MOLECULAR DIVERSITY OF *bla*-TEM HOMOLOGOUS GENES OF COW AND CHICKEN FARM SOILS IN PERAK

By

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A dissertation submitted to Department of Biological Science,

Faculty of Science

Universiti Tunku Abdul Rahman,

in partial fulfillment of the requirements for the degree of Master of Science in

May 2019

ABSTRACT

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The prevalence of antibiotic-resistant microorganisms has become a global concern both in health care and agribusiness settings. Regardless of the risk revealed by antibiotic resistance, limited information is available regarding the diversity, distribution and origins of resistance genes, especially among environmental bacteria in their natural settings. The aim of this research was to investigate the prevalence and diversity of β-lactamase genes in two different soil ecotypes from animal farm setting via culture-dependent and independent approaches. In this study, soil samples representing ecotypes of cow and chicken farms were collected. A total of fifteen morphologically different bacterial isolates were obtained and characterised. The isolates identified using the API 20 E test and 16S rDNA sequencing in this study belonged to the families *Enterobacteriaceae* and *Pseudomonadaceae*. The resistance patterns amongst the isolates varied significantly and phenotypic characteristics of NSBL, ESBL, CMT, pAmpC, and IRT phenotypes were observed. Among the nine β-lactamase genes characterised using PCR, the resistant genes were

confined to the *bla*-TEM and *bla*-SHV families, with high similarities towards TEM-1 and TEM-116. To further investigate the diversity of the *bla*-TEM genes, culture-independent approach was performed. High percentage of variants was observed in both animal farm soil samples in which a total of 122 recombinant *bla*-TEM homologous genes were obtained for cow and chicken farm soils, whereby 32 % were identical to TEM-1 and 4 % to TEM-116. The remaining recombinants (64 %) were demonstrated to be different variants of the known *bla*-TEM family and were further characterised. Further analysis showed that these variants involved 64 different amino acid substitutions, with up to three amino acid residue modifications. Seven *bla*-TEM protein sequences resembled TEM-176, whereby a change of amino acid from alanine (A) to valine (V) at position 224 was observed, and five resembled TEM-215, whereby a change from histidine (H) to arginine (R) at position 153 was observed. The remaining 66 *bla*-TEM protein sequences have at least one amino acid substitution at different positions, however, these substitutions at their respective positions do not correlate with the sequences from the existing database. These observations seemed to suggest that the substitutions may represent novel *bla*-TEM variants that have arisen through several mutational events. Phylogenetic analysis was carried out to investigate the inferred evolutionary relationships among the gene sequences. Ladder-type phylogenetic topologies were observed in both soil ecotypes, thus suggesting observations that are consistent with the presence of *bla*-TEM protein sequences with divergent mutations. The results obtained in this study seemed to suggest the prevalence of novel *bla*-TEM variants in enhancing or maintaining the enzymatic activity in the farm soil ecotypes studied. Resistance genes residing in environmental reservoirs pose serious

threat to human health and these results can be used to enhance the understanding of the emergence and dissemination of novel antibiotic resistance from the natural reservoir to the clinical setting, which may aid the development of inhibitors of resistance mechanisms.

ACKNOWLEDGEMENTS

The journey of taking Master of Science is not an easy path to me. I would like to take this opportunity to thank everyone who has been helping me in this journey.

First of all, I would like to express my gratitude towards my supervisor, Dr. Choo Quok Cheong for his patience, guidance and advice throughout this project. It is my honour with him as my supervisor. Not to be forgotten, thanks to my co-supervisor, Dr. Chew Choy Hoong and her guidance in my dissertation writing and as my co-supervisor. I would also like to thank UTAR Research Scholarship Scheme (RSS) for their financial support in this research.

Most importantly, I would like to thank my family for their unconditional support throughout my study. I want to say many thanks to my lovely bench-mates (Wong Hong Kin, Melissa Ong Hui Ling, Tham Yong Yao and Esther Thou), seniors and D213 lab mates for their encouragement and friendships. It is my pleasure and blessing to get to know all of you. Last but not least, I would like to thank lab officers from Department of Biological Science, Department of Biomedical Science and Department of Chemical Science for their assistance.

FACULTY OF SCIENCE

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SUMMISION OF DISSERTATION

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I understand that the University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may to be made accessible to UTAR community and public.

Yours truly,

_____

Joseph Yong

APPROVAL SHEET

This dissertation entitled "MOLECULAR DIVERSITY OF *bla*-TEM HOMOLOGOUS GENES OF COW AND CHICKEN FARM SOILS IN PERAK" was prepared by JOSEPH YONG MING WEