, CMY-1-like, FOX, and MOX (Meini *et al.*, 2019). Isolates carrying pAmpC genes are resistant not only to cefoxitin but also to cefotaxime and ceftazidime (Philippon, Arlet and Jacoby, 2002). Our study found that 21 isolates carrying $bla_{\text{CMY-2 group}}$ or $bla_{\text{DHA}}$ exhibited resistance to cefotaxime (100%, 21/21), ceftazidime (76.2%, 16/21), and both (cefotaxime & ceftazidime) (76.2%, 16/21) (Table 4.8), highlighting the CMY-2 group as the main mediator of resistance to extended spectrum cephalosporin in *E. coli*. These findings are consistent with a study

conducted in Columbia, which also did not apply antimicrobials enrichment and characterised ESBL/AmpC gene in *E. coli* isolated from poultry faeces (Castellanos *et al.*, 2017).

In a previous study conducted on a German broiler farm, it was found that 21.73% (78/359) of *E. coli* isolates carried *bla*$_{\text{CMY}}$ (Laube *et al.*, 2013). Similarly, in our study, we observed that 29% of *E. coli* isolates carried *bla*$_{\text{CMY-2 group}}$ (Table 4.5). The prevalence of CMY-2-producing *E. coli* has been investigated in poultry faecal samples in several countries, including Spain, Belgium, Greece, Columbia, Finland, Nigeria, Tunisia, and Japan (Blanc *et al.*, 2006; Smet *et al.*, 2008; Kameyama *et al.*, 2013; Castellanos *et al.*, 2017; Hassen *et al.*, 2020; Päivärinta *et al.*, 2020; Athanasakopoulou *et al.*, 2021; Ejikeugwu *et al.*, 2021). This raised the question of why *bla*$_{\text{CMY-2}}$ is common in *E. coli* from broiler despite the rare use of cephalosporins in broiler production (Nilsson *et al.*, 2014). A longitudinal study investigating the epidemiology of ESBL/AmpC-producing *E. coli* in the Dutch broiler production pyramid revealed that *bla*$_{\text{CMY-2}}$ was the only ESBL/AmpC gene detected at the top of the production pyramid, suggesting that primary breeding companies from UK, Ireland and US may be the source of AmpC resistance genes (C. M. Dierikx *et al.*, 2013). This could potentially explain the common detection of *bla*$_{\text{CMY-2}}$-carrying *E. coli* in our study if the poultry breed in the farms was imported from oversea sources.

Our finding showed a similar trend, with a high prevalence of *bla*$_{\text{DHA}}$ found in *Klebsiella spp.* (36.8%, 7/19) and a lower prevalence in *E. coli* (3.2%, 1/31) (Table 4.5). This finding is consistent with the statement that the DHA-1 enzyme is the dominant cephalosporinase type in *K. pneumoniae* (Hennequin *et al.*, 2018). Through genome analysis, it has been observed that SHV-11, a broad spectrum β-lactamase similar to SHV-1, is frequently described in *K. pneumoniae* producing DHA-1 (Hennequin, Ravet

and Robin, 2018). This can explain the co-existence of *bla*$_{DHA}$ and *bla*$_{SHV}$ in *Klebsiella spp.* in Table 4.8. There are two types of DHA, namely DHA-1 and DHA-2. DHA-1 is resistant to cephamycins and oxyimino-cephalosporins, while DHA-2 is intermediate in resistance to cefoxitin but susceptible to cefotaxime or ceftazidime (Philippon, Arlet and Jacoby, 2002). Based on Table 4.8, *E. coli* and *Klebsiella spp.* carrying *bla*$_{DHA}$ showed resistance to cefoxitin, and cefotaxime or ceftazidime, indicating that the DHA type in our study is more likely to be DHA-1 rather than DHA-2. *K. pneumoniae* producing DHA-1 and *E. coli* producing DHA-1 have been detected in poultry originating from Cambodia and Vietnam, respectively (Nguyen *et al.*, 2016; Atterby *et al*., 2019).

The ACT-1 and MIR-1 plasmid-mediated AmpC β-lactamases are thought to have originated from *Enterobacter* species (Philippon, Arlet and Jacoby, 2002; Rottman *et al*., 2002). ACT-1 and MIR-1 share a high degree of similarity, with a 91.4% amino acid identity (Philippon, Arlet and Jacoby, 2002). Although MIR/ACT enzymes are rarely described in poultry, they have been observed in clinical settings (Mohd Khari *et al.*, 2016). In our study, only one *Enterobacter spp.* isolate was found to carry *bla*$_{MIR/ACT}$, as shown in Table 4.5.

CMY-2, along with other variants such as CMY-3, CMY-4, CMY-5, CMY-6, CMY-7, LAT-1, LAT-2, LAT-3, LAT-4, and BIL-1, exhibited high sequence similarity, with approximately 99% similarity among these variants (Pérez-Pérez and Hanson, 2002). Certain variants within this group, such as CMY-2, BIL-1, and LAT-2 are identical, while LAT-1 and LAT-4, as well as LAT-3 and CMY-6, also share identical sequences (Philippon, Arlet and Jacoby, 2002). In our study, we are unable to distinguish CMY-2 and other similar variants using the primer sequence designed by Hasman *et al.* Similarly, the primers targeting DHA and MIR were unable to

differentiate between DHA-1 and DHA-2, and MIR-1 and ACT-1 (Hasman *et al.*, 2005). Hence, sequencing is needed to confirm the specific variant type within the CMY-2 group, as well as for DHA and MIR/ACT.

Resistance and virulence are both important mechanisms for bacterial survival under adverse conditions. They share common features as they are associated with infections, and their dissemination and co-selection are mediated by horizontal gene transfer (Beceiro, Tomás and Bou, 2013). Our initial aim was to screen more virulence genes representative of all the bacterial isolates. However, due to small sample sizes for *Proteus spp., Salmonella spp., Enterobacter spp.* and *Shigella spp.*, we focused on investigating only nine virulence genes associated with different *E. coli* pathotypes. In this study, the virulence genes including *F18, STa, LT, eae, est, AIDA-I,* and *aspU* were not detected in all isolates (Table 4.6). It has been observed that avian pathogenic *E. coli* does not typically harbour the *eae* and *est* genes (Al-Marri *et al.*, 2021). Similarly, in our study, no *E. coli* strains derived from poultry were found to carry *eae* and *est* genes.

The heat-stable toxin EAST1 is encoded by *astA* gene, which can be located on either chromosome or plasmids. EAST1 is a peptide consisting of 38 amino acids (Veilleux and Dubreuil, 2006). In our study, *EAST1* was detected in 16.1% (5/31) of *E. coli* isolates and 5.3% (1/19) of *Klebsiella spp.* isolates (Table 4.6). However, a study conducted in Portugal reported a higher percentage of *E. coli* carrying *astA*, with 36.1% (22/61) of *E. coli* derived from healthy broilers faeces testing positive for *astA* (Paixão *et al.*, 2016). The lower percentage of *E. coli* carrying *EAST1* in our study compared to Portugal could be attributed to several factors, such as differences in the poultry health status (diseased/healthy broilers in Malaysia versus healthy broilers in Portugal), endemic factor (Malaysia versus Portugal), sampling locations (farms versus

slaughterhouse), the number of isolates (31 *E. coli* in Malaysia versus 66 *E. coli* in Portugal), and random chance. EAST1 shares 50% homology with the enterotoxigenic domain of STa, another heat-stable enterotoxin found in ETEC (Kuhnert, Boerlin and Frey, 2000; Veilleux and Dubreuil, 2006). It is suggested that the mechanism of action of EAST1 is identical to that of STa (Dubreuil, 2019). STa binding to the surface of enterocytes activates guanylate cyclase, leading to the accumulation of cGMP and massive loss of electrolyte and water. EAST1 has been found in various pathotypes of *E. coli*, including ETEC, EPEC, EHEC, and EAEC, and has been investigated in farm animals such as swine and cattle (Kuhnert, Boerlin and Frey, 2000; Veilleux and Dubreuil, 2006).

The study conducted by Batisson *et al*. found that the pAA proteins are involved in the formation of attaching/effacing lesions and contribute to the pathogenicity of EPEC in pigs (Batisson *et al.*, 2003). Additionally, Vidotto, Florian and Ono reported that pAA may be a virulence factor in *E. coli* associated with diarrhoea in piglets (Vidotto, Florian and Ono, 2013). In our study, two *E. coli* strains were found to harbour *pAA*, indicating its emergence and distribution in poultry (Table 4.6). The presence of both *EAST1* and *pAA* in the *E. coli* strains isolated from poultry samples suggests that they are potentially virulent and could pose a health threat to humans through the food chain.

In 2005, Carattoli and colleagues developed PCR-based replicon typing, which targets the replicons of the major plasmid incompatibility groups in *Enterobacteriaceae*, including HI2, HI1, I1, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, FIIs, FrepB, K, and B/O (Carattoli *et al.*, 2005). In our study, we screened 13 plasmid replicons using PBRT. Among 31 *E. coli* isolates, IncFIB (71%) and IncFrepB (64.5%) were the predominant types, followed by IncI1 (29%) and IncN (29%) (Table 4.7). The primers targeting

FrepB can recognise a heterogeneous group of IncF-plasmids, including FI, FII, FIII, FIV, FV, and FVI plasmids (Carattoli *et al.*, 2005). Our findings are consistent with a study conducted in the United States that investigated the distribution of plasmid replicons in 92 *E. coli* strains originated from faecal swabs of apparently healthy poultry. The study revealed a high frequency of IncFrepB (67%) and IncFIB (51.1%), followed by IncI1 (17.4%) and IncN (10.9%) (Johnson *et al.*, 2007). In addition, our finding also showed similarity to a study conducted in Turkey, where IncFIB (87.7%), IncFrepB (64.9%) and IncI1 (64.9%) were found among 154 ESBL/pAmpC-producing *E. coli* strains derived from broiler faecal samples (Aslantaş, 2020).

IncF plasmids are capable of carrying multiple replicons for initiation of replication, and typical multi-replicon plasmids often include the FII replicon in combination with FIA and FIB replicons (Rozwandowicz *et al.*, 2018). In our study, we found that the FIB-FrepB combination (equivalent to FIB-FII) was present in 54.8% (17/31) of *E. coli* isolates, while three *E. coli* isolates carried the FIA-FIB-FrepB combination (Table 4.8). This finding is consistent with studies conducted in Turkey and Belgium, which also reported the detection of the FIB-FrepB combination in *E. coli* isolated from poultry faecal samples (Lambrecht *et al*., 2017; Aslantaş, 2020). The multi-replicon nature of IncF plasmids allows plasmids with a narrow host range to carry out replication in a broad host range, while also allowing for the acquisition of plasmids carrying incompatible replicons when replication is controlled by a compatible one. The replicon can undergo genetic alternation if it does not participate in the control of replication, which can lead to advent of new compatible variants. This feature may contribute to the high prevalence of the FIB-FrepB combination and IncF plasmids in faecal samples observed in our study, as well as their successful dissemination among enteric bacteria (Woodford *et al.*, 2009; Villa *et al.*, 2010). On

the other hand, IncF plasmid contains addiction systems that eliminate the cells without the plasmid, allowing for stable maintenance of IncF plasmids in *E. coli* within the gastrointestinal tract of humans and animals, even in the absence of antimicrobial pressure (Woodford *et al.*, 2009; Bevan, Jones and Hawkey, 2017). This may also contribute to the high frequency of IncF plasmids observed in our study.

In our poultry samples, IncF, IncN and IncI1 plasmids were frequently detected in *Klebsiella spp.* and *E. coli*, while other replicons such as IncK, IncB/O, IncHI2, IncHI1, IncX, IncA/C, and IncY were less common (Table 4.7). It has been reported that IncF and IncN plasmids often carry genes that confer resistance to extended-spectrum β-lactams, quinolones and aminoglycosides, suggesting their role in the dissemination of antibiotic resistance and virulence genes within *Enterobacteriaceae* populations (Rozwandowicz *et al.*, 2018). However, our study did not specifically examine the presence of these genes. Further investigation using whole genome sequencing would be necessary to identify the specific resistance and virulence genes carried by these plasmids, determine their location on either the chromosome or plasmids, and elucidate the genetic elements involved in their mobilisation.

The global dissemination of CTX-M genes is largely facilitated by horizontal gene transfer, often mediated by conjugative plasmids (Bevan, Jones and Hawkey, 2017). These genes are commonly located on plasmids belonging to various Inc groups, including IncFII, IncFI, IncI, IncA/C, IncHI2, IncL/M, and IncP (Carattoli, 2009). For instance, previous studies have reported the presence of CTX-M β-lactamase on IncF plasmids carrying variants like CTX-M-55, CTX-M-27, and CTX-M-14, as well as on IncI1 plasmid harbouring CTX-M-1 in *E. coli* isolated from poultry (García-Fernández *et al.*, 2008; Liu *et al.*, 2013; Rozwandowicz *et al.*, 2018). The detection of IncI1, IncFrepB, and IncFIB plasmids in *E. coli* isolates carrying CTX-M genes (Table 4.8)

suggests that these plasmids may also contribute to the dissemination of CTX-M ESBLs among poultry via horizontal gene transfer.

The *bla*$_{\text{CMY-2}}$ gene has been identified on various plasmid groups, including IncI1, IncK, IncA/C, and IncF (Carattoli, 2009; Naseer *et al*., 2010; Börjesson *et al*., 2013; C. Dierikx *et al*., 2013). Previous studies have shown that IncI1 and IncK plasmids are commonly associated with CMY-2 genes in *E. coli* isolated from poultry, and that the *bla*$_{\text{CMY-2}}$ can be horizontally transferred with the assistance of these plasmids (Bergenholtz *et al.*, 2009; Börjesson *et al.*, 2013; C. Dierikx *et al*., 2013; Agersø *et al*., 2014; Huijbers *et al.*, 2014; Hansen *et al.*, 2016). Additionally, IncB/O plasmids have been found to carry resistance gene such as CMY-2 (Rozwandowicz *et al.*, 2018). Our study found that IncI1, IncK, and IncB/O plasmids were commonly detected in 55.6% (5/9) of *E. coli* isolates carrying CMY-2 gene (Table 4.8), suggesting their potential role in the spread of this resistance gene. However, it is important to note that some studies have reported the presence of chromosomally encoded CMY-2 in *E. coli* isolated from both humans and animals, and whole genome sequencing has revealed the integration of the CMY-2 gene into chromosome of *P. mirabilis*, which may explain the absence of plasmid observed in CMY-2 group-producing *Proteus spp.* in our study (Table 4.8) (Mac Aogáin, Rogers and Crowley, 2015; Fang *et al.*, 2015; Pietsch *et al.*, 2018).

*K. pneumoniae* is known to be a preferred host for DHA-1 carrying plasmids. These plasmids can belong to different Inc groups, including R, A/C2, L/M, FII(k), HIB, HI2, and FIB, and are often hybrids of different Inc groups (Hennequin, Ravet and Robin, 2018). DHA-1 plasmids carry several resistance genes, including those resistant to β-lactam (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{KPC}}$), aminoglycosides, fluoroquinolones, phenicols, rifampicine, sulfonamides, trimethoprim, tetracyclines,

and macrolides. It is concerning that DHA-1 plasmid can accommodate multiple resistance genes, particularly carbapenemase genes (Hennequin, Ravet and Robin, 2018). In our study, 36.8% (7/19) of *Klebsiella spp.* were found to carry DHA-1 (Table 4.5) and if multidrug-resistant DHA-1 plasmids are present in our *Klebsiella spp.*, it would be highly alarming.

Virulence mechanisms can be encoded on both chromosomes or MGEs (Cepas and Soto, 2020). Plasmids, phages and transposons are known to carry numerous virulence genes in *E. coli* (Kuhnert, Boerlin and Frey, 2000). For example, the EAST1 gene has been identified on plasmids in certain studies, and genes encoding pAA were found on plasmids along with other virulence and tetracycline resistance genes (Yamamoto and Echeverria, 1996; Yamamoto and Nakazawa, 1997; Leclerc *et al.*, 2007). Furthermore, IncF plasmids are known to harbour various virulence traits, such as toxins, adhesion factors, haemolysin, Type II and III secretion systems, *Salmonella* plasmid virulence, and iron uptake systems (Villa *et al.*, 2010). In Table 4.8, some isolates (isolate A6, B28, 19-1, 33-1, and 48-1) carrying *EAST1* and *pAA* were associated with the FIB and FIC replicons, suggesting a potential role of IncF plasmid in the dissemination of these virulence genes.

The presence of the insertion sequence *IS1414* in pathogenic *E. coli* strains, has been reported to carry the EAST1 gene in *Salmonella*, indicating the possibility of intergeneric transfer of virulence genes through this insertion sequence (Bacciu *et al.*, 2004). This study implies that horizontal transfer of virulence factors could be linked to the presence of *astA* (encoding EAST1) in *Salmonella* and *K. pneumoniae* (Paiva De Sousa and Dubreuil, 2001; Nguyen Thi *et al.*, 2003). The mobilisation of *EAST1* between different bacterial populations via MGEs could explain the detection of this gene in *Klebsiella spp.* as well (Table 4.8).

Among all isolates, isolates 34-1 & 49-1 are of particular concern, being *bla*$_{\text{CTX-M}}$ producing bacteria associated with both IncF and IncI1 plasmids simultaneously. The association between the *bla*$_{\text{CTX-M}}$ gene and IncF/IncI1 plasmids has been well studied in *E. coli*-derived from human and poultry (Accogli *et al.*, 2013; Zurfluh *et al*., 2014; Seo and Lee, 2021; Zeng *et al*., 2021; Negeri *et al.*, 2023). Clonal expansion and horizontal gene transfer may contribute to the widespread dissemination of *bla*$_{\text{CTX-M}}$ (Negeri *et al.*, 2023). Given that several antimicrobial resistance genes are plasmid-mediated and a plasmid could harbor multiple resistance genes, this connectivity allows for the linkage of multiple plasmids and the manifestation of a multidrug resistance phenotype (Puangseree *et al.*, 2022). Further studies could focus on isolate 33-1, 53-1, and 59-1, as these three isolates contain multiple plasmids. Investigating whether they carry multiple resistance genes would be beneficial.

The detection of ESBL/AmpC producers in poultry faecal samples in our study (40.7%, 24/59) is concerning (Table 4.8), as these isolates can become more prevalent through vertical transmission and horizontal transmission, as well as recirculation in farms. Evidence suggests that recirculation of resistant strains from previous production cycles can lead to poultry contamination, even after intensive cleaning and disinfections procedures (C. M. Dierikx *et al.*, 2013). Moreover, ESBL/AmpC producing *E. coli* has been shown to transmit and persist within broiler flock even in the absence of selective pressure from antibiotics (Huijbers *et al.*, 2016). Additionally, ESBL/AmpC producers commonly co-carry resistance genes against multiple classes of antibiotics, such as aminoglycosides, quinolones/fluoroquinolone, trimethoprim-sulfametoxazole, chloramphenicol, sulfonamide, or tetracycline (Gniadkowski, 2001; Philippon, Arlet and Jacoby, 2002). The multidrug resistant phenotype of ESBL/pAmpC producers poses a significant health threat as it severely limits therapeutic options (Pitout and

Laupland, 2008; Abdalhamid *et al.*, 2017; Rensing *et al.*, 2019). There is a concern that these resistant bacteria could spread from poultry to humans through various routes, which could have significant health implications.

## CONCLUSION

The high occurrence of ESBL/AmpC-producing *Enterobacteriaceae* in poultry is a concerning trend. The distribution of plasmid replicons in isolates from poultry could drive the spread of resistance among *Enterobacteriaceae* in humans, animals, and the environment. This study highlights that poultry may serve as a reservoir for antibiotic-resistant bacteria and virulence determinants. It is crucial to enhance surveillance of antibiotic usage and antibiotic resistance to mitigate the emergence of antibiotic resistance and minimise the spread of infections caused by multidrug-resistant organisms.

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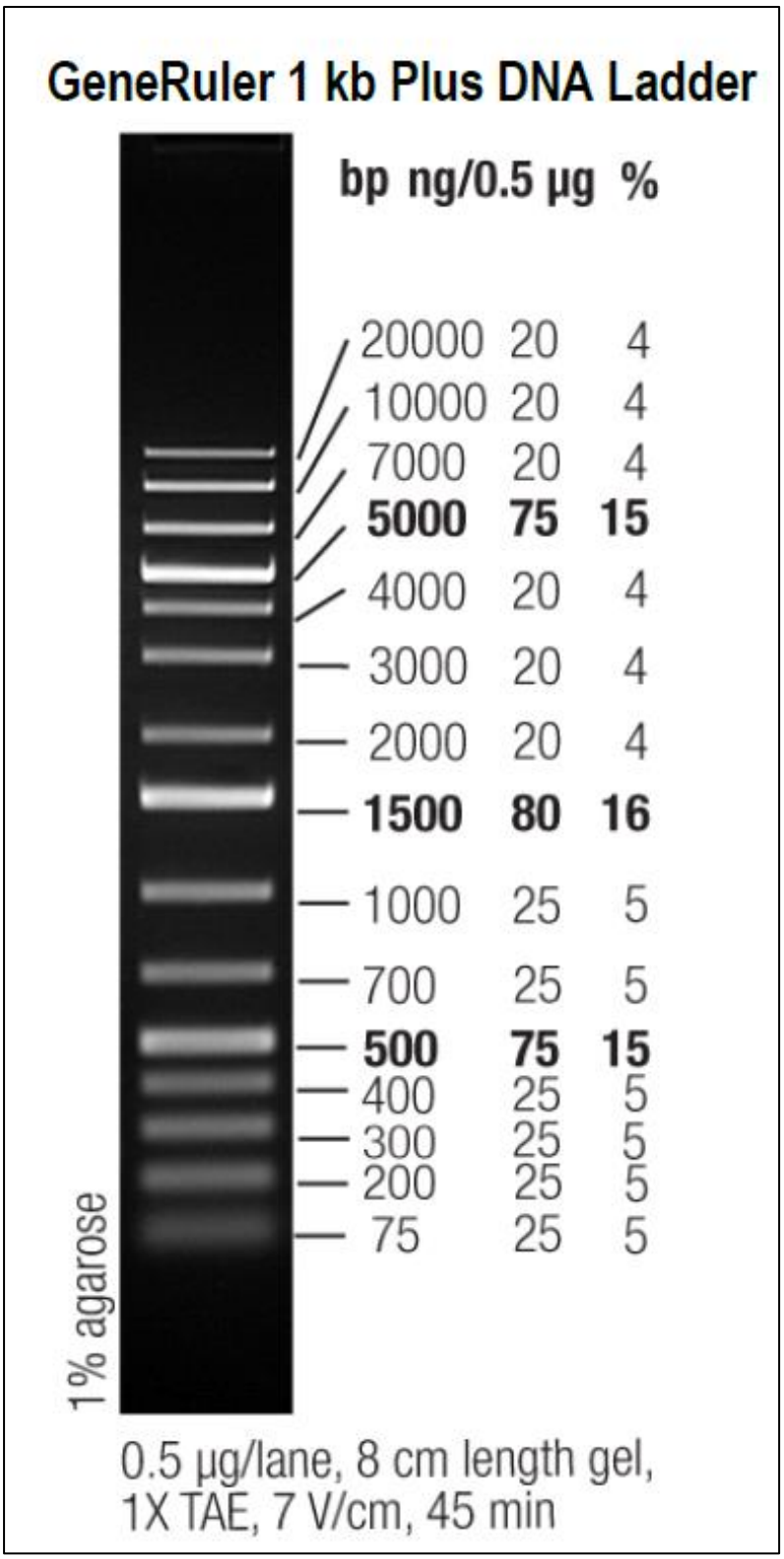
**APPENDICES**

**Appendix 1: Size reference of GeneRuler 1kb Plus DNA Ladder**

The figure presents the size reference of 1kb DNA ladder in agarose gel electrophoresis.



**Appendix 2: Additional information about primers targeting plasmid replicons**

The table below displays additional information on the primers used for plasmid replicon typing, including annealing position, accession number, and target site (The table is adapted from Carattoli *et al.*, 2005).

| Plasmid replicon | Primer | Annealing position | Target site | Accession number |
|---|---|---|---|---|
| I1 | I1-F | 93-111 | RNAI | M20413 |
| | I1-R | 214-234 | | |
| FIC | FIC-F | 912-933 | repA2 | AH003523 |
| | FIC-R | 1149-1171 | | |
| FIA | FIA-F | 937-959 | iterons | J01724 |
| | FIA-R | 1375-1398 | | |
| FIB | FIB-F | 2221-2244 | repA | M26308 |
| | FIB-R | 2903-2923 | | |
| N | N-F | 31781-31800 | repA | NC_003292 |
| | N-R | 32321-32340 | | |
| X | X-F | 46-73 | ori γ | Y00768 |
| | X-R | 385-414 | | |
| K | K-F | 447-468 | RNAI | M93063 |
| | K-R | 592-611 | | |
| A/C | A/C-F | 784-807 | repA | X73674 |
| | A/C-R | 1226-1249 | | |
| HI1 | HI1-F | 24008- 24030 | parA-parB | AF250878 |
| | HI1-R | 24458- 24478 | | |

| | | | | |
|---|---|---|---|---|
| HI2 | HI2-F | 260083-260107 | iterons | BX664015 |
| | HI2-R | 259461- 259482 | | |
| Y | Y-F | 1075-1099 | repA | K02380 |
| | Y-R | 1814-1839 | | |
| $F_{repB}$ | $F_{repB}$-F | 1696-1715 | RNAI/repA | AY234375 |
| | $F_{repB}$-R | 1946-1965 | | |
| B/O | B/O-F | 446-467 | RNAI | M28718 |
| | B/O-R | 585-605 | | |

**Appendix 3: The partial 16S rRNA sequence amplified by primer 341F and 785R in six isolates derived from poultry faecal samples**

The partial 16S rRNA sequence amplified by primer 341F and 785R in isolate **33-1** (436bp)

```
  1 GGNATGGGCG CAGCCTGATG CAGCCATGCC GCGTGTATGA AGAAGGCCTT
 51 CGGGTTGTAA AGTACTTTCA GCGGGGAGGA AGGGAGTAAA GTTAATACCT
101 TTGCTCATTG ACGTTACCCG CAGAAGAAGC ACCGGCTAAC TCCGTGCCAG
151 CAGCCGCGGT AATACGGAGG GTGCAAGCGT TAATCGGAAT TACTGGGCGT
201 AAAGCGCACG CAGGCGGTTT GTTAAGTCAG ATGTGAAATC CCCGGGCTCA
251 ACCTGGGAAC TGCATCTGAT ACTGGCAAGC TTGAGTCTCG TAGAGGGGGG
301 TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG AGGAATACCG
351 GTGGCGAAGG CGGCCCCCTG GACGAAGACT GACGCTCAGG TGCGAAAGCG
401 TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCAG
```

---

The partial 16S rRNA sequence amplified by primer 341F and 785R in isolate **B7** (434bp)

```
  1 GANTGGGCGC AGCCTGATGC AGCCATGCCG CGTGTGTGAA GAAGGCCTTC
 51 GGGTTGTAAA GCACTTTCAG CGGGGAGGAA GGCGTTAAGG TTAATAACCT
101 TGGCGATTGA CGTTACCCGC AGAAGAAGCA CCGGCTAACT CCGTGCCAGC
151 AGCCGCGGTA ATACGGAGGG TGCAAGCGTT AATCGGAATT ACTGGGCGTA
201 AAGCGCACGC AGGCGGTCTG TCAAGTCGGA TGTGAAATCC CCGGGCTCAA
251 CCTGGGAACT GCATTCGAAA CTGGCAGGCT AGAGTCTTGT AGAGGGGGGT
301 AGAATTCCAG GTGTAGCGGT GAAATGCGTA GAGATCTGGA GGAATACCGG
351 TGGCGAAGGC GGCCCCCTGG ACAAAGACTG ACGCTCAGGT GCGAAAGCGT
401 GGGGAGCAAA CAGGATTAGA TACCTTGGTA GTCA
```

---

The partial 16S rRNA sequence amplified by primer 341F and 785R in isolate **A12** (439bp)

```
  1 TTTGGNTGGG GCGCAGCCTG ATGCAGCCAT GCCGCGTGTA TGAAGAAGGC
 51 CTTAGGGTTG TAAAGTACTT TCAGCGGGGA GGAAGGTGAT AAGGTTAATA
101 CCCTTATCAA TTGACGTTAC CCGCAGAAGA AGCACCGGCT AACTCCGTGC
151 CAGCAGCCGC GGTAATACGG AGGGTGCAAG CGTTAATCGG AATTACTGGG
201 CGTAAAGCGC ACGCAGGCGG TCAATTAAGT CAGATGTGAA AGCCCCGAGC
251 TTAACTTGGG AATTGCATCT GAAACTGGTT GGCTAGAGTC TTGTAGAGGG
301 GGGTAGAATT CCATGTGTAG CGGTGAAATG CGTAGAGATG TGGAGGAATA
351 CCGGTGGCGA AGGCGGCCCC CTGGACAAAG ACTGACGCTC AGGTGCGAAA
401 GCGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCAG
```

---

The partial 16S rRNA sequence amplified by primer 341F and 785R in isolate **B8** (439bp)

```
  1 TTTGGAATGG GCGCAGCCTG ATGCAGCCAT GCCGCGTGTA TGAAGAAGGC
 51 CTTCGGGTTG TAAAGTACTT TCAGCGGGGA GGAAGGTGTT GTGGTTAATA
101 ACCGCAGCAA TTGACGTTAC CCGCAGAAGA AGCACCGGCT AACTCCGTGC
151 CAGCAGCCGC GGTAATACGG AGGGTGCAAG CGTTAATCGG AATTACTGGG
201 CGTAAAGCGC ACGCAGGCGG TCTGTCAAGT CGGATGTGAA ATCCCCGGGC
251 TCAACCTGGG AACTGCATTC GAAACTGGCA GGCTTGAGTC TTGTAGAGGG
301 GGGTAGAATT CCAGGTGTAG CGGTGAAATG CGTAGAGATC TGGAGGAATA
351 CCGGTGGCGA AGGCGGCCCC CTGGACAAAG ACTGACGCTC AGGTGCGAAA
401 GCGTGGGGAG CAAACAGGAT TAGATACCCT GGTAGTCAG
```

---

The partial 16S rRNA sequence amplified by primer 341F and 785R in isolate **B25** (437bp)

```
  1 TTGGATGGGC GCAGCCTGAT GCAGCCATGC CGCGTGTATG AAGAAGGCCT
 51 TCGGGTTGTA AAGTACTTTC AGCGGGGAGG AAGGCGTTAA GGTTAATAAC
101 CTTGTCGATT GACGTTACCC GCAGAAGAAG CACCGGCTAA CTCCGTGCCA
151 GCAGCCGCGG TAATACGGAG GGTGCAAGCG TTAATCGGAA TTACTGGGCG
201 TAAAGCGCAC GCAGGCGGTC TGTCAAGTCG GATGTGAAAT CCCCGGGCTC
251 AACCTGGGAA CTGCATTCGA AACTGGCAGG CTAGAGTCTT GTAGAGGGGG
301 GTAGAATTCC AGGTGTAGCG GTGAAATGCG TAGAGATCTG GAGGAATACC
351 GGTGGCGAAG GCGGCCCCCT GGACAAAGAC TGACGCTCAG GTGCGAAAGC
401 GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCAG
```

---

The partial 16S rRNA sequence amplified by primer 341F and 785R in isolates **32-2B** (430bp)

```
  1 AGGGGCGCAG CCTGATGCAG CCATGCCGCG TGTATGAAGA AGGCCTTCGG
 51 GTTGTAAAGT ACTTTCAGCG GGGAGGAAGG GAGTAAAGTT AATACCTTTG
101 CTCATTGACG TTACCCGCAG AAGAAGCACC GGCTAACTCC GTGCCAGCAG
151 CCGCGGTAAT ACGGAGGGTG CAAGCGTTAA TCGGAATTAC TGGGCGTAAA
201 GCGCACGCAG GCGGTTTGTT AAGTCAGATG TGAAATCCCC GGGCTCAACC
251 TGGGAACTGC ATCTGATACT GGCAAGCTTG AGTCTCGTAG AGGGGGGTAG
301 AATTCCAGGT GTAGCGGTGA AATGCGTACA GATCTGGAGG AATACCGGTG
351 GCGAAGGCGG CCCCCTGGAC CAAGACTGAC GCTCAGGTGC GAAAGCGTGG
401 GGAGCAAACA GGATTAGATA CCCTGGTAGT
```

---

**Appendix 4: Result of Antimicrobial Susceptibility test (cefepime and cefotaxime)**

The table below displays the result of the antimicrobial susceptibility test against cefepime and cefotaxime in 59 *Enterobacteriaceae* isolates, including the triplicate reading of the zone of inhibition and interpretive category.

| Isolate | Cefepime - 30µg (FEP 30) | | | | | Cefotaxime - 30µg (CTX 30) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Zone of Inhibition (mm) | | | | IC | Zone of Inhibition (mm) | | | | IC |
| | R1 | R2 | R3 | Average | | R1 | R2 | R3 | Average | |
| A6 | 25 | 24 | 25 | 24.67 | SDD | 18 | 17 | 18 | 17.67 | R |
| A7 | 29 | 28 | 28 | 28.33 | S | 27 | 27 | 26 | 26.67 | S |
| A17 | 25 | 25 | 25 | 25.00 | S | 21 | 22 | 22 | 21.67 | R |
| B3 | 27 | 27 | 27 | 27.00 | S | 26 | 26 | 26 | 26.00 | S |
| B5 | 28 | 28 | 28 | 28.00 | S | 26 | 27 | 28 | 27.00 | S |
| B6 | 25 | 26 | 26 | 25.67 | S | 25 | 26 | 25 | 25.33 | I |
| B15 | 26 | 26 | 25 | 25.67 | S | 25 | 26 | 25 | 25.33 | I |
| B17 | 25 | 25 | 25 | 25.00 | S | 25 | 27 | 27 | 26.33 | S |
| B18 | 27 | 28 | 27 | 27.33 | S | 27 | 26 | 25 | 26.00 | S |
| B20 | 29 | 28 | 29 | 28.67 | S | 28 | 28 | 27 | 27.67 | S |
| B21 | 25 | 26 | 26 | 25.67 | S | 26 | 26 | 25 | 25.67 | S |
| B24 | 25 | 25 | 25 | 25.00 | S | 16 | 16 | 16 | 16.00 | R |
| B26 | 26 | 25 | 26 | 25.67 | S | 27 | 27 | 27 | 27.00 | S |
| B28 | 26 | 26 | 25 | 25.67 | S | 18 | 18 | 18 | 18.00 | R |
| B29 | 25 | 25 | 25 | 25.00 | S | 25 | 24 | 24 | 24.33 | I |
| B31 | 26 | 26 | 26 | 26.00 | S | 27 | 27 | 26 | 26.67 | S |
| B32 | 26 | 26 | 26 | 26.00 | S | 26 | 26 | 26 | 26.00 | S |
| B33 | 27 | 26 | 26 | 26.33 | S | 26 | 27 | 26 | 26.33 | S |
| B34 | 26 | 26 | 26 | 26.00 | S | 27 | 27 | 28 | 27.33 | S |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 16-1 | 24 | 23 | 23 | 23.33 | SDD | 15 | 16 | 15 | 15.33 | R |
| 18-1 | 26 | 26 | 26 | 26.00 | S | 26 | 26 | 26 | 26.00 | S |
| 19-1 | 25 | 25 | 25 | 25.00 | S | 25 | 25 | 25 | 25.00 | I |
| 33-1 | 25 | 25 | 25 | 25.00 | S | 19 | 19 | 19 | 19.00 | R |
| 34-1 | 17 | 17 | 18 | 17.33 | R | 10 | 10 | 9 | 9.67 | R |
| 47-1 | 26 | 27 | 27 | 26.67 | S | 18 | 17 | 17 | 17.33 | R |
| 48-1 | 25 | 25 | 25 | 25.00 | S | 18 | 18 | 18 | 18.00 | R |
| 49-1 | 17 | 18 | 17 | 17.33 | R | 10 | 10 | 10 | 10.00 | R |
| 50-1 | 26 | 26 | 26 | 26.00 | S | 24 | 25 | 25 | 24.67 | I |
| 53-1 | 25 | 25 | 25 | 25.00 | S | 18 | 17 | 17 | 17.33 | R |
| 55-1 | 26 | 26 | 26 | 26.00 | S | 25 | 24 | 25 | 24.67 | I |
| 59-1 | 25 | 25 | 25 | 25.00 | S | 17 | 17 | 17 | 17.00 | R |
| A5 | 22 | 23 | 23 | 22.67 | SDD | 24 | 25 | 25 | 24.67 | I |
| A13 | 26 | 25 | 25 | 25.33 | S | 20 | 19 | 20 | 19.67 | R |
| A15 | 27 | 28 | 27 | 27.33 | S | 21 | 21 | 20 | 20.67 | R |
| A16 | 26 | 25 | 27 | 26.00 | S | 26 | 26 | 25 | 25.67 | I |
| A18 | 25 | 26 | 25 | 25.33 | S | 21 | 21 | 20 | 20.67 | R |
| B4 | 29 | 29 | 28 | 28.67 | S | 28 | 28 | 28 | 28.00 | S |
| B7 | 25 | 25 | 25 | 25.00 | S | 25 | 24 | 23 | 24.00 | I |
| B10 | 25 | 25 | 25 | 25.00 | S | 20 | 21 | 21 | 20.67 | R |
| B13 | 25 | 25 | 25 | 25.00 | S | 21 | 21 | 20 | 20.67 | R |
| B14 | 26 | 26 | 26 | 26.00 | S | 27 | 27 | 26 | 26.67 | S |
| B19 | 25 | 26 | 26 | 25.67 | S | 25 | 25 | 25 | 25.00 | I |
| B22 | 25 | 26 | 25 | 25.33 | S | 26 | 26 | 26 | 26.00 | S |
| B27 | 26 | 27 | 27 | 26.67 | S | 26 | 26 | 25 | 25.67 | S |
| 4-1 | 25 | 25 | 25 | 25.00 | S | 23 | 24 | 23 | 23.33 | I |
| 8-1 | 25 | 24 | 24 | 24.33 | SDD | 19 | 18 | 19 | 18.67 | R |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 10-1 | 25 | 26 | 25 | 25.33 | S | 20 | 20 | 20 | 20.00 | R |
| 36-1 | 24 | 24 | 23 | 23.67 | SDD | 16 | 16 | 16 | 16.00 | R |
| 43-1 | 25 | 26 | 25 | 25.33 | S | 18 | 17 | 18 | 17.67 | R |
| 62-1 | 25 | 25 | 25 | 25.00 | S | 16 | 15 | 15 | 15.33 | R |
| A12 | 25 | 26 | 25 | 25.33 | S | 21 | 20 | 21 | 20.67 | R |
| 7-2 | 28 | 27 | 27 | 27.33 | S | 29 | 28 | 29 | 28.67 | S |
| 9-1 | 26 | 25 | 25 | 25.33 | S | 28 | 27 | 28 | 27.67 | S |
| 14-1 | 25 | 26 | 26 | 25.67 | S | 29 | 28 | 29 | 28.67 | S |
| B8 | 30 | 30 | 30 | 30 | S | 26 | 26 | 27 | 26.33 | S |
| 2-2B | 25 | 25 | 25 | 25 | S | 24 | 23 | 23 | 23.33 | I |
| 15-1B | 28 | 27 | 27 | 27.33 | S | 27 | 27 | 27 | 27.00 | S |
| B25 | 26 | 26 | 25 | 25.67 | S | 24 | 23 | 24 | 23.67 | I |
| 32-2B | 26 | 25 | 26 | 25.67 | S | 25 | 25 | 25 | 25 | I |

*IC: Interpretive Category; R1/R2/R3: Reading 1/2/3; S: Susceptible; SDD: Susceptible-dose dependent; I: Intermediate; R: Resistant

**Appendix 5: Result of Antimicrobial Susceptibility test (ceftazidime and cefoxitin)**

The table below shows the result of the antimicrobial susceptibility test against ceftazidime and cefoxitin in 59 *Enterobacteriaceae* isolates, including the triplicate reading of the zone of inhibition and interpretive category.

| Isolate | Ceftazidime - 30µg (CAZ 30) | | | | | Cefoxitin - 30µg (FOX 30) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Zone of Inhibition (mm) | | | | IC | Zone of Inhibition (mm) | | | | IC |
| | R1 | R2 | R3 | Average | | R1 | R2 | R3 | Average | |
| A6 | 16 | 15 | 15 | 15.33 | R | 9 | 10 | 9 | 9.33 | R |
| A7 | 24 | 24 | 24 | 24.00 | S | 22 | 21 | 22 | 21.67 | S |
| A17 | 18 | 18 | 18 | 18.00 | I | - | - | - | - | R |
| B3 | 23 | 24 | 24 | 23.67 | S | 20 | 20 | 20 | 20 | S |
| B5 | 25 | 26 | 26 | 25.67 | S | 20 | 20 | 20 | 20 | S |
| B6 | 25 | 24 | 24 | 24.33 | S | 20 | 20 | 20 | 20 | S |
| B15 | 22 | 22 | 23 | 22.33 | S | 18 | 18 | 20 | 18.67 | S |
| B17 | 24 | 24 | 23 | 23.67 | S | 19 | 19 | 18 | 18.67 | S |
| B18 | 24 | 23 | 23 | 23.33 | S | 21 | 21 | 21 | 21.00 | S |
| B20 | 25 | 25 | 25 | 25.00 | S | 22 | 21 | 22 | 21.67 | S |
| B21 | 25 | 25 | 24 | 24.67 | S | 19 | 19 | 19 | 19.00 | S |
| B24 | 14 | 13 | 14 | 13.67 | R | 9 | 10 | 9 | 9.33 | R |
| B26 | 25 | 25 | 25 | 25.00 | S | 22 | 22 | 22 | 22.00 | S |
| B28 | 16 | 15 | 15 | 15.33 | R | 9 | 9 | 9 | 9.00 | R |
| B29 | 22 | 23 | 22 | 22.33 | S | 21 | 22 | 21 | 21.33 | S |
| B31 | 22 | 23 | 23 | 22.67 | S | 21 | 22 | 22 | 21.67 | S |
| B32 | 24 | 23 | 24 | 23.67 | S | 21 | 21 | 21 | 21.00 | S |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| B33 | 22 | 23 | 23 | 22.67 | S | 23 | 23 | 23 | 23.00 | S |
| B34 | 23 | 24 | 24 | 23.67 | S | 22 | 21 | 21 | 21.33 | S |
| 16-1 | 13 | 14 | 13 | 13.33 | R | 7 | 7 | 8 | 7.33 | R |
| 18-1 | 24 | 25 | 25 | 24.67 | S | 22 | 22 | 21 | 21.67 | S |
| 19-1 | 23 | 23 | 23 | 23.00 | S | 20 | 20 | 19 | 19.67 | S |
| 33-1 | 17 | 18 | 18 | 17.67 | I | 11 | 11 | 12 | 11.33 | R |
| 34-1 | 20 | 20 | 19 | 19.67 | I | 22 | 22 | 22 | 22.00 | S |
| 47-1 | 17 | 17 | 16 | 16.67 | R | 10 | 10 | 10 | 10.00 | R |
| 48-1 | 18 | 18 | 18 | 18.00 | I | 9 | 9 | 9 | 9.00 | R |
| 49-1 | 22 | 22 | 22 | 22.00 | S | 20 | 20 | 20 | 20.00 | S |
| 50-1 | 23 | 22 | 22 | 22.33 | S | 20 | 20 | 20 | 20.00 | S |
| 53-1 | 15 | 15 | 16 | 15.33 | R | 9 | 9 | 9 | 9.00 | R |
| 55-1 | 23 | 24 | 23 | 23.33 | S | 22 | 22 | 22 | 22.00 | S |
| 59-1 | 15 | 15 | 14 | 14.67 | R | 9 | 9 | 10 | 9.33 | S |
| A5 | 26 | 25 | 26 | 25.67 | S | 20 | 20 | 19 | 19.67 | S |
| A13 | 16 | 15 | 15 | 15.33 | R | - | - | - | - | R |
| A15 | 17 | 17 | 17 | 17.00 | R | - | - | - | - | R |
| A16 | 22 | 23 | 23 | 22.67 | S | 18 | 18 | 18 | 18.00 | S |
| A18 | 16 | 17 | 16 | 16.33 | R | - | - | - | - | R |
| B4 | 26 | 26 | 27 | 26.33 | S | 23 | 23 | 23 | 23 | S |
| B7 | 22 | 21 | 23 | 22.00 | S | 19 | 18 | 18 | 18.33 | S |
| B10 | 17 | 16 | 17 | 16.67 | R | - | - | - | - | R |
| B13 | 16 | 17 | 16 | 16.33 | R | - | - | - | - | R |
| B14 | 24 | 23 | 23 | 23.33 | S | 20 | 20 | 20 | 20.00 | S |
| B19 | 22 | 21 | 21 | 21.33 | S | 18 | 18 | 18 | 18.00 | S |
| B22 | 23 | 23 | 23 | 23.00 | S | 21 | 22 | 21 | 21.33 | S |
| B27 | 21 | 21 | 22 | 21.33 | S | 19 | 19 | 18 | 18.67 | S |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 4-1 | 21 | 21 | 21 | 21.00 | S | 19 | 20 | 20 | 19.67 | S |
| 8-1 | 17 | 16 | 17 | 16.67 | R | 14 | 13 | 14 | 13.67 | R |
| 10-1 | 18 | 18 | 18 | 18.00 | I | 13 | 14 | 13 | 13.33 | R |
| 36-1 | 11 | 11 | 11 | 11.00 | R | - | - | - | - | R |
| 43-1 | 16 | 16 | 17 | 16.33 | R | 10 | 10 | 10 | 10.00 | R |
| 62-1 | 12 | 12 | 12 | 12.00 | R | - | - | - | - | R |
| A12 | 19 | 21 | 21 | 20.33 | I | 14 | 14 | 14 | 14 | R |
| 7-2 | 26 | 26 | 27 | 26.33 | S | 21 | 21 | 20 | 20.67 | S |
| 9-1 | 25 | 24 | 26 | 25.00 | S | 21 | 20 | 21 | 20.67 | S |
| 14-1 | 25 | 25 | 25 | 25.00 | S | 22 | 21 | 21 | 21.33 | S |
| B8 | 22 | 22 | 23 | 22.33 | S | 21 | 22 | 22 | 21.67 | S |
| 2-2B | 20 | 20 | 20 | 20.00 | I | 20 | 21 | 21 | 20.67 | S |
| 15-1B | 24 | 23 | 24 | 23.67 | S | 24 | 23 | 23 | 23.33 | S |
| B25 | 21 | 20 | 21 | 20.67 | S | - | - | - | - | R |
| 32-2B | 23 | 22 | 22 | 22.33 | S | 20 | 21 | 20 | 20.33 | S |

*IC: Interpretive Category; R1/R2/R3: Reading 1/2/3; "-": No zone of inhibition; S: Susceptible; SDD: Susceptible-dose dependent; I: Intermediate; R: Resistant

**Appendix 6: Result of Double disk synergy test**

Twenty-three cefotaxime resistant *Enterobacteriaceae* isolates collected from Malaysia's poultry farm were selected to conduct double disk synergy test to detect ESBL production. The positive ESBL production was represented by showing a keyhole-shape zone produced by enhancing the inhibition zones of the cephalosporin disk towards the direction of amoxicillin/clavulanic acid disk. The table shows the result of double disk synergy test.

| **Isolate** | **CTX-AMC synergy** | | **CAZ-AMC synergy** | |
|---|---|---|---|---|
| | **30mm** | **20mm** | **30mm** | **20mm** |
| A6 | - | - | - | - |
| A17 | - | - | - | - |
| B24 | - | - | - | - |
| B28 | - | - | - | - |
| 16-1 | - | - | - | - |
| 33-1 | - | - | - | - |
| 34-1 | - | + | - | - |
| 47-1 | - | - | - | - |
| 48-1 | - | - | - | - |
| 49-1 | - | + | - | - |
| 53-1 | - | - | - | - |
| 59-1 | - | - | - | - |
| A13 | - | - | - | - |
| A15 | - | - | - | - |

| | | | | |
|---|---|---|---|---|
| A18 | - | - | - | - |
| B10 | - | - | - | - |
| B13 | - | - | - | - |
| 8-1 | - | - | - | - |
| 10-1 | - | - | - | - |
| 36-1 | - | - | - | - |
| 43-1 | - | - | - | - |
| 62-1 | - | - | - | - |
| A12 | - | - | - | - |

*“+” means detection of synergy; “-” means no synergy

**Appendix 7: Result of detection of β-lactamase genes**

The following table presents the result of screening six β-lactamase genes in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm using PCR.

| Isolate | β-lactamase gene | | | | | |
|---|---|---|---|---|---|---|
| | **TEM** | **SHV** | **CTX-M** | **CMY-2** | **DHA** | **MIR/ACT** |
| A6 | + | - | - | + | - | - |
| A7 | - | - | - | - | - | - |
| A17 | - | + | - | - | + | - |
| B3 | + | - | - | - | - | - |
| B5 | + | - | - | - | - | - |
| B6 | + | - | - | - | - | - |
| B15 | + | - | - | - | - | - |
| B17 | + | - | - | - | - | - |
| B18 | + | - | - | - | - | - |
| B20 | + | - | - | - | - | - |
| B21 | + | - | - | - | - | - |
| B24 | + | - | - | + | - | - |
| B26 | + | - | - | - | - | - |
| B28 | + | - | - | + | - | - |
| B29 | + | - | - | - | - | - |
| B31 | + | - | - | - | - | - |
| B32 | + | - | - | - | - | - |
| B33 | + | - | - | - | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| B34 | + | - | - | - | - | - |
| 16-1 | + | - | - | + | - | - |
| 18-1 | + | - | - | - | - | - |
| 19-1 | - | - | - | - | - | - |
| 33-1 | + | - | - | + | - | - |
| 34-1 | + | - | + | - | - | - |
| 47-1 | + | - | - | + | - | - |
| 48-1 | + | - | - | + | - | - |
| 49-1 | + | - | + | - | - | - |
| 50-1 | + | - | - | - | - | - |
| 53-1 | - | - | - | + | - | - |
| 55-1 | + | - | - | - | - | - |
| 59-1 | + | - | - | + | - | - |
| A5 | + | + | - | - | - | - |
| A13 | - | + | - | - | + | - |
| A15 | - | + | - | - | + | - |
| A16 | - | + | - | - | - | - |
| A18 | - | + | - | - | + | - |
| B4 | + | - | - | - | - | - |
| B7 | - | - | - | - | - | - |
| B10 | - | + | - | - | + | - |
| B13 | - | + | - | - | + | - |
| B14 | - | + | - | - | - | - |
| B19 | + | + | - | - | - | - |

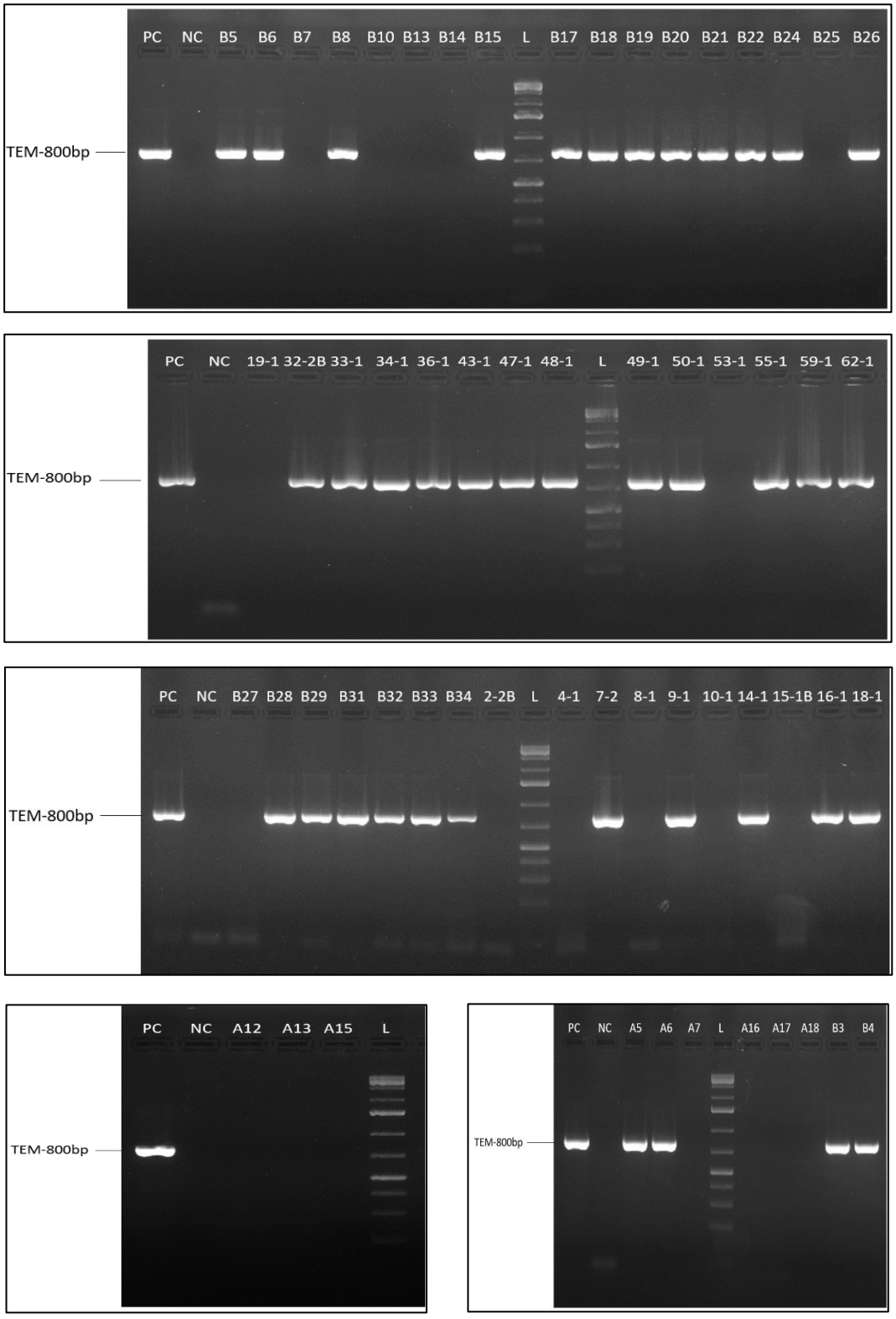
| | | | | | | |
|---|---|---|---|---|---|---|
| B22 | + | - | - | - | - | - |
| B27 | - | + | - | - | - | - |
| 4-1 | - | + | - | - | - | - |
| 8-1 | - | + | - | + | - | - |
| 10-1 | - | + | - | + | - | - |
| 36-1 | + | + | - | - | + | - |
| 43-1 | + | - | - | + | - | - |
| 62-1 | + | + | - | - | + | - |
| A12 | - | - | - | + | - | - |
| 7-2 | + | - | - | - | - | - |
| 9-1 | + | - | - | - | - | - |
| 14-1 | + | - | - | - | - | - |
| B8 | + | - | - | - | - | - |
| 2-2B | - | - | - | - | - | - |
| 15-1B | - | - | - | - | - | - |
| B25 | - | - | - | - | - | + |
| 32-2B | + | - | - | - | - | - |

*“+” means positive detection of interest gene, “-” means absence of interest gene

**Appendix 8: Gel image of screening β-lactamase genes**

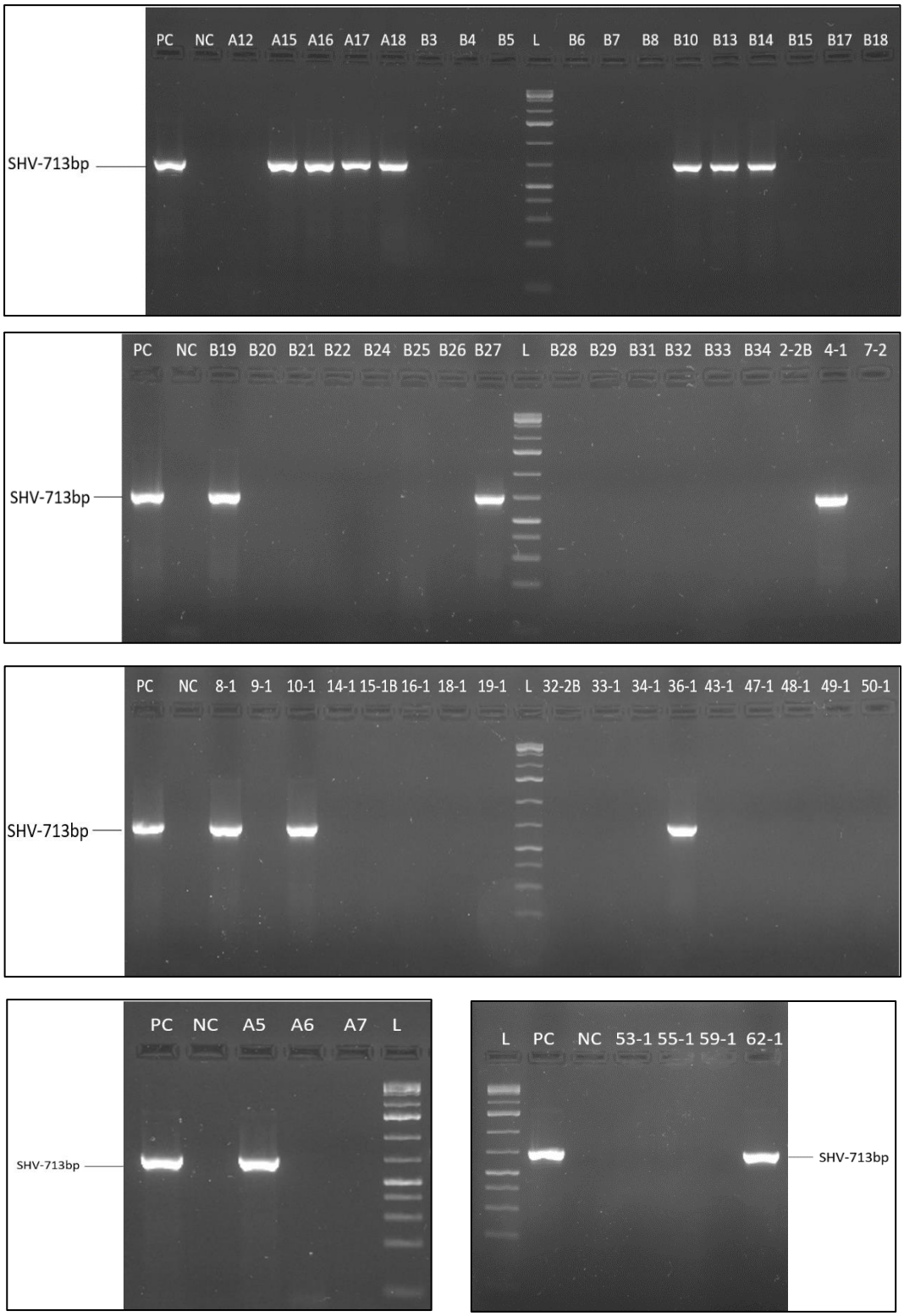
The gel images, captured after PCR-amplification and agarose gel electrophoresis at 70V, 400mA for 50 minutes, are displayed. Six β-lactamase genes were amplified, including (a) TEM (800bp), (b) SHV (713bp), (c) CTX-M (593bp), (d) CMY-2 (758bp), (e) DHA (405bp), and (f) MIR/ACT (302bp).

(a) TEM (800bp)



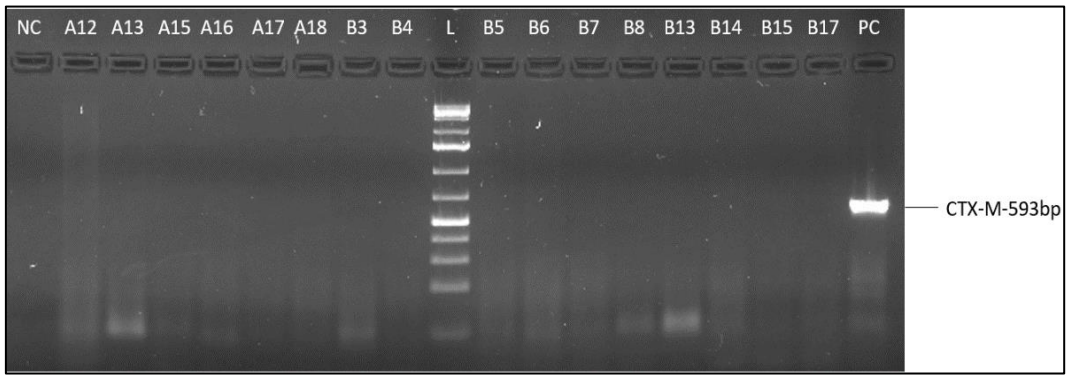

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

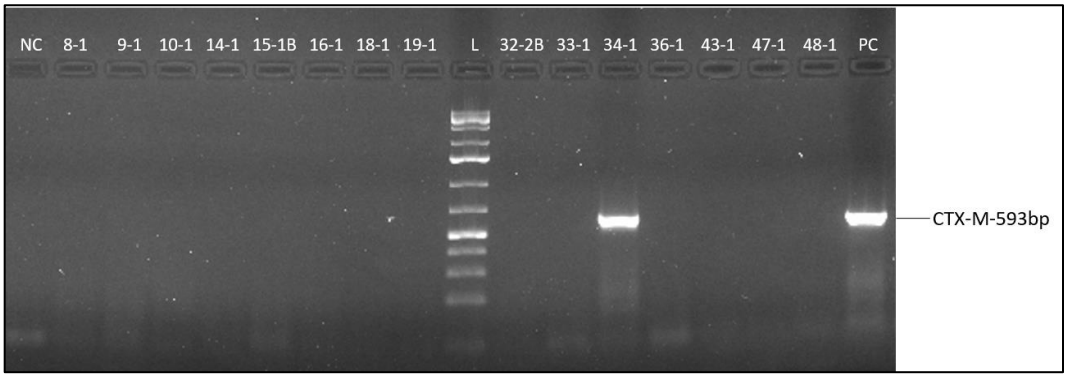
(b) SHV (713bp)

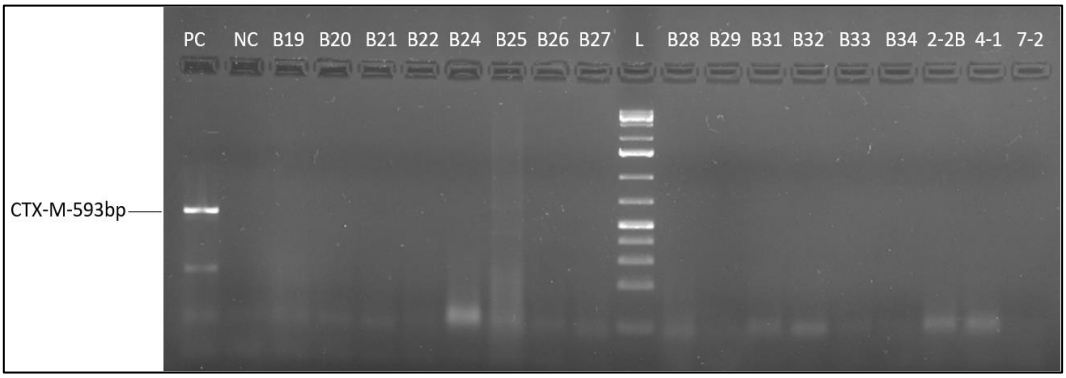


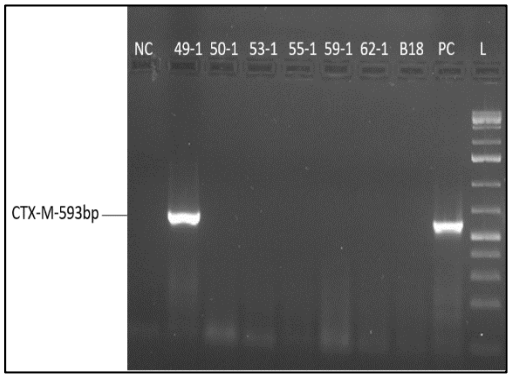

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

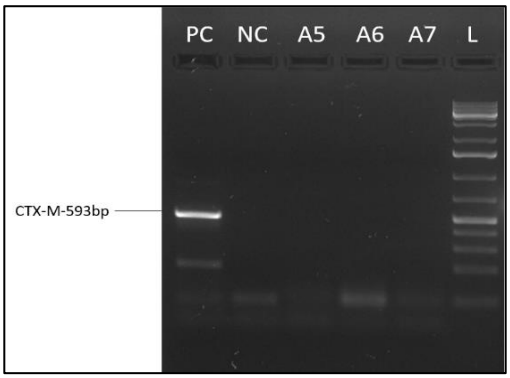
(c) CTX-M (593bp)

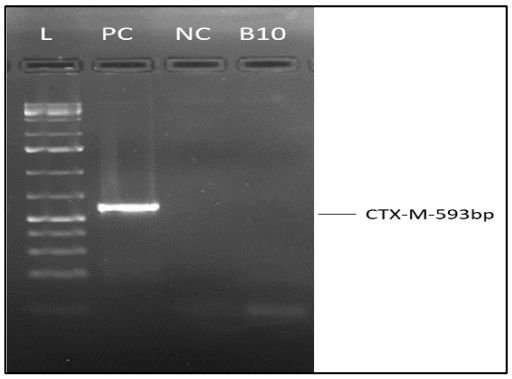

NC A12 A13 A15 A16 A17 A18 B3 B4 L B5 B6 B7 B8 B13 B14 B15 B17 PC
CTX-M-593bp


NC 8-1 9-1 10-1 14-1 15-1B 16-1 18-1 19-1 L 32-2B 33-1 34-1 36-1 43-1 47-1 48-1 PC
CTX-M-593bp


PC NC B19 B20 B21 B22 B24 B25 B26 B27 L B28 B29 B31 B32 B33 B34 2-2B 4-1 7-2
CTX-M-593bp

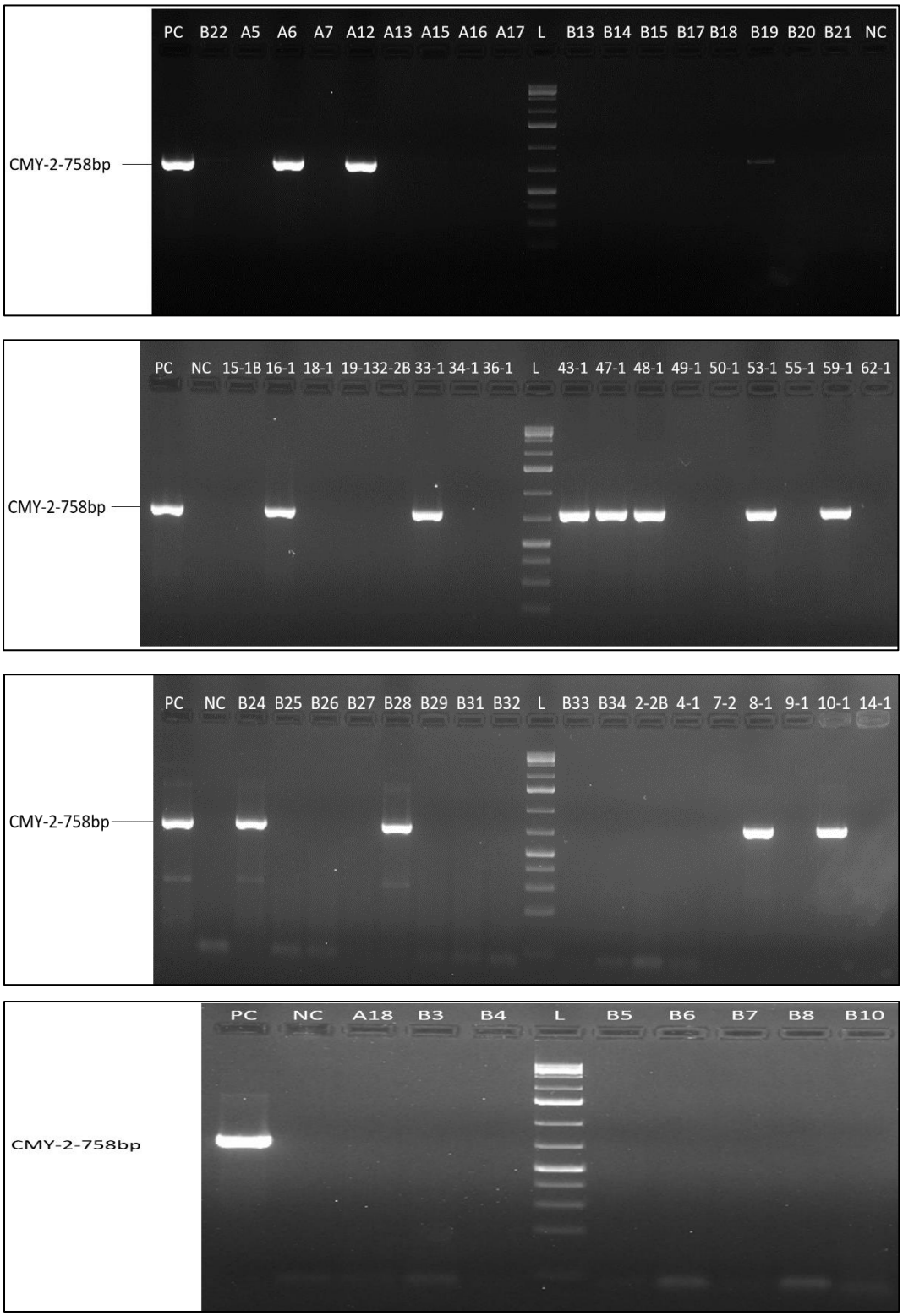

NC 49-1 50-1 53-1 55-1 59-1 62-1 B18 PC L
CTX-M-593bp


PC NC A5 A6 A7 L
CTX-M-593bp



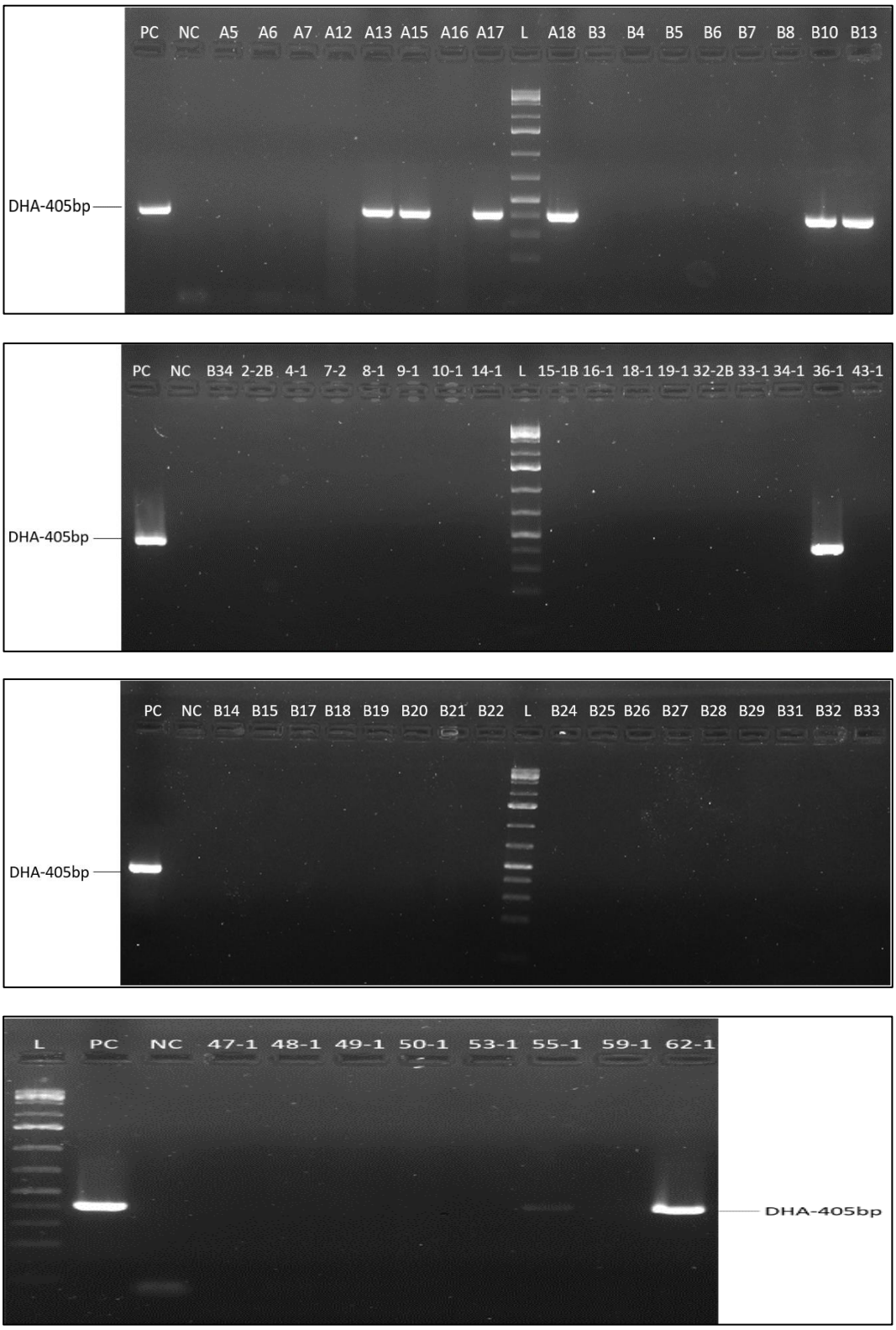

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

(d) CMY-2 (758bp)



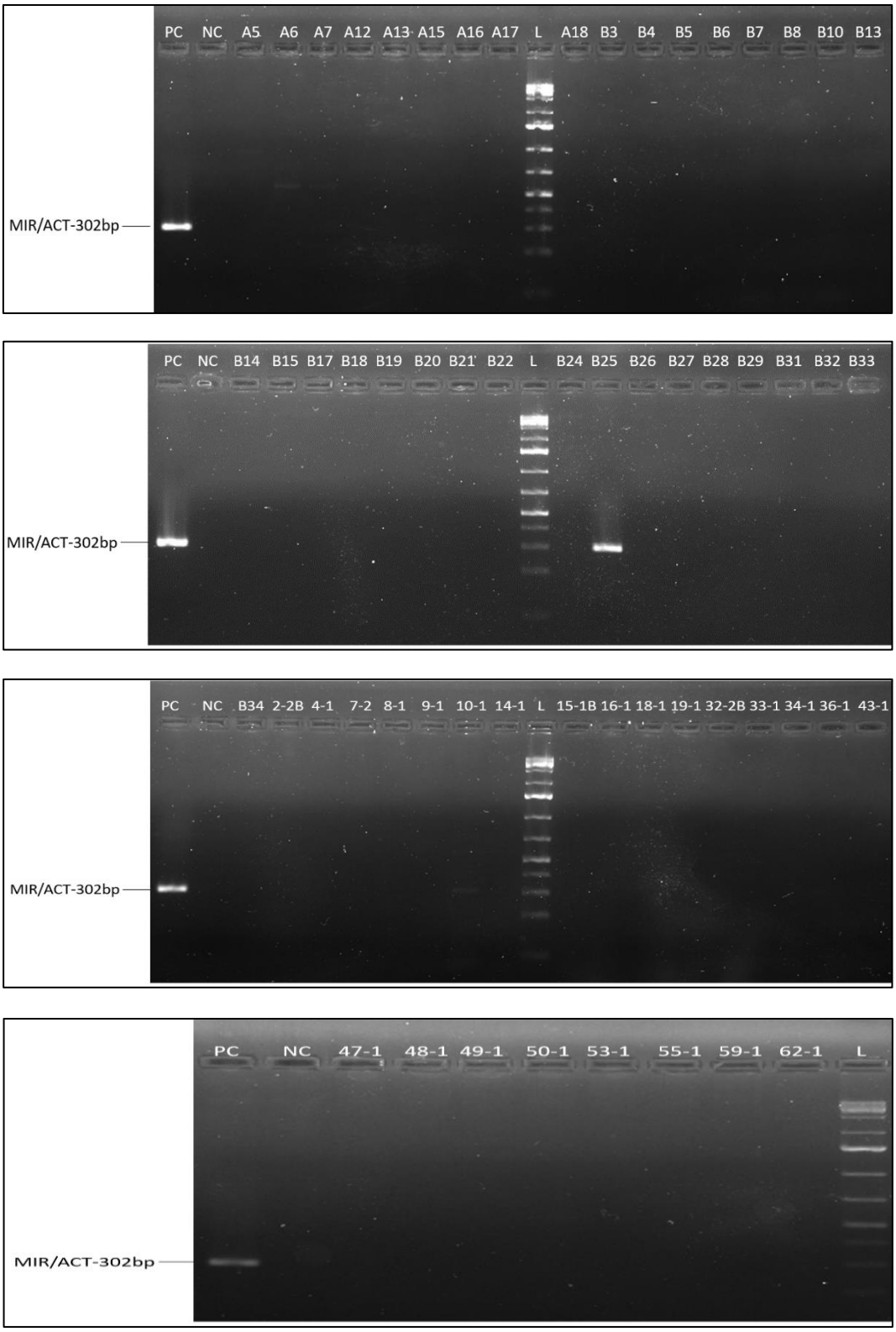

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

(e) DHA (405bp)




*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

(f) MIR/ACT (302bp)




*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

**Appendix 9: Results of screening virulence genes**

The table shows the result of screening nine virulence genes in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm.

| Isolate | Virulence genes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **EAST1** | **pAA** | **F18** | **Sta** | **LT** | **eae** | **est** | **AIDA** | **aspU** |
| A6 | + | - | - | - | - | - | - | - | - |
| A7 | - | - | - | - | - | - | - | - | - |
| A17 | - | - | - | - | - | - | - | - | - |
| B3 | - | - | - | - | - | - | - | - | - |
| B5 | - | - | - | - | - | - | - | - | - |
| B6 | - | - | - | - | - | - | - | - | - |
| B15 | - | - | - | - | - | - | - | - | - |
| B17 | - | - | - | - | - | - | - | - | - |
| B18 | - | - | - | - | - | - | - | - | - |
| B20 | - | - | - | - | - | - | - | - | - |
| B21 | - | - | - | - | - | - | - | - | - |
| B24 | - | - | - | - | - | - | - | - | - |
| B26 | - | - | - | - | - | - | - | - | - |
| B28 | + | - | - | - | - | - | - | - | - |
| B29 | - | - | - | - | - | - | - | - | - |
| B31 | - | - | - | - | - | - | - | - | - |
| B32 | + | - | - | - | - | - | - | - | - |
| B33 | - | - | - | - | - | - | - | - | - |
| B34 | - | - | - | - | - | - | - | - | - |
| 16-1- | - | - | - | - | - | - | - | - | - |
| 18-1- | - | + | - | - | - | - | - | - | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 19-1- | - | + | - | - | - | - | - | - | - |
| 33-1 | + | - | - | - | - | - | - | - | - |
| 34-1 | - | - | - | - | - | - | - | - | - |
| 47-1 | - | - | - | - | - | - | - | - | - |
| 48-1 | + | - | - | - | - | - | - | - | - |
| 49-1 | - | - | - | - | - | - | - | - | - |
| 50-1 | - | - | - | - | - | - | - | - | - |
| 53-1 | - | - | - | - | - | - | - | - | - |
| 55-1 | - | - | - | - | - | - | - | - | - |
| 59-1 | - | - | - | - | - | - | - | - | - |
| A5 | - | - | - | - | - | - | - | - | - |
| A13 | - | - | - | - | - | - | - | - | - |
| A15 | + | - | - | - | - | - | - | - | - |
| A16 | - | - | - | - | - | - | - | - | - |
| A18 | - | - | - | - | - | - | - | - | - |
| B4 | - | - | - | - | - | - | - | - | - |
| B7 | - | - | - | - | - | - | - | - | - |
| B10 | - | - | - | - | - | - | - | - | - |
| B13 | - | - | - | - | - | - | - | - | - |
| B14 | - | - | - | - | - | - | - | - | - |
| B19 | - | - | - | - | - | - | - | - | - |
| B22 | - | - | - | - | - | - | - | - | - |
| B27 | - | - | - | - | - | - | - | - | - |
| 4-1- | - | - | - | - | - | - | - | - | - |
| 8-1- | - | - | - | - | - | - | - | - | - |
| 10-1- | - | - | - | - | - | - | - | - | - |
| 36-1 | - | - | - | - | - | - | - | - | - |

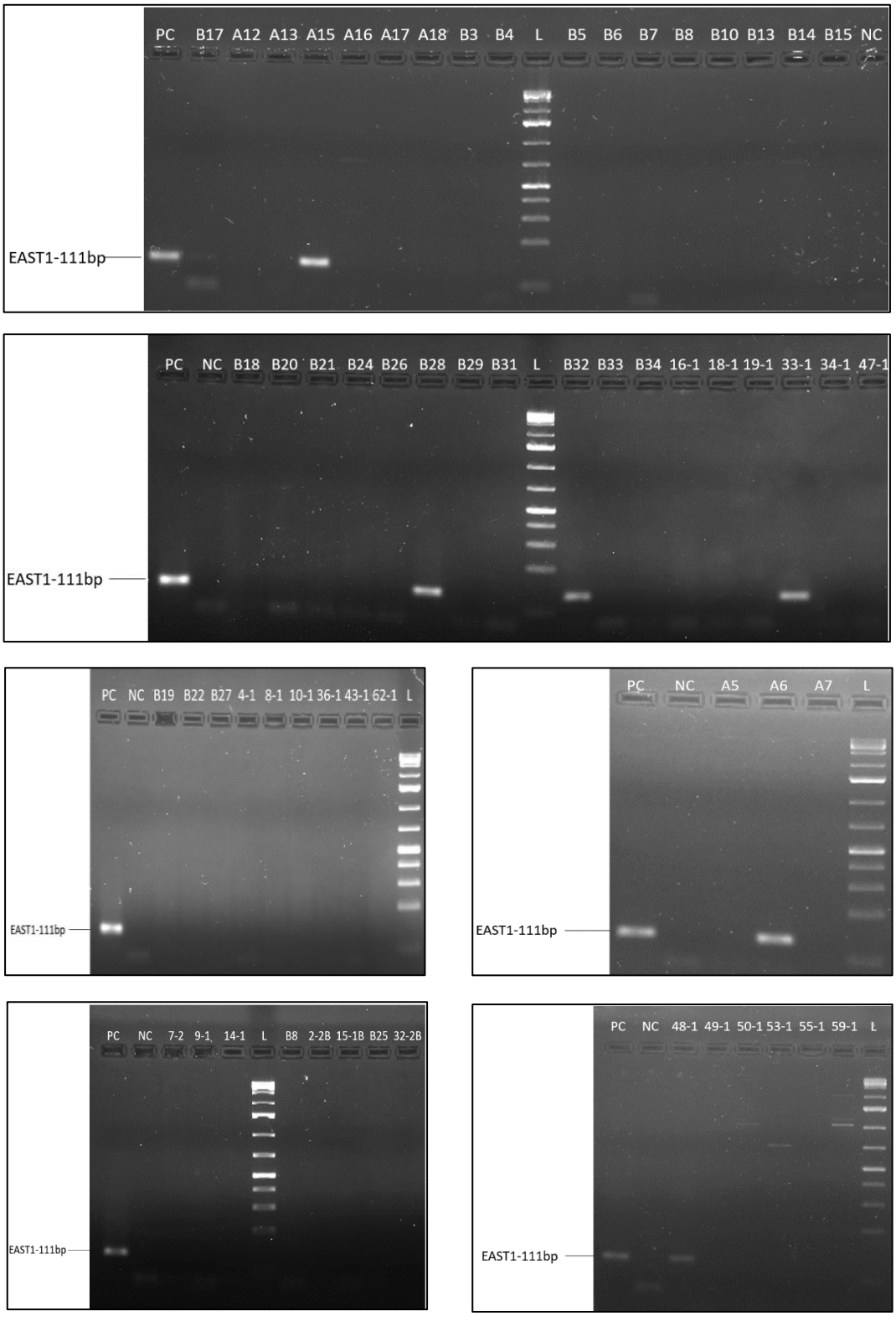
| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 43-1 | - | - | - | - | - | - | - | - | - |
| 62-1 | - | - | - | - | - | - | - | - | - |
| A12 | - | - | - | - | - | - | - | - | - |
| 7-2- | - | - | - | - | - | - | - | - | - |
| 9-1- | - | - | - | - | - | - | - | - | - |
| 14-1- | - | - | - | - | - | - | - | - | - |
| B8 | - | - | - | - | - | - | - | - | - |
| 2-2B | - | - | - | - | - | - | - | - | - |
| 15-1B | - | - | - | - | - | - | - | - | - |
| B25 | - | - | - | - | - | - | - | - | - |
| 32-2B | - | - | - | - | - | - | - | - | - |

*“+” means positive detection of interest gene, “-” means absence of interest gene

**Appendix 10: Gel images of screening virulence genes**

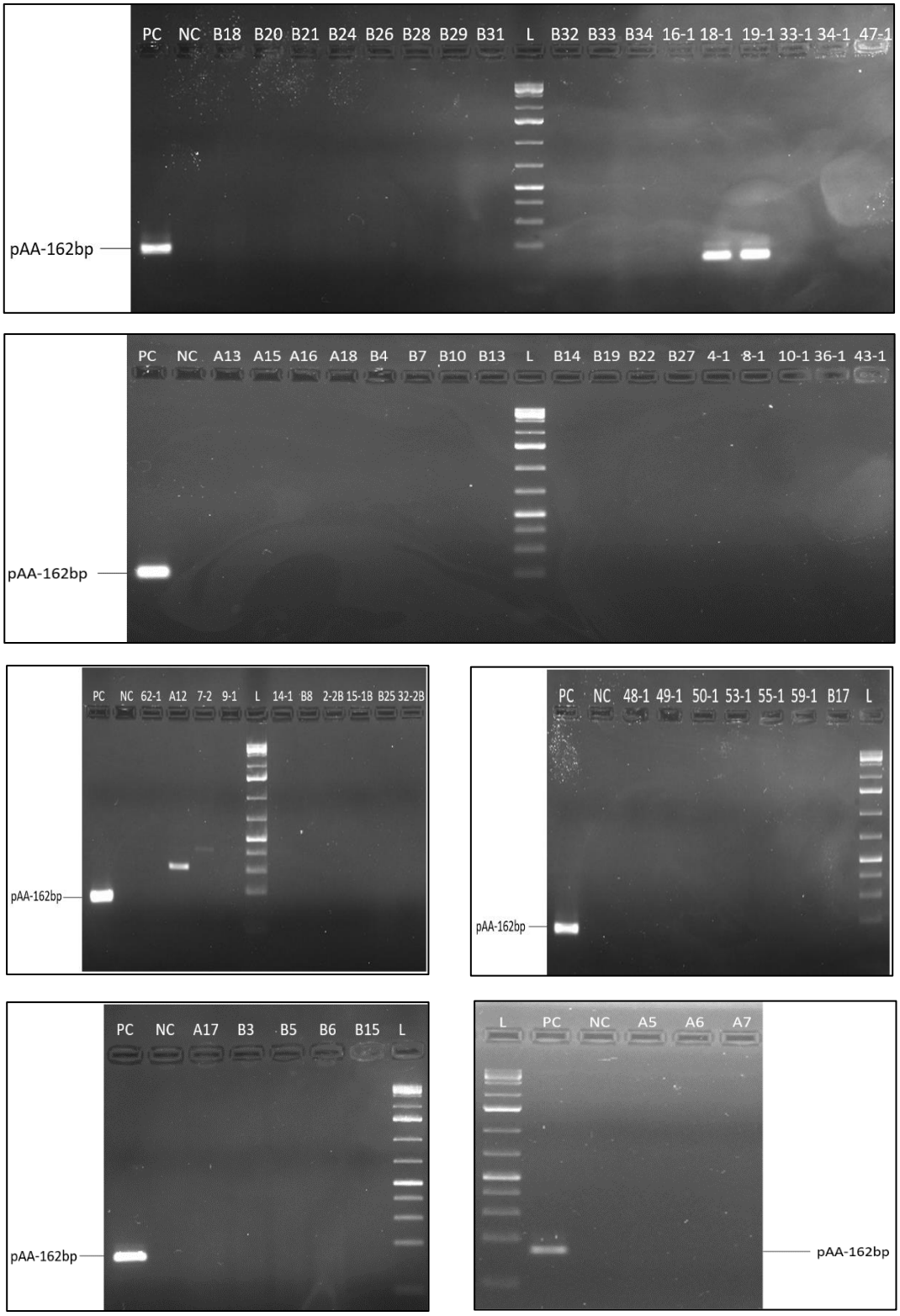
The following gel images display the screening results for (a) EAST1 (111bp) and (b) pAA (162bp), obtained after performing PCR amplification and agarose gel electrophoresis at 70V, 400mA for 50 minutes.

(a) EAST1 (111bp)




*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

(b) pAA (162bp)




*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

## Appendix 11: Results of PCR-based replicon typing

The table displays the result of screening thirteen plasmid replicon types in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm via PCR. The replicons were amplified using seven panel, including two simplex PCR (FrepB, B/O), four duplex PCR (I1&FIC, FIA&FIB, N&X, K&A/C) and one multiplex PCR (HI1&HI2&Y).

| **Isolate** | **Plasmid replicon typing** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Panel 1** | | **Panel 2** | | **Panel 3** | | **Panel 4** | | **Panel 5** | | | **Panel 6** | **Panel 7** |
| | **I1** | **FIC** | **FIA** | **FIB** | **N** | **X** | **K** | **A/C** | **HI1** | **HI2** | **Y** | **FrepB** | **B/O** |
| A6 | - | - | - | + | - | - | + | - | - | - | - | - | + |
| A7 | - | - | - | + | - | - | - | - | + | - | - | + | - |
| A17 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B3 | - | - | + | + | - | - | - | - | - | - | - | + | - |
| B5 | - | - | + | + | - | - | - | - | - | - | - | + | - |
| B6 | - | - | + | + | - | - | - | - | - | - | - | + | - |
| B15 | - | - | - | - | - | - | - | - | - | - | - | + | - |
| B17 | - | - | - | - | - | - | - | - | - | - | + | + | - |
| B18 | - | - | - | + | + | - | - | - | - | - | - | + | - |
| B20 | - | - | - | + | + | - | - | - | - | - | - | + | - |
| B21 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| B24 | + | - | - | - | - | - | - | - | - | - | - | - | - |
| B26 | - | - | - | + | + | - | - | - | - | - | - | + | - |
| B28 | - | - | - | + | - | - | + | - | + | - | - | - | + |
| B29 | - | - | - | + | - | - | - | - | - | - | - | + | - |
| B31 | - | - | - | + | + | - | - | - | - | - | - | + | - |
| B32 | - | - | - | - | - | + | - | - | - | - | - | - | - |
| B33 | + | - | - | + | + | - | - | - | - | - | - | + | - |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B34 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 16-1 | + | - | - | + | - | - | - | - | - | - | - | + | - |
| 18-1 | - | - | - | - | + | - | - | - | - | + | - | - | - |
| 19-1 | - | + | - | + | - | - | - | - | - | - | - | + | - |
| 33-1 | - | - | + | + | + | - | + | - | - | - | - | + | + |
| 34-1 | + | - | - | + | - | - | - | - | - | - | - | + | - |
| 47-1 | + | - | - | + | - | - | - | - | - | - | - | - | - |
| 48-1 | + | - | - | + | - | - | - | - | - | - | - | + | - |
| 49-1 | + | - | - | - | - | - | - | - | - | - | - | + | - |
| 50-1 | - | - | - | + | - | - | - | - | - | + | - | - | - |
| 53-1 | - | - | - | + | - | - | + | + | - | - | - | + | + |
| 55-1 | + | - | - | + | - | - | - | - | - | - | - | + | - |
| 59-1 | + | - | - | + | - | - | + | - | - | + | - | - | + |
| A5 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A13 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A15 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| A16 | + | - | - | - | + | - | - | - | - | - | - | - | - |
| A18 | - | - | - | - | + | - | + | - | - | - | - | - | - |
| B4 | - | - | - | - | + | - | - | - | + | - | - | - | - |
| B7 | - | - | - | + | + | - | - | - | - | - | - | - | - |
| B10 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B13 | + | - | - | - | - | - | - | - | - | - | - | - | - |
| B14 | - | - | - | + | + | - | - | - | - | - | - | - | - |
| B19 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| B22 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B27 | - | - | - | + | + | - | - | - | - | - | - | - | - |
| 4-1 | - | - | - | - | - | - | - | - | + | - | - | - | - |
| 8-1 | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 10-1 | + | - | - | - | - | - | - | - | - | - | - | - | - |

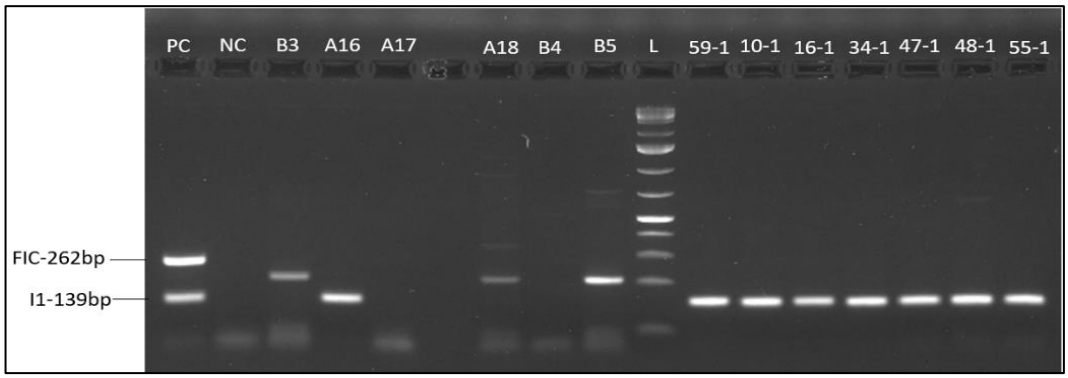
| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 36-1 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 43-1 | + | - | - | + | - | - | - | - | - | - | - | - | - |
| 62-1 | - | - | - | - | + | - | - | - | - | - | - | - | - |
| A12 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 7-2 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 9-1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 14-1 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B8 | - | - | - | - | + | - | - | - | + | - | - | - | - |
| 2-2B | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 15-1B | - | - | - | - | - | - | - | - | - | - | - | - | - |
| B25 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 32-2B | - | - | - | + | - | - | - | - | + | - | - | - | - |

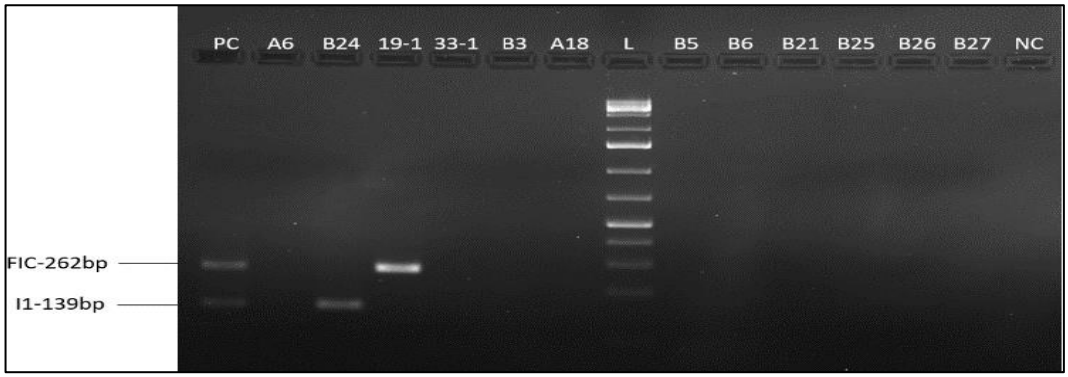
*“+” means positive detection of interest gene, “-” means absence of interest gene

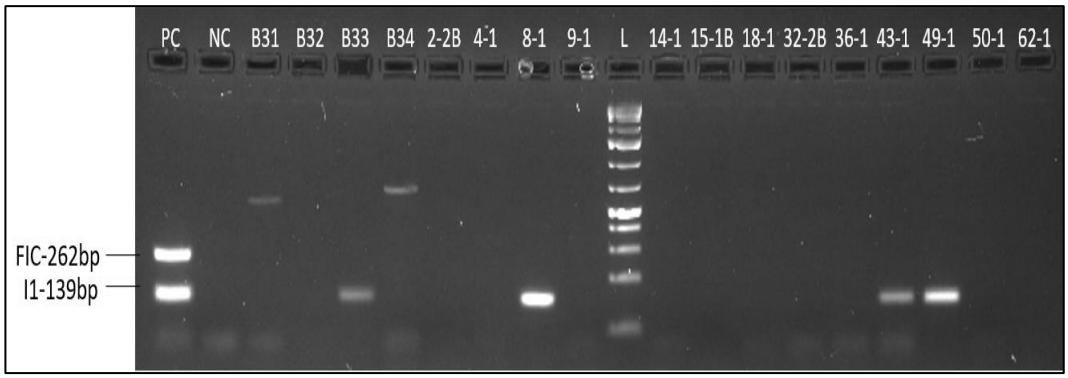
## Appendix 12: Gel images of plasmid replicon typing

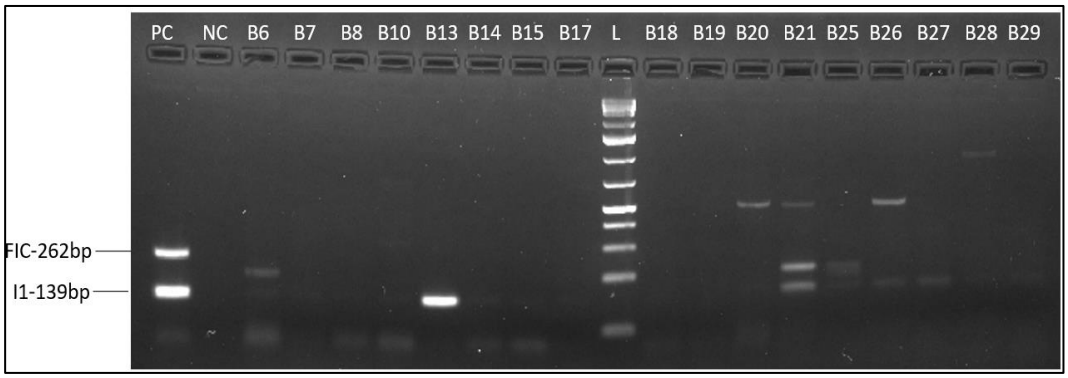
The gel images were captured following screening of thirteen plasmid replicon types in 59 *Enterobacteriaceae* isolates collected from Malaysia's poultry farm using PCR. The replicons were amplified using seven panels, including: (a) Panel 1 - Duplex PCR (I1-139bp; FIC-262bp), (b) Panel 2 - Duplex PCR (FIA-462bp; FIB-702bp), (c) Panel 3 - Duplex PCR (N-559bp; X-376bp), (d) Panel 4 - Duplex PCR (K-160bp; A/C-465bp), (e) Panel 5 – Multiplex PCR (HI1-471bp; HI2-644bp; Y-765bp), (f) Panel 6 - Simplex PCR (FrepB–270bp), and (g) Panel 7 - Simplex PCR (B/O – 159bp).

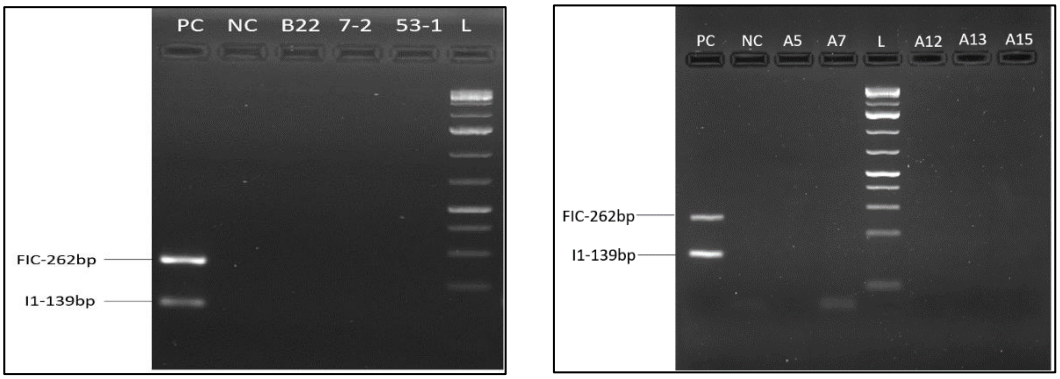
a) Panel 1 - Duplex PCR (I1-139bp; FIC-262bp)



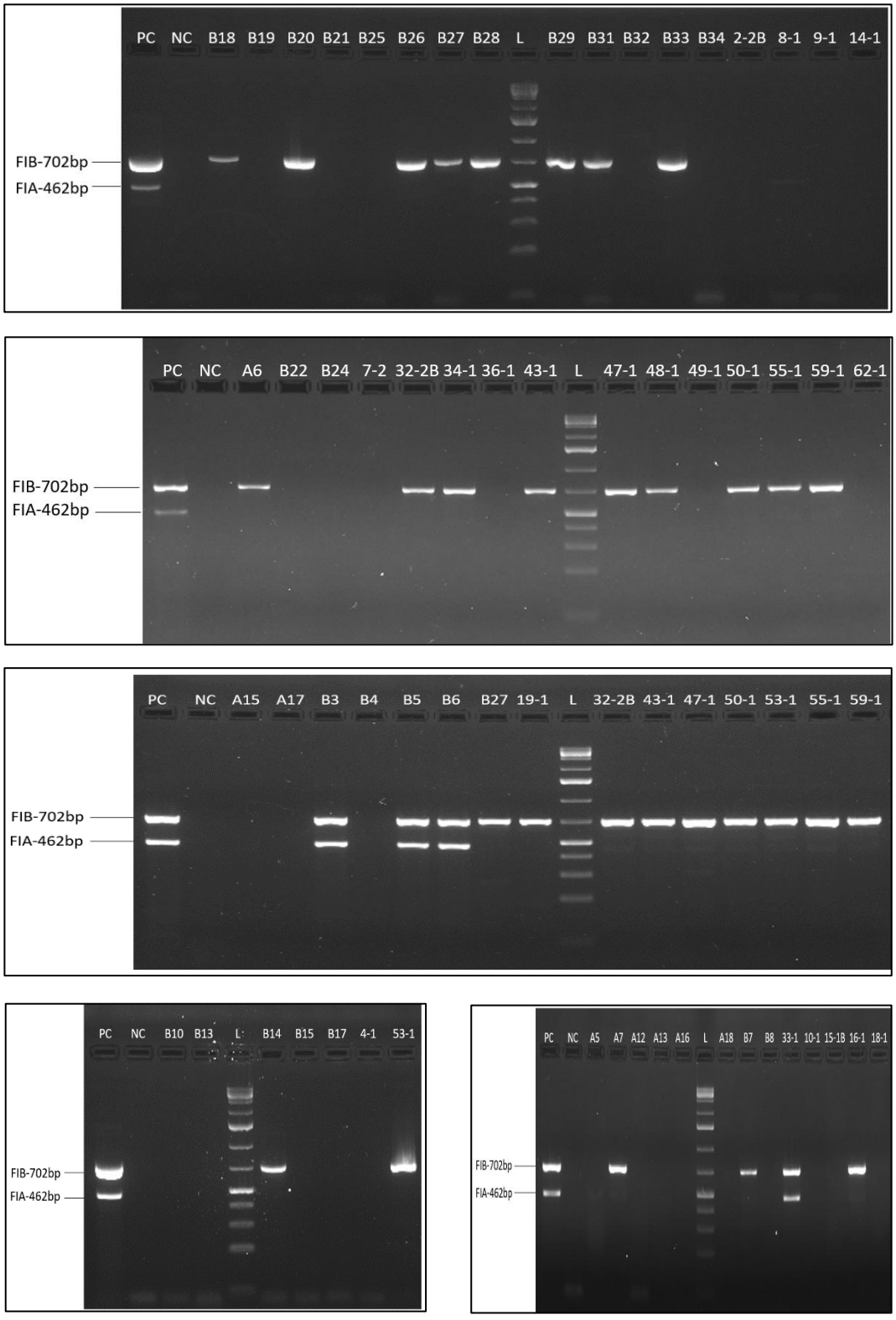





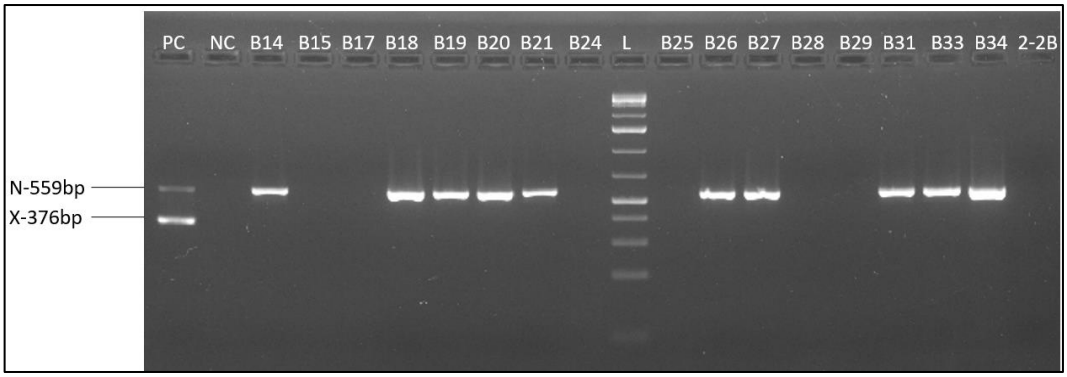

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

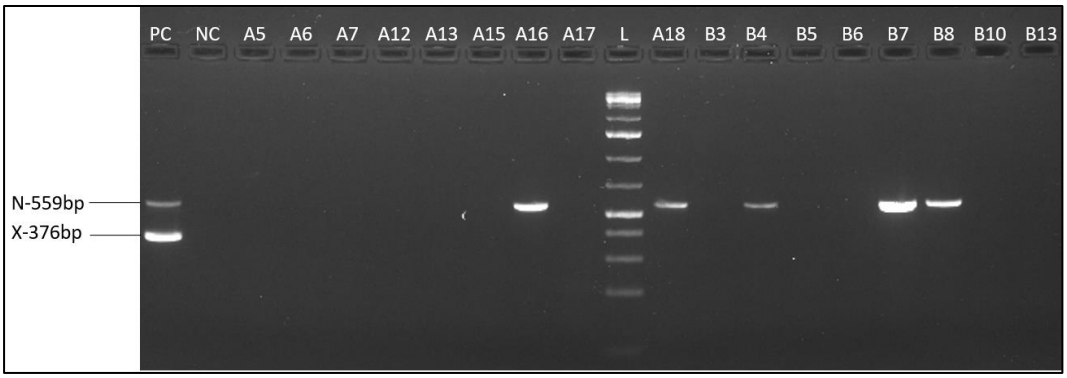
(b) Panel 2 - Duplex PCR (FIA-462bp; FIB-702bp)

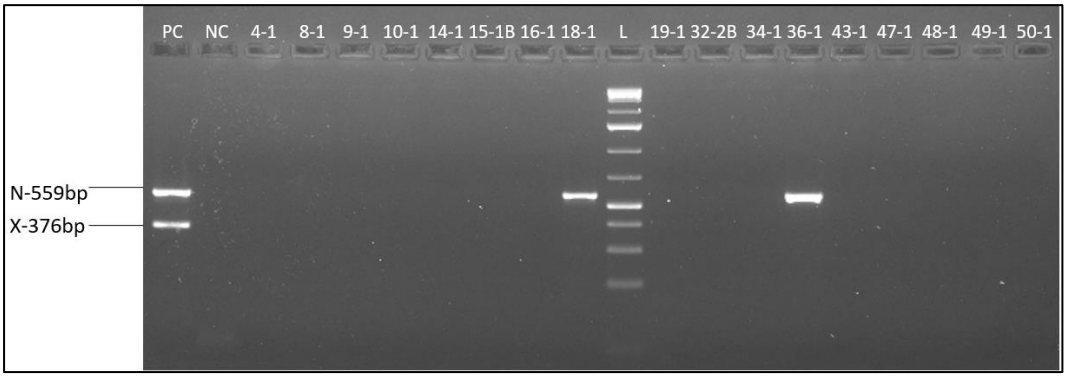


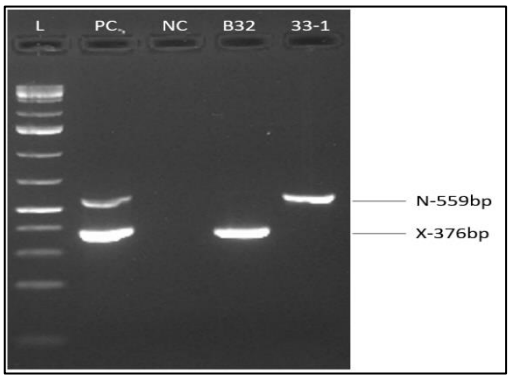

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

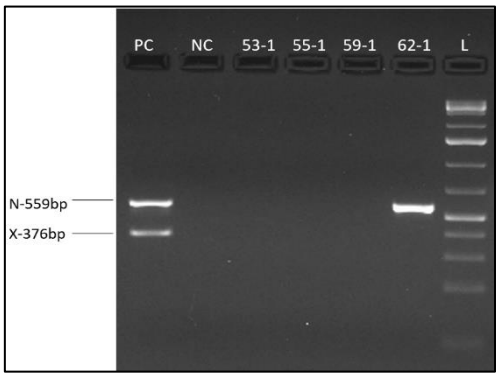
(c) Panel 3 - Duplex PCR (N-559bp; X-376bp)

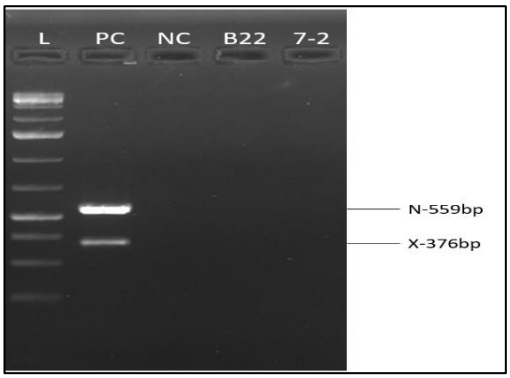


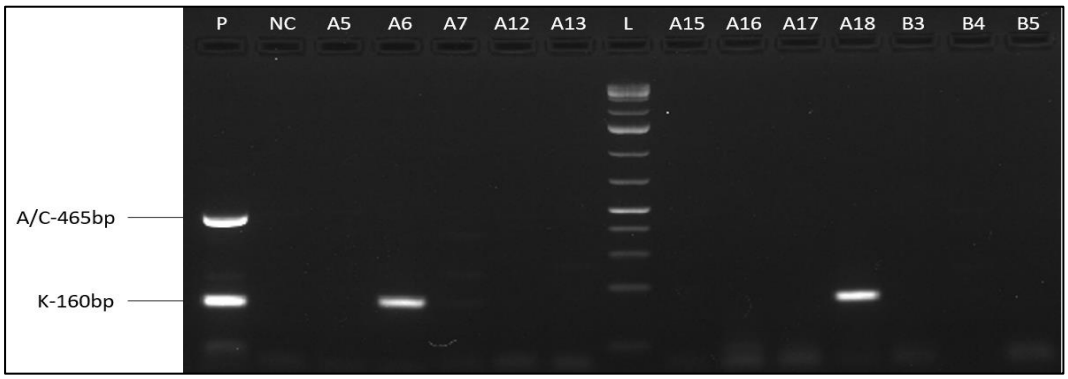



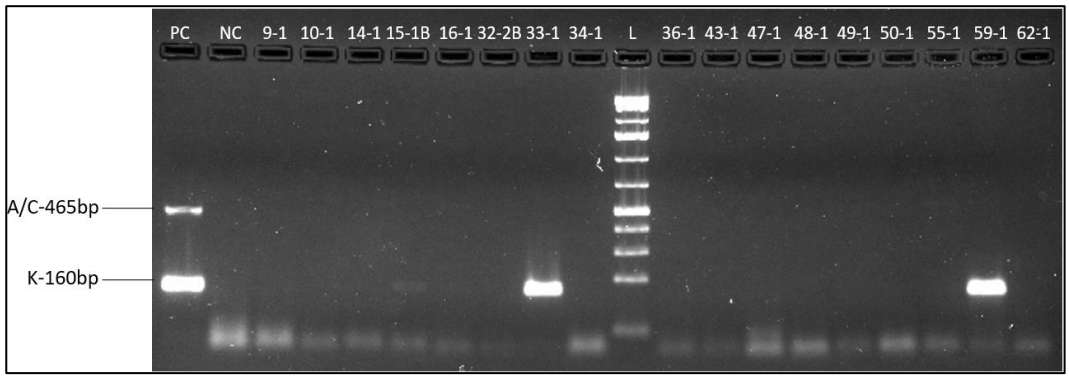



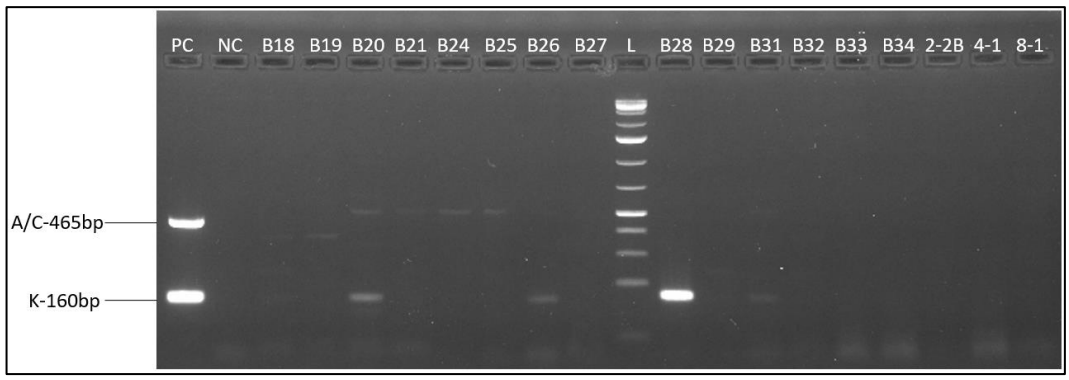


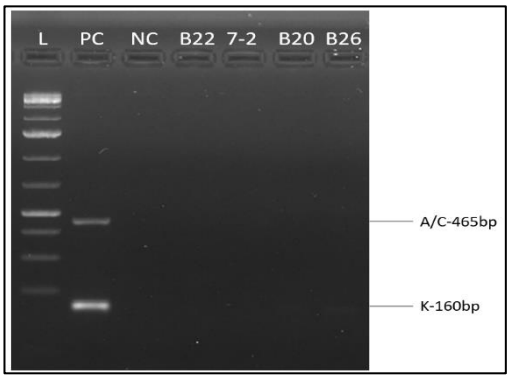

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

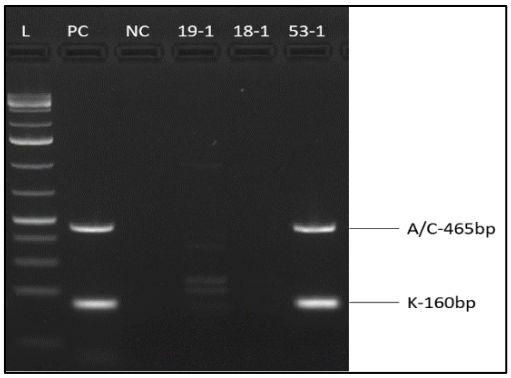
(d) Panel 4 - Duplex PCR (K-160bp; A/C-465bp)

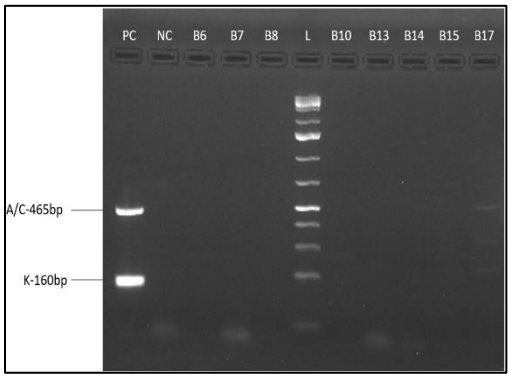


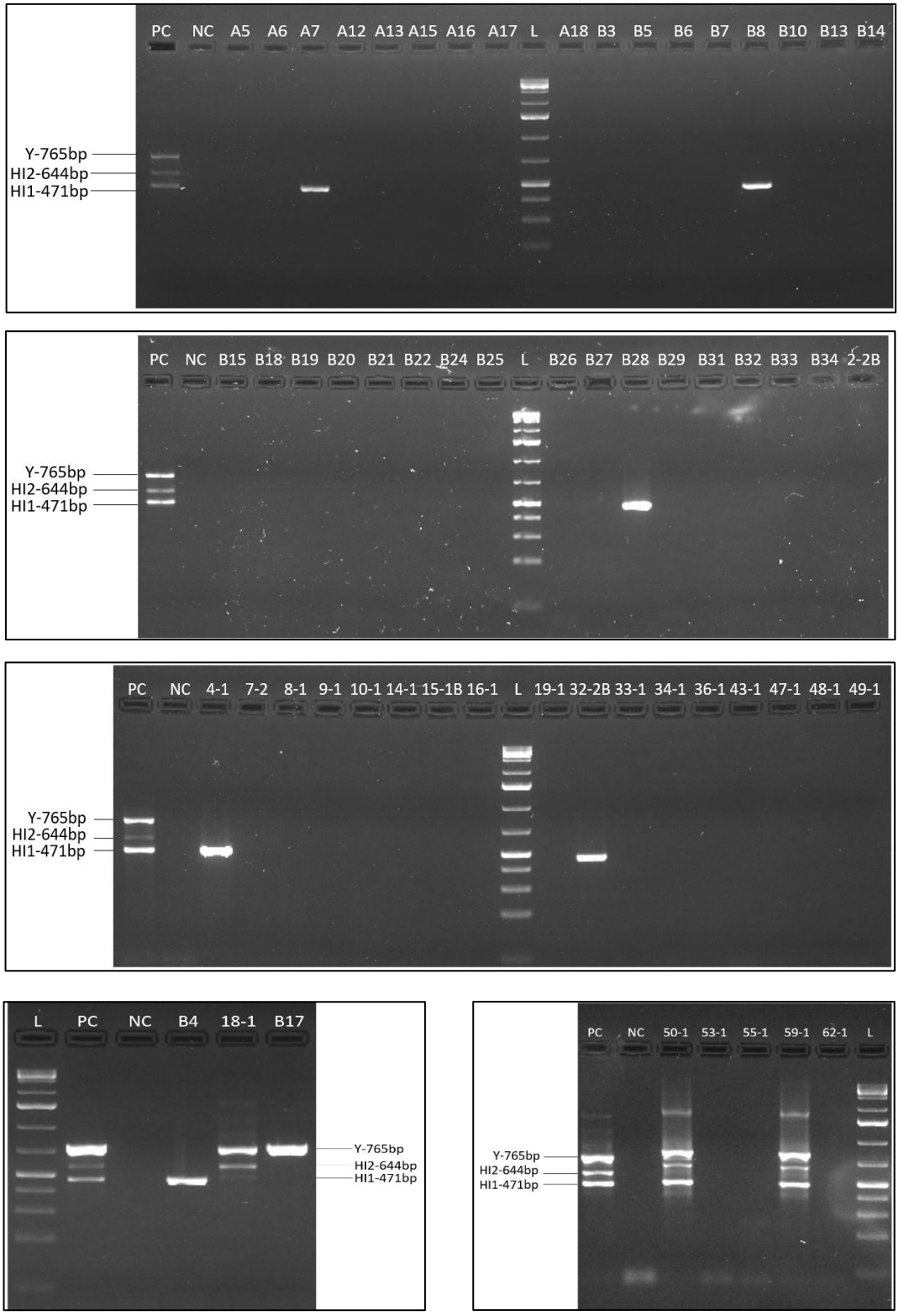





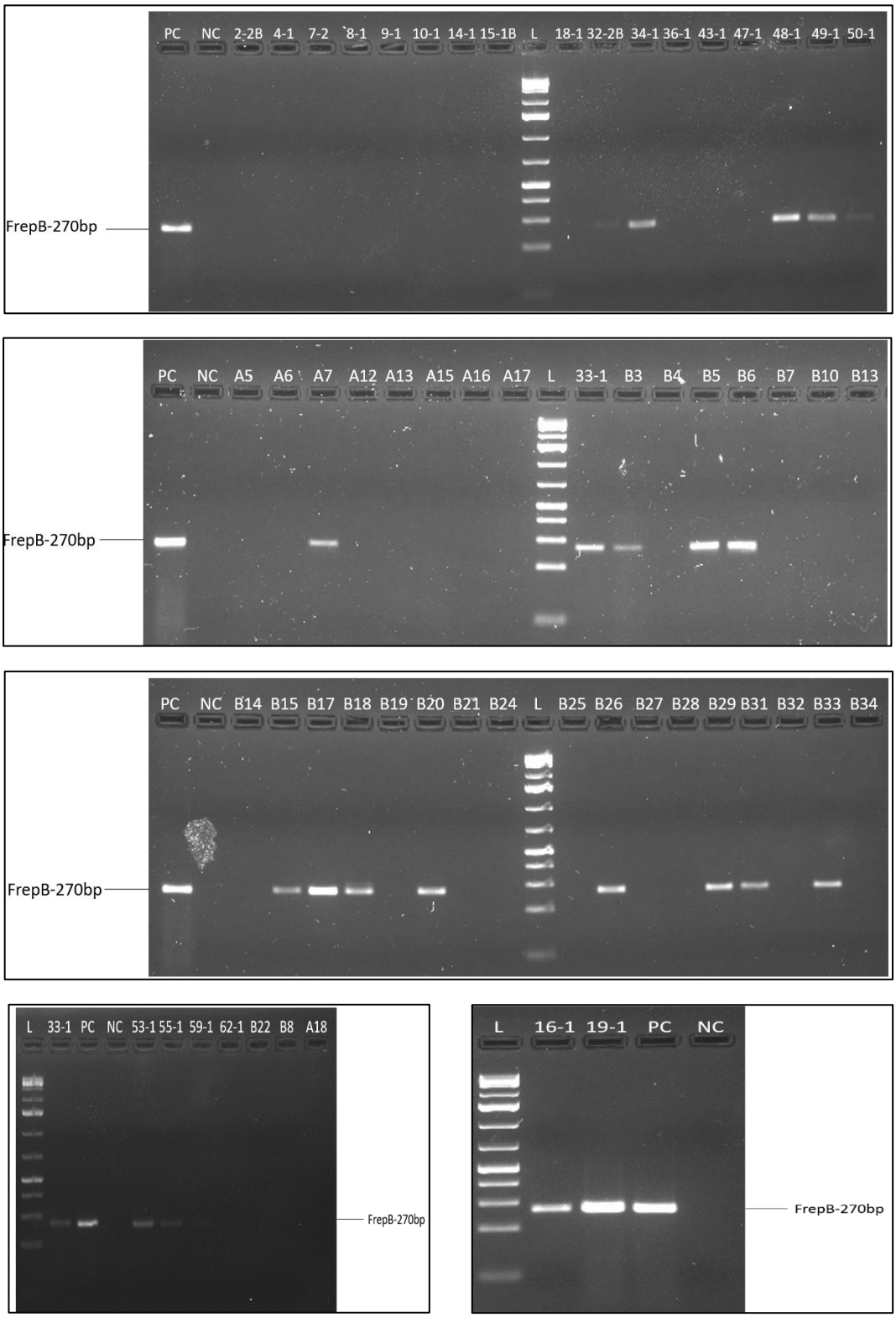

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

(e) Panel 5 - Multiplex PCR (HI1-471bp; HI2-644bp; Y-765bp)



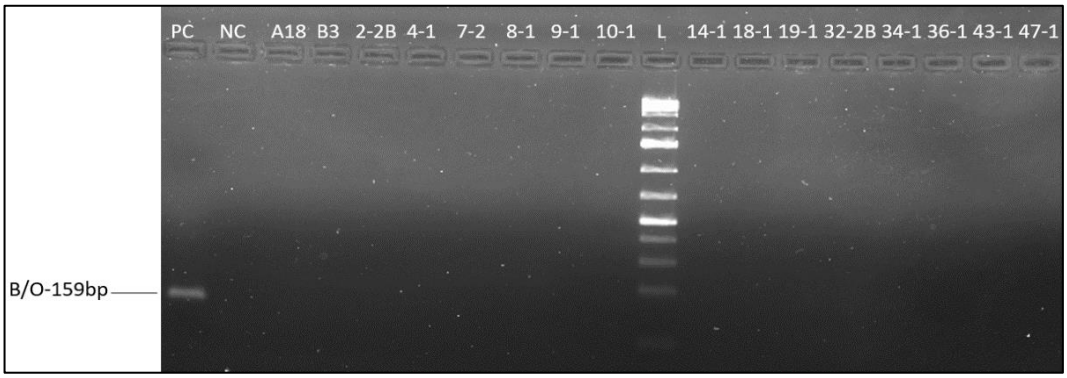

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

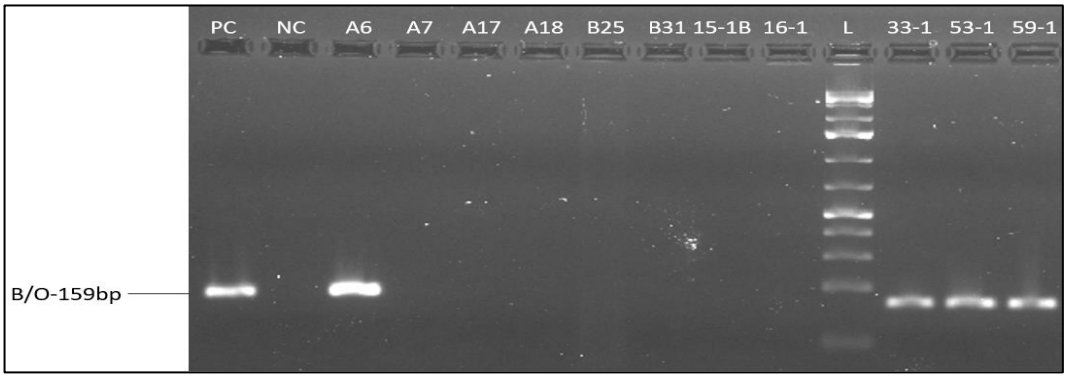
(f) Panel 6 - Simplex PCR (FrepB–270bp)

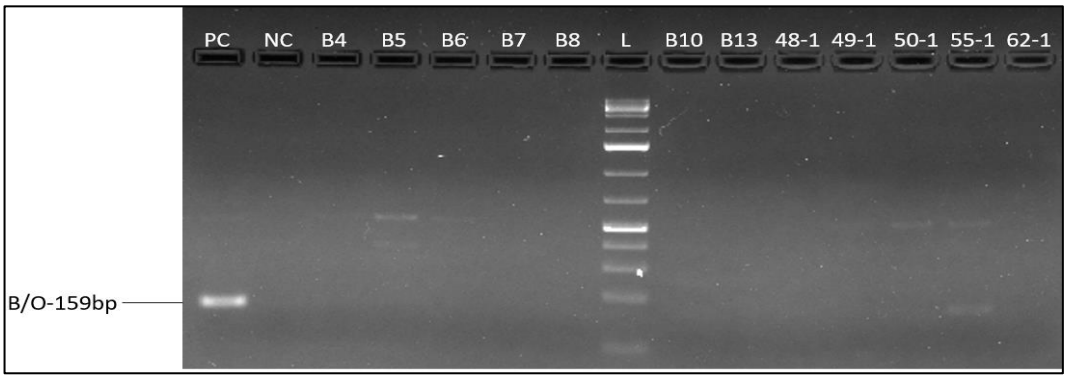


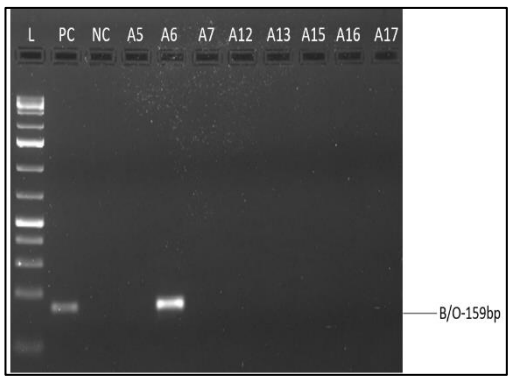

*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

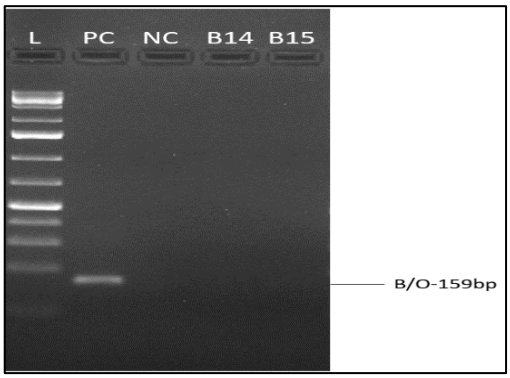
(g) Panel 7 - Simplex PCR (B/O – 159bp)

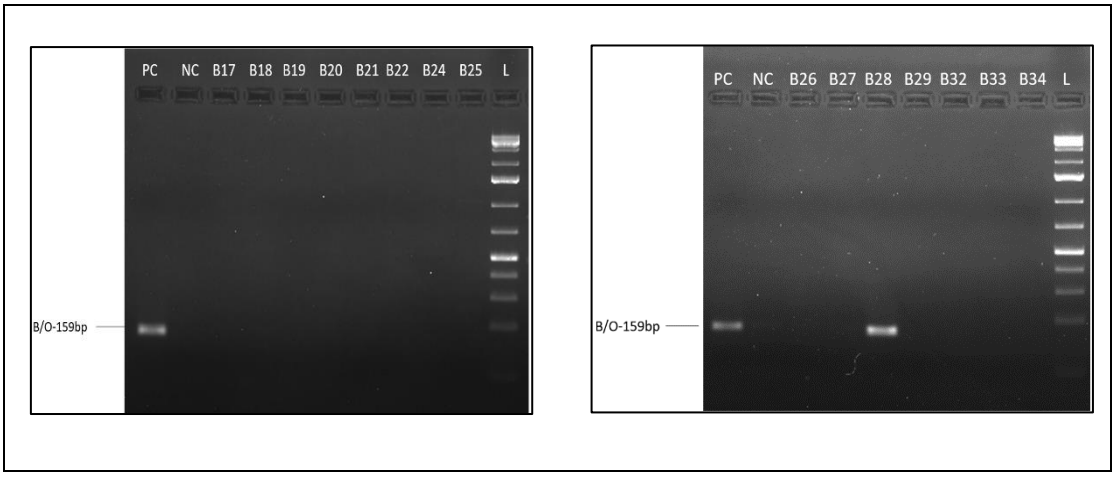





*L: GeneRuler 1kb DNA Ladder Plus; PC: Positive control; NC: Negative control

## Appendix 13: Publication of research paper

Hui-Shee Tan, Pan Yan, Hollysia Alda Agustie, Hwei-San Loh, Nabin Rayamajhi, Chee-Mun Fang. Characterisation of ESBL/AmpC-Producing *Enterobacteriaceae* isolated from poultry farms in Peninsular Malaysia. *Letters in Applied Microbiology*, 2022; ovac044, https://doi.org/10.1093/lambio/ovac044

The published paper comprises 10 pages and the page breakdown is provided below.

Page 1

*Letters in Applied Microbiology*, 2023, 76, 1–10
https://doi.org/10.1093/lambio/ovac044
Advance access publication date: 15 December 2022
Research Article

LETTERS IN APPLIED MICROBIOLOGY
OXFORD UNIVERSITY PRESS

# Characterisation of ESBL/AmpC-Producing *Enterobacteriaceae* isolated from poultry farms in Peninsular Malaysia

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### Abstract

Extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases (AmpCs)-producing *Enterobacteriaceae* have been increasingly reported and imposing significant threat to public. Livestock production industry might be the important source for clinically important ESBL-producing *Enterobacteriaceae*. This study aims to investigate the resistance profile, phenotypic ESBL production, beta-lactamase genes, virulence factors, and plasmid replicon types among 59 *Enterobacteriaceae* strains isolated from poultry faecal samples in Malaysia's commercial poultry farm. There were 38.7% and 32.3% of *Escherichia coli* resistant to cefotaxime and cefoxitin, respectively, while *Klebsiella* spp. demonstrated resistance rate of 52.6% to both mentioned antimicrobials. Majority of the *E. coli* isolates carried $bla_{TEM}$ and $bla_{CMY\text{-}2\ group}$. $bla_{SHV}$ was the most prevalent gene detected in *Klebsiella* spp., followed by $bla_{DHA}$ and $bla_{TEM}$. Resistance to extended spectrum cephalosporin in our isolates was primarily mediated by plasmid mediated AmpC beta-lactamase such as CMY-2 group and DHA enzyme. The CTX-M genes were found in two ESBL-producing *E. coli*. IncF, IncI1, and IncN plasmids were most frequently detected in *E. coli* and *Klebsiella* spp. The virulence factor, including EAST1 and pAA were identified at low frequency. This study highlights the poultry as a reservoir of resistance and virulence determinants and prevalence of plasmids in *Enterobacteriaceae* might drive their dissemination.

### Significance and impact of study:

This study indicated that poultry could become a reservoir of antibiotic resistant and virulent bacteria. A relatively high prevalence of plasmid-mediated AmpC beta-lactamase in *E. coli* and *Klebsiella* spp. was investigated in this study, comparing to ESBL enzymes. Frequent detection of plasmid replicons in *E. coli* and *Klebsiella* spp. might play a role in spread of resistance and virulence from poultry to food chain, human, and environment.

**Keywords:** extended-spectrum beta-lactamases (ESBLs), AmpC beta-lactamases (AmpCs), plasmid replicon, virulence genes, poultry

## Introduction

Antimicrobial resistance is global public health concern. Imprudent use of antimicrobials in livestock is one of the driving forces behind the increasing rate of resistance. In addition to therapeutic purpose, antibiotics could be applied as prophylaxis and growth promoter in animals. This may impose the selective pressure on both commensal and pathogenic microorganisms, which can disseminate among humans via direct contact, food chain, or environmental pollution of farm effluents (Roca et al. 2015). Beta-lactam ($\beta$-lactam) is one of the most important classes of antimicrobial agents for combating bacterial infection. $\beta$-lactams can be categorized to several classes, including penicillins, cephalosporins, carbapenems, and monobactams. Resistance to $\beta$-lactams is predominantly due to the production of beta-lactamase ($\beta$-lactamase), which can inactivate the drug by hydrolysing $\beta$-lactam ring. Extended-spectrum beta-lactamase (ESBL) and AmpC-beta-lactamase (AmpC) are enzymes capable of conferring resistance to extended-spectrum $\beta$-lactams (Smet et al. 2010).

The bacteria's ability to disseminate genetic materials through horizontal gene transfer plays an important role in development of antimicrobial resistance and virulence (Stokes and Gillings 2011). The genes encoding beta-lactamase such as ESBL and AmpC are associated with mobile genetic elements (MGEs) such as insertion sequences (ISs), integrons, transposons, and plasmids (Smet et al. 2010). Resistance genes can be disseminated across different species through MGEs. The horizontal transfer of resistance via MGEs has been investigated between commensals and pathogens in human, animal, and environment setting. *Escherichia coli* may serve as an important contributor to the spread of antibiotic resistance because it is an important animal commensal that can persist in the environment, with presence of some pathogenic variants (Stokes and Gillings 2011).

From 2000 s, ESBL/AmpC-producing *Enterobacteriaceae*, which was initially described in human medical practice have been increasingly detected in farm animals, particularly in

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poultry. This leads to the emergence of hypothesis that animals might become sources or reservoirs of infection, contributing to the spread of resistant bacteria. (Ewers et al. 2012). The prevalence of ESBL producers has been reported to be 67.9% among poultry meat products in Malaysia (Guo et al. 2021). However, the phenotypic and molecular characteristics of resistant isolates from poultry in Malaysia remains unknown. Thus, the present study was preformed to characterize the antimicrobial resistance profile, ESBL production, beta-lactamase genes, virulence genes, and plasmid replicon among *Enterobacteriaceae* isolates collected from poultry in Malaysia.

## Materials and methods

### Sample collection

The faecal samples were collected from three commercial poultry farms in West coast, Peninsular Malaysia. Farms 1 and 2 have three to five sheds while Farm 3 has two big sheds with temperature control system. Diseased broiler chicken samples (under enrofloxacin treatment) were collected at Farms 1 and 2 while the samples were collected from healthy broiler chickens in Farm 3. Farms 1 and 2 raised broiler chickens with three–seven weeks old while Farm 3 raised younger broiler chickens with 10–12 days old. A total of 100 faecal samples were collected from randomly selected chickens and 30–40 samples were obtained from each farm. The faecal swabs were stored at 4°C and subjected to microbiological analysis within 24 h after sample collection. The faecal swabs were pooled in 20 mL of Luria-Bertani (LB) broth and each enriched sample was streaked onto MacConkey agar plate to select *Enterobacteriaceae*. All samples were positive for *Enterobacteriaceae* isolates and one enteric isolate were isolated from each sample. A total of 59 stock cultures of *Enterobacteriaceae* isolated from poultry faecal samples were used in this study. All isolates were archived in glycerol stocks, which were stored at −80°C for further usage.

### Bacterial identification

The Gram stain, catalase and oxidase tests were performed for initial identification. Oxidase-negative isolates were subjected to biochemical identification by API® 20E (bioMérieux, France). Bacterial suspension was added into tubes and some tubes were covered by mineral oil. Some reagents were added to the tubes after 18 h of incubation, followed by reading result to obtain numerical profile. A seven-digit number was input into API 20E software to allow bacterial identification. Selected isolates were subjected for 16S rRNA sequencing to further confirm their identity by using 341F (5′-CCTACGGGAGGCAGCAG-3′) and 785R (5′- GACTACCAGGGTATCTAATCC-3′) (Klindworth et al. 2013). PCR products were sent for sequencing (Apical Scientific, Singapore) and sequences were subjected to analysis through Basic Local Alignment Search Tool (BLAST) (Comparing to Accession number KY962911.1, OM816745.1, OL958647.1, OM666543.1, and MT525340.1). Gene encoding lactose permease (*lacY*), which is hallmark of *E. coli* was screened to differentiate *E. coli* from *Shigella* spp. via *lacY*-F (5′- ACCAGACCCAGCACCAGATAAG-3′) and *lacY*-R (5′-GCACCTACGATGTTTTTGACCA-3′) (Horakova et al. 2008).

### Antimicrobial susceptibility test to $\beta$-lactams and phenotypic ESBL detection

The antimicrobial susceptibility was determined by disc diffusion method following standard procedure of the Clinical and Laboratory Standards Institute (CLSI) by using cefepime (FEP, 30 $\mu$g, 4rd generation cephalosporins), cefotaxime (CTX, 30 $\mu$g, 3rd generation cephalosporins), ceftazidime (CAZ, 30 $\mu$g, 3rd generation cephalosporins), and cefoxitin (FOX, 30 $\mu$g, cephamycin) (Oxoid, UK). The results of antimicrobial susceptibility test were interpreted according to CLSI criteria (CLSI 2021). All CTX-resistant isolates were tested for ESBL production by the double-disk synergy test (DDST) by placing a disc of amoxicillin/clavulanic acid (AMC, 20/10 $\mu$g) and a 30-$\mu$g disc of cephalosporin (cefotaxime and ceftazidime) at the distance of 20–30 mm (centre to centre) on a Mueller–Hinton agar plates inoculated with the bacterial suspension of 0.5 McFarland turbidity. The test was interpreted as positive when there is enhancement of inhibition zones of cephalosporin disk toward the direction of AMC disc. *E. coli* ATCC® 25 922 was used as a quality control strain.

### DNA extraction

A single colony was picked from a fresh bacterial culture on LB agar and suspended in 100 $\mu$l of sterile water. The cells were heated at 99°C for 10 min followed by centrifugation at 13 000 rpm for 2 min to spin down the cell debris. The supernatant was stored at −20°C for following usage as template in the polymerase chain reactions.

### Detection of beta-lactamase gene

Genes encoding TEM, SHV, CTX-M, CMY-2, DHA, and MIR/ACT type beta-lactamase were examined in all isolates through PCR using universal primers for the corresponding gene families. The information regarding primers and PCR condition was shown in Table 1. Each 20 $\mu$L of PCR reaction mixture consisted of 1x $NH_4^+$ Reaction Buffer (Bioline, UK), 0.2 mM dNTPs (Promega, US), 1 mM $MgCl_2$ (Bioline, UK), 0.1 unit/$\mu$L Taq DNA polymerase (Bioline, UK), and various concentration range of primers and 1 $\mu$L template DNA. DNA amplification was carried out under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60°C–63°C for 30 sec, elongation at 72°C for 30–60 sec, and final extension at 72°C for 5 min. PCR products were visualized on 2% agarose gel electrophoresis.

### Detection of virulence gene and plasmid replicon typing

Some *E. coli* clones can acquire specific virulence determinants, which enable them to cause various diseases. Nine virulence factors, including *EAST1*, *pAA*, *F18*, *Sta*, *LT*, *eae*, *est*, *AIDA-I*, and *aspU* were screened by PCR assay. The targeted virulence factors include adhesins (*pAA*, *F18*, *eae*, and *AIDA-I*), toxins (*EAST1*, *Sta*, *LT*, and *est*), and secreted protein (*aspU*). Plasmid incompatibility (Inc) groups were determined by PCR-based replicon typing (PBRT). In this study, the plasmid replicons of all isolates were examined by using 13 pairs of primers targeting replicon I1, FIC, FIA, FIB, N, X, K, A/C, HI1, HI2, Y, FrepB, and B/O (Carattoli et al. 2005). These are major plasmid incompatibility groups circulating among the

**Table 1.** Primers used for screening virulence gene and plasmid replicon and corresponding PCR condition.

| Target's gene | Primer's name | Sequence (5′-3′) | Amplicon size (bp) | Primer concentration ($\mu$M) | $T_{annealing}$ (°C) | $t_{elongation}$ (second) | Reference |
|---|---|---|---|---|---|---|---|
| **Beta-lactamase genes** | | | | | | | |
| $bla_{TEM}$ | TEM-F | CATTTCCGTGTCGCCCTTATTC | 800 | 0.4 | 60 | 60 | (Dallenne et al. 2010) |
| | TEM-R | CGTTCATCCATAGTTGCCTGAC | | | | | |
| $bla_{SHV}$ | SHV-F | AGCCGCTTGAGCAAATTAAAC | 713 | 0.4 | 60 | 60 | (Dallenne et al. 2010) |
| | SHV-R | ATCCCGCAGATAAATCACCAC | | | | | |
| $bla_{CTX-M}$ | CTX-M-F | ATGTGCAGTACCAGTAAGGTGATGGC | 593 | 0.4 | 63 | 40 | (Hasman et al. 2005) |
| | CTX-M-R | TGGGTAAAGTAGGTCACCAGAACCAGCGG | | | | | |
| $bla_{CMY-2\ group}$ | CMY-2-F | GCACTTAGCCACCTATACGGCAG | 758 | 0.5 | 62 | 60 | (Hasman et al. 2005) |
| | CMY-2-R | GCTTTTCAAGAATGCGCCAGG | | | | | |
| $bla_{DHA}$ | DHA-F | AACTTTCACAGGTGTGCTGGGT | 405 | 0.6 | 63 | 30 | (Pérez-Pérez and Hanson 2002) |
| | DHA-R | CCGTACGCATACTGGCTTTGC | | | | | |
| $bla_{MIR/ACT}$ | MIR/ACT-F | TCGGTAAAGCCGATGTTGCGG | 302 | 0.5 | 63 | 30 | (Pérez-Pérez and Hanson 2002) |
| | MIR/ACT-R | CTTCCACTGCGGCTGCCAGTT | | | | | |
| **Virulence genes** | | | | | | | |
| *EAST1* | EAST1-F | CCATCAACACAGTATATCCGA | 111 | 0.25 | 58 | 60 | (Rayamajhi et al. 2011) |
| | EAST1-R | GGTCGCGAGTGACGGCTTTGT | | | | | |
| *pAA* | pAA-F | CCATAAAGACAGCTTCAGTGAAAA | 162 | 0.45 | 58 | 60 | (Rayamajhi et al. 2011) |
| | pAA-R | GTATTACTGGTACCACCACCATCA | | | | | |
| *F18* | F18-F | CTTTCACATTGCGTGTGGAG | 441 | 0.25 | 55 | 60 | (Rayamajhi et al. 2011) |
| | F18-R | ATTCGACGCCTTAACCTCCT | | | | | |
| *STa* | Sta-F | GAAACAACATGACGGGAGGT | 229 | 0.25 | 55 | 60 | (Rayamajhi et al. 2011) |
| | Sta-R | GCACAGGCAGATTACAACA | | | | | |
| *LT* | LT-F | GGTTTCTGCGTTAGGTGGAA | 605 | 0.25 | 55 | 60 | (Rayamajhi et al. 2011) |
| | LT-R | GGGACTTCGACCTGAAATGT | | | | | |
| *eae* | *eae*-F | CCCGAATTCGGCACAAGCATAAGC | 881 | 0.25 | 60 | 60 | (Rayamajhi et al. 2011) |
| | *eae*-R | CCCGGATCCGTCTCGCCAGTATTCG | | | | | |
| *est* | *est*-F | TTAATAGCACCCGGTACAAGCAGG | 147 | 0.25 | 55 | 60 | (Rayamajhi et al. 2011) |
| | *est*-R | CCTGACTCTTCAAAAGAGAAAATTAC | | | | | |
| *AIDA-I* | AIDA-I-F | TGGTGGGAAAACCACTGCTA | 771 | 0.25 | 55 | 60 | (Rayamajhi et al. 2011) |
| | AIDA-I-R | TAGCCGCCATCACTAACCAG | | | | | |
| *aspU* | *aspU*-F | GCCTTTGCGGGTGGTAGCGG | 282 | 0.25 | 60 | 60 | (Rayamajhi et al. 2011) |
| | *aspU*-R | AACCCATTCGGTTAGAGCAC | | | | | |

**Table 1.** Continued

| Target's gene | Primer's name | Sequence (5′-3′) | Amplicon size (bp) | Primer concentration ($\mu$M) | $T_{anneal}$ (°C) | $t_{elongate}$ (second) | Reference |
|---|---|---|---|---|---|---|---|
| **Plasmid replicons** | | | | | | | |
| IncI1 | I1-F | CGAAAGCCGGACGGCAGAA | 139 | 0.35 | 63 | 60 | (Carattoli et al. 2005) |
| | I1-R | TCGTCGTTCCGCCAAGTTCGT | | | | | |
| IncFIC | FIC-F | GTGAACTGGCAGATGAGGAAGG | 262 | 0.25 | 63 | 60 | (Carattoli et al. 2005) |
| | FIC-R | TTCTCCTCGTCGCCAAACTAGAT | | | | | |
| IncFIA | FIA-F | CCATGCTGGTTCTAGAGAAGGTG | 462 | 0.25 | 63 | 60 | (Carattoli et al. 2005) |
| | FIA-R | GTATATCCTTACTGGCTTCCGCAG | | | | | |
| IncFIB | FIB-F | GGAGTTCTGACACACGATTTTCTG | 702 | 0.25 | 63 | 60 | (Carattoli et al. 2005) |
| | FIB-R | CTCCCGTCGCTTCAGGGCATT | | | | | |
| IncN | N-F | GTCTAACGAGCTTACCGAAG | 559 | 0.35 | 60 | 60 | (Carattoli et al. 2005) |
| | N-R | GTTTCAACTCTGCCAAGTTC | | | | | |
| IncX | X-F | AACCTTAGAGGCTATTTAAGTTGCTGAT | 376 | 0.25 | 60 | 60 | (Carattoli et al. 2005) |
| | X-R | TGAGAGTCAATTTTATCTCATGTTTTAGC | | | | | |
| IncK | K-F | GCGGTCCGGAAAGCCAGAAAAC | 160 | 0.35 | 63 | 60 | (Carattoli et al. 2005) |
| | K-R | TCTTTCACGAGCCCGCCAAA | | | | | |
| IncA/C | A/C-F | GAGAACCAAAGACAAAGACCTGGA | 465 | 0.35 | 63 | 60 | (Carattoli et al. 2005) |
| | A/C-R | ACGACAAACCTGAATTGCCTCCTT | | | | | |
| IncHI1 | HI1-F | GGAGCGATGGATTACTTCAGTAC | 471 | 0.25 | 63 | 45 | (Carattoli et al. 2005) |
| | HI1-R | TGCCGTTTCACCTCGTGAGTA | | | | | |
| IncHI2 | HI2-F | TTTCTCCTCGAGTCACCTGTTAACAC | 644 | 0.35 | 63 | 45 | (Carattoli et al. 2005) |
| | HI2-R | GGCTCACTACCGTTGTCATCCT | | | | | |
| IncY | Y-F | AATTCAAACAACACTGTGCAGCCTG | 765 | 0.15 | 63 | 45 | (Carattoli et al. 2005) |
| | Y-R | GCGAGAATGGACGATTACAAAACTTT | | | | | |
| IncFrepB | $F_{repB}$-F | TGATCGTTTAAGGAATTTTG | 270 | 0.25 | 52 | 60 | (Carattoli et al. 2005) |
| | $F_{repB}$-R | GAAGATCAGTCACACCATCC | | | | | |
| IncB/O | B/O-F | GCGGTCCGGAAAGCCAGAAAAC | 159 | 0.45 | 63 | 45 | (Carattoli et al. 2005) |
| | B/O-R | TCTGCGTTCCGCCAAGTTCGA | | | | | |

*$T_{anneal}$: annealing temperature; $t_{elongate}$: elongation duration.

*Enterobacteriaceae*. The primers and PCR conditions were illustrated in Table 1.

## Results and discussion

### Bacterial identification

A total of 59 *Enterobacteriaceae* isolates from poultry origin were examined in this study. *E. coli* ($n = 31$) was the most common type of *Enterobacteriaceae* isolated from the poultry faecal samples, followed by *Klebsiella* spp. ($n = 19$), *Proteus* spp. ($n = 4$), *Salmonella* spp. ($n = 3$), *Enterobacter* spp. ($n = 1$), and *Shigella* spp. ($n = 1$) (Fig. 1a). Generally, *E. coli* (87.5%) was dominant species identified in poultry faeces while *Klebsiella pneumoniae* (2.8%) and *Enterobacter cloacae* (6.9%) were found at less frequency (Moawad et al. 2018). Due to relatively smaller sample sizes in *Proteus* spp., *Salmonella* spp., *Enterobacter* spp., and *Shigella* spp., their results should not be considered as representative for population in term of prevalence of ESBL producers, beta-lactamase gene, virulence gene, and plasmid replicon. Further study with increased sample size and collection locations are required to provide representative result.

### Susceptibility to $\beta$-lactams and ESBL production

As shown in Fig. 1a, *Klebsiella* spp. isolates demonstrated higher resistance rate to cefotaxime, ceftazidime, and cefoxitin than *E. coli* isolates. Two *E. coli* isolates (6.5%, 2/31) has been interpreted as ESBL producers by double disk synergy test (DDST). The prevalence rate is lower as compared to similar studies conducted in Thailand (Boonyasiri et al. 2014) where the ESBL prevalence among *E. coli* was reported to be 24.2% (32/132). The selective method (1 $\mu$g/ml ceftriaxone in Thailand) had been applied to determine the ESBL prevalence by using antibiotic to select resistant isolates. The pre-enrichment with cephalosporin can increase the detection efficiency of ESBL-producers (Schauss et al. 2015). Direct comparison of our ESBL prevalence rate might not be appropriate as all isolates in this study were selected randomly without using the antimicrobial enrichment. This may underestimate the prevalence of ESBL/AmpC producers in poultry samples. The representative results were demonstrated in Fig. 1b–e.

### Detection of beta-lactamase genes

In Fig. 2a, $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ were the most prevalent in *E. coli* and *Klebsiella* spp., respectively. TEM- and SHV-type ESBLs are mutant enzymes derived from TEM-1, TEM-2, and SHV-1 enzymes with few or one amino acid substitutions. However, TEM-1, TEM-2, and SHV-1 are not considered as ESBLs (Smet et al. 2010). TEM and SHV families detected in isolates without carrying AmpC genes in this study are more likely to be broad-spectrum TEM/SHV (TEM-1 and SHV-1) or inhibitor-resistant type instead of ESBL-type of TEM/SHV as they did not confer resistance to third/fourth generation cephalosporin (Table 2). The synergy effect of DDST is hidden when there is co-existence of AmpC $\beta$-lactamase and ESBL (Kaur et al. 2013). Thus, sequencing is needed to determine the type of $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ in the AmpC-carrying isolates. *K. pneumoniae* producing $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$, derived from chicken, exhibited multidrug resistance (Elmonir et al. 2021). $bla_{\text{CTX-M}}$ was recovered from two ESBL producers as determined by DDST previously (Table 2). CTX-M-15 and CTX-M-14 are dominant CTX-M ESBLs present in poultry in Asia (Ewers et al. 2012). Further investigation such as sequencing is required to identify the variant of CTX-M enzyme in this study.

Our isolates, which were resistant to cefoxitin were all positive for gene encoding plasmid-mediated AmpC beta-lactamases (pAmpC). In our study, CMY-2 group was main mediator for resistance to extended spectrum cephalosporin in *E. coli*. *E. coli* carrying CMY-2 gene was found at every level of the broiler production pyramid, including grandparent flock, parent flock, and broiler, suggesting vertical transmission of resistance (Dierikx et al. 2013). This rises a concern as it may enter food chain during slaughter. DHA was dominant pAmpC in *Klebsiella* spp. (Fig. 2a) and $bla_{\text{MIR/ACT}}$ was detected in the *Enterobacter* spp. (Table 2). $bla_{\text{DHA}}$-producing *E. coli* isolated from chicken origin was reported as well as coexistence of AmpC gene and quinolone resistance gene on the same plasmids (Abd El-Aziz and Gharib 2015). The representative results were displayed in Fig. 2b.

### Detection of virulence genes

Resistance and virulence are important mechanisms for bacterial survival under adverse conditions. They share common features that both are associated with infections and their dissemination, and co-selection is mediated by horizontal gene transfer (Beceiro et al. 2013). Our initial aim was to screen more virulence genes representative of all the bacterial isolates; however, due to small sample sizes of *Proteus* spp., *Salmonella* spp., *Enterobacter* spp., and *Shigella* spp., only nine virulence genes associated with different *E. coli* pathotypes, were investigated. *F18* (fimbriae), *Sta* (Heat stable enterotoxin a), *LT* (Heat labile enterotoxin), *eae* (intimin), *est* (Heat stable enterotoxin-ST I), *AIDA-I* (adhesin involved in diffuse adherence), and *aspU* (Cryptic secreted protein/EAEC-secreted protein U) were not identified in any of isolates in this study. The gene encoding *EAST1* (Heat stable enterotoxin) was found in 16.1% (5/31) of *E. coli* and 5.3% (1/19) of *Klebsiella* spp. *EAST1* and *pAA* were not detected in *Proteus* spp., *Salmonella* spp., *Enterobacter* spp., and *Shigella* spp. (Table 2). *EAST1* is heat-stable toxin encoded by *astA* and associated with diarrhoeal disease in human, cattle and swine (Dubreuil 2019). *astA* has been detected in Avian Faecal *E. coli* (isolates collected from faeces of healthy broilers) and Avian Pathogenic *E. coli* (isolates collected from organs of broiler with colibacillosis) (Paixão et al. 2016). IS1414, which is insertion sequence identified in pathogenic *E. coli* strains, was reported to carry *EAST1* gene in *Salmonella*, suggesting intergeneric transfer of virulence genes via insertion sequence (Bacciu et al. 2004). Mobilization of *EAST1* between different bacterial population via MGEs might explain the detection of this gene in *Klebsiella* spp. (Table 2). On the other hand, two (6.5%, 2/31) *E. coli* isolates were found to carry *pAA* (porcine attaching and effacing-associated factor). *pAA* is a type of adhesins that may be a virulence factor in *E. coli* from piglets with diarrhoea (Vidotto et al. 2013).

### Plasmid replicon typing and its association with resistance gene

In Table 3, IncFIB and IncFrepB were the most frequent replicon types in *E. coli*, followed by IncI1 and IncN. This finding is consistent with the study in United States that investigated distribution of plasmid replicon in *E. coli* originated from faecal swabs of apparently healthy poultry (Johnson et al. 2007). IncF plasmid contains addiction systems, which allows stable

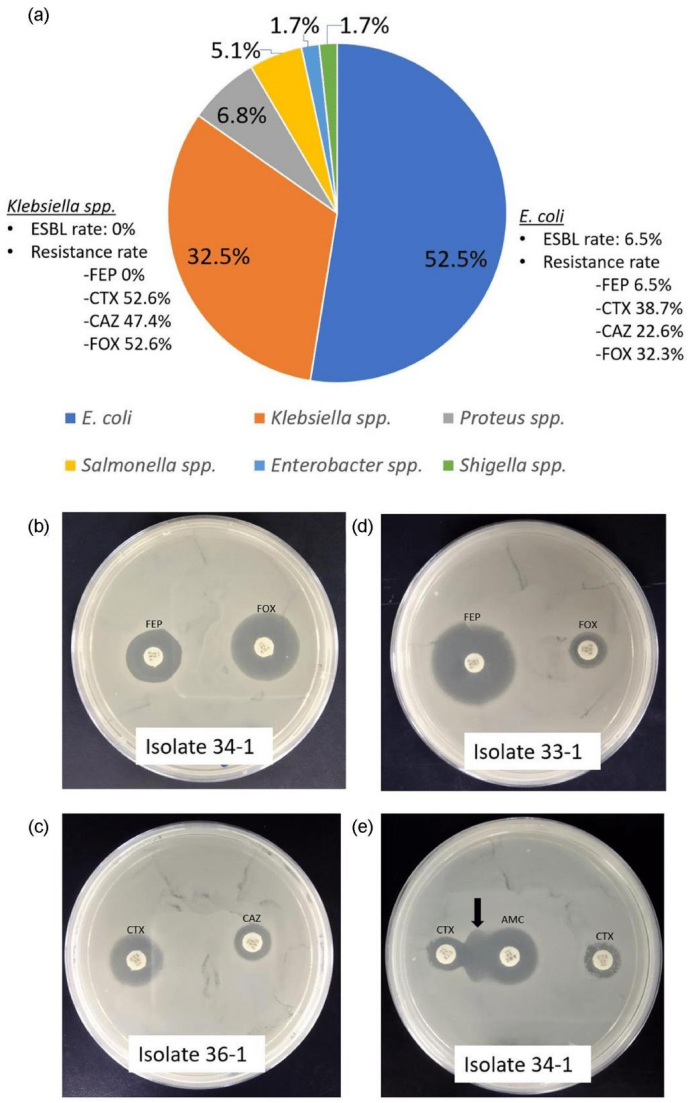


**Figure 1.** (a) Percentage of various *Enterobacteriaceae* isolates obtained from poultry faecal samples and ESBL and resistance rates of *E. coli* and *Klebsiella* spp.; (b–d) Representative results from the antimicrobial susceptibility; and (e) double disc synergy tests. (b) *E. coli* (Isolate 34–1) resistant to cefepime (ZOI = 17 mm). (c) *Klebsiella* spp. (Isolate 36–1) resistant to cefotaxime (ZOI = 16 mm) and ceftazidime (ZOI = 11 mm). (d) *E. coli* (Isolate 33–1) resistant to cefoxitin (ZOI = 11 mm). Zone Diameter Breakpoints for *Enterobacterales* to interpret as resistant phenotype in cefepime (ZOI ≤ 18 mm), cefotaxime (ZOI ≤ 22 mm), ceftazidime (ZOI ≤ 17 mm), and cefoxitin (ZOI ≤ 14 mm) (CLSI 2021). (e) *E. coli* (Isolate 34–1) interpreted as ESBL producer because the inhibition zone was enhanced between discs of cephalosporin and amoxicillin/clavulanic acid (synergy is indicated by arrow) when these two discs were positioned at distance of 20 mm (centre to centre). *ZOI: Zone of inhibition. AMC: Amoxicillin/clavulanic acid; FEP: Cefepime; CTX: Cefotaxime; CAZ: Ceftazidime; and FOX: Cefoxitin.

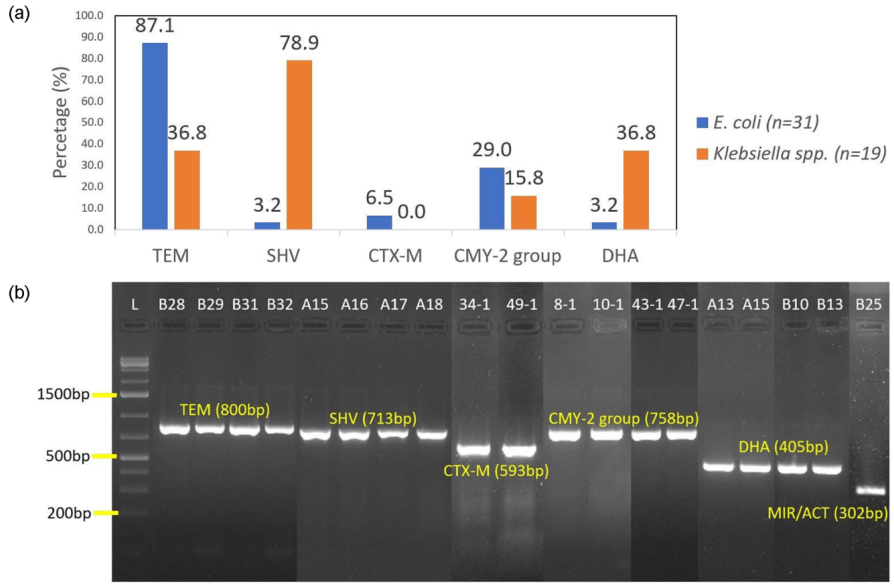


**Figure 2.** (a) Prevalence of beta-lactamase genes among the isolated *E. coli* and *Klebsiella* spp. ∗Blue bar indicates *E. coli* and orange bar indicates *Klebsiella* spp. (b) PCR profile of the respective detected beta-lactamase genes. All PCR products were resolved on 2% TBE agarose gels. The top labels indicate the isolate reference. L: GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific, US).

maintenance of IncF plasmids in *E. coli* within the gastrointestinal tract of humans and animals under condition without antimicrobial pressure (Bevan et al. 2017). This might explain the high frequency of IncF plasmid in our study. IncF plasmids can carry more than one replicon for initiation of replication. The typical multi-replicon IncF plasmids can harbour the FII replicon together with FIA and FIB replicons (Rozwandowicz et al. 2018). Our study showed that FIB-FrepB (same to FIB-FII) combination was detected in 54.8% (17/31) of *E. coli* (Table 2).

Among our poultry samples, IncF, IncN, and IncI1 plasmids was commonly recovered from *Klebsiella* spp. and *E. coli* while some replicons, including IncK, IncB/O, IncHI2, IncHI1, IncX, IncA/C, and IncY were found at low frequency (Table 3). IncF and IncN plasmids have been frequently reported to carry genes conferring resistance to extended-spectrum $\beta$-lactams, quinolones, and aminoglycosides (Rozwandowicz et al. 2018). Plasmids detected in *Enterobacteriaceae* might involve in dissemination of resistance and virulence genes, which were not examined in this study among different bacterial population. Whole genome sequencing should be performed to further elucidate various resistance and virulence genes, location of these genes (either chromosome or plasmids), and genetic element of mobilization.

The global dissemination of CTX-M genes mainly depends on horizontal gene transfer via conjugative plasmid (Bevan et al. 2017). IncF plasmids carrying CTX-M-55, CTX-M-27, and CTX-M-14, and IncI1 plasmid harbouring CTX-M-1 have been described in *E. coli* from poultry (Liu et al. 2013, Rozwandowicz et al. 2018). Plasmid IncI1, IncFrepB and IncFIB, which were detected in the *E. coli* isolates carrying CTX-M gene (Table 2) might play a role in dissemination of CTX-M ESBLs among poultry via horizontal gene transfer. On the other hands, other study has found CMY-2 genes located on IncI1 and IncK plasmids among *E. coli* from poultry origin (Huijbers et al. 2014). Our study has consistent finding that IncI1, IncK, and IncB/O were commonly detected in 55.6% (5/9) of *E. coli* carrying CMY-2 group (Table 2), suggesting the role of these plasmids in spreading resistance gene.

## Conclusion

The wide distribution of ESBL/AmpC-producing *Enterobacteriaceae* in faecal isolates from poultry is a worrisome trend. Prevalent distribution of plasmid replicons among poultry-derived isolates could drive the dissemination of resistance among *Enterobacteriaceae* in human, animals and the environment. This study implicates poultry as a reservoir of antibiotic-resistant bacteria and virulence determinants. Strengthening surveillance of antibiotic use and antibiotic resistance is essential to reduce the emergence of antibiotic resistance and decrease the spread of infections caused by multidrug resistant organisms.

**Table 2.** Characteristics profile of 59 *Enterobacteriaceae* isolates derived from poultry in Malaysia.

| Isolate | Species | Resistance phenotype to beta-lactam* | Phenotypic ESBL production† | Beta-lactamase genes‡ | Virulence genes§ | Plasmid replicons (Inc)¶ |
|---|---|---|---|---|---|---|
| A6 | *E. coli* | FOX, CTX, and CAZ | – | TEM and CMY-2 group | EAST1 | FIB, K, and B/O |
| A7 | *E. coli* | – | ND | – | – | FIB, HI1, and FrepB |
| A17 | *E. coli* | FOX and CTX | – | SHV and DHA | – | – |
| B3 | *E. coli* | – | ND | TEM | – | FIA, FIB, and FrepB |
| B5 | *E. coli* | – | ND | TEM | – | FIA, FIB, and FrepB |
| B6 | *E. coli* | – | ND | TEM | – | FIA, FIB, and FrepB |
| B15 | *E. coli* | – | ND | TEM | – | FrepB |
| B17 | *E. coli* | – | ND | TEM | – | Y, FrepB |
| B18 | *E. coli* | – | ND | TEM | – | FIB, N, and FrepB |
| B20 | *E. coli* | – | ND | TEM | – | FIB, N, and FrepB |
| B21 | *E. coli* | – | ND | TEM | – | N |
| B24 | *E. coli* | FOX, CTX, and CAZ | – | TEM and CMY-2 group | – | I1 |
| B26 | *E. coli* | – | ND | TEM | – | FIB, N, and FrepB |
| B28 | *E. coli* | FOX, CTX, and CAZ | – | TEM and CMY-2 group | EAST1 | FIB, K, HI1, and B/O |
| B29 | *E. coli* | – | ND | TEM | – | FIB and FrepB |
| B31 | *E. coli* | – | ND | TEM | – | FIB, N, and FrepB |
| B32 | *E. coli* | – | ND | TEM | EAST1 | X |
| B33 | *E. coli* | – | ND | TEM | – | I1, FIB, N, and FrepB |
| B34 | *E. coli* | – | ND | TEM | – | N |
| 16–1 | *E. coli* | FOX, CTX, and CAZ | – | TEM and CMY-2 group | – | I1, FIB, and FrepB |
| 18–1 | *E. coli* | – | ND | TEM | pAA | HI2, and N |
| 19–1 | *E. coli* | – | ND | – | pAA | FIC, FIB, and FrepB |
| 33–1 | *E. coli* | FOX and CTX | – | TEM and CMY-2 group | EAST1 | FIA, FIB, K, B/O, N, and FrepB |
| 34–1 | *E. coli* | FEP and CTX | + | TEM and CTX-M | – | I1, FIB, and FrepB |
| 47–1 | *E. coli* | FOX, CTX, and CAZ | – | TEM and CMY-2 group | – | I1, and FIB |
| 48–1 | *E. coli* | FOX and CTX | – | TEM and CMY-2 group | EAST1 | I1, FIB, and FrepB |
| 49–1 | *E. coli* | FEP and CTX | + | TEM and CTX-M | – | I1, and FrepB |
| 50–1 | *E. coli* | – | ND | TEM | – | FIB and HI2 |
| 53–1 | *E. coli* | FOX, CTX, and CAZ | – | CMY-2 group | – | FIB, K, A/C, B/O, and FrepB |
| 55–1 | *E. coli* | – | ND | TEM | – | I1, FIB, and FrepB |
| 59–1 | *E. coli* | FOX, CTX, and CAZ | – | TEM and CMY-2 group | – | I1, FIB, K, HI2, and B/O |
| A5 | *Klebsiella* spp. | – | ND | TEM and SHV | – | – |
| A13 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | SHV and DHA | – | – |
| A15 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | SHV and DHA | EAST1 | – |
| A16 | *Klebsiella* spp. | – | ND | SHV | – | I1, N |
| A18 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | SHV and DHA | – | K, N |
| B4 | *Klebsiella* spp. | – | ND | TEM | – | HI1, N |
| B7 | *Klebsiella* spp. | – | ND | – | – | FIB, N |
| B10 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | SHV and DHA | – | – |
| B13 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | SHV and DHA | – | I1 |
| B14 | *Klebsiella* spp. | – | ND | SHV | – | FIB, N |
| B19 | *Klebsiella* spp. | – | ND | TEM and SHV | – | N |
| B22 | *Klebsiella* spp. | – | ND | TEM | – | – |
| B27 | *Klebsiella* spp. | – | ND | SHV | – | FIB, N |
| 4–1 | *Klebsiella* spp. | – | ND | SHV | – | HI1 |
| 8–1 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | SHV and CMY-2 group | – | I1 |
| 10–1 | *Klebsiella* spp. | FOX and CTX | – | SHV and CMY-2 group | – | I1 |
| 36–1 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | TEM, SHV, and DHA | – | N |
| 43–1 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | TEM, CMY-2 group | – | I1, FIB |
| 62–1 | *Klebsiella* spp. | FOX, CTX, and CAZ | – | TEM, SHV, and DHA | – | N |
| A12 | *Proteus* spp. | FOX and CTX | – | CMY-2 group | – | – |
| 7–2 | *Proteus* spp. | – | ND | TEM | – | – |
| 9–1 | *Proteus* spp. | – | ND | TEM | – | – |
| 14–1 | *Proteus* spp. | – | ND | TEM | – | – |
| B8 | *Salmonella* spp. | – | ND | TEM | – | HI1, N |
| 2–2B | *Salmonella* spp. | – | ND | – | – | – |
| 15–1B | *Salmonella* spp. | – | ND | – | – | – |
| B25 | *Enterobacter* spp. | FOX | ND | MIR/ACT | – | – |
| 32–2B | *Shigella* spp. | – | ND | TEM | – | FIB and HI1 |

*Antimicrobial susceptibility test. FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; and FOX, cefoxitin; "-" means not resistant to all four beta-lactams.
†ESBL screening by double disc synergy test. "-" means negative result (no synergy observed); "+" means positive result (synergy between cephalosporin and amoxicillin/clavulanic acid observed); and ND: Not determined (Only the isolates resistant to cefotaxime subjected for screening of ESBL production by double disc synergy test.)
‡Molecular detection of beta-lactamase genes. "-" means none of six genes (TEM, SHV, CTX-M, and CMY-2 group, DHA, MIR/ACT) detected.
§Molecular detection of virulence genes. "-" means none of nine virulence factors identified.
¶Plasmid replicon typing. Incompatibility group (Inc); "-" means none of thirteen replicons detected.

**Table 3.** Distribution of plasmid replicon among 59 *Enterobacteriaceae* of poultry origin.

| Bacterial types | Total No. | No. of Positive isolates (%) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I1 | FIC | FIA | FIB | N | X | K | A/C | HI1 | HI2 | Y | FrepB | B/O |
| *Escherichia coli* | 31 | 9 (29.0) | 1 (3.2) | 4 (12.9) | 22 (71.0) | 9 (29.0) | 1 (3.2) | 5 (16.1) | 1 (3.2) | 2 (6.5) | 3 (9.7) | 1 (3.2) | 20 (64.5) | 5 (16.1) |
| *Klebsiella* spp. | 19 | 5 (26.3) | 0 (0) | 0 (0) | 4 (21.1) | 9 (47.4) | 0 (0) | 1 (5.3) | 0 (0) | 2 (10.5) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Proteus* spp. | 4 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Salmonella* spp. | 3 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (33.3) | 0 (0) | 0 (0) | 0 (0) | 1 (33.3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Enterobacter* spp. | 1 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| *Shigella* spp. | 1 | 0 (0) | 0 (0) | 0 (0) | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Grand Total | 59 | 14 (23.7) | 1 (1.7) | 4 (6.8) | 27 (45.8) | 19 (32.2) | 1 (1.7) | 6 (10.2) | 1 (1.7) | 6 (10.2) | 3 (5.1) | 1 (1.7) | 20 (33.9) | 5 (8.5) |

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## Conflict of interest

The authors declare no conflict of interest.

## Authors contribution

Hui-Shee Tan (Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing), Pan Yan (Supervision, Writing – review & editing), Hollysia Alda Agustie (Data curation, Formal analysis, Investigation, Methodology, Project administration), Hwei-San Loh (Conceptualization, Data curation, Project administration, Validation, Writing – original draft, Writing – review & editing), Nabin Rayamajhi (Conceptualization, Data curation, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing) and Chee-Mun Fang (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing). C.M.F, N. R., and H.S.T conceived the study. H.S.T carried out the study and wrote the first draft. All authors reviewed and revised the draft.

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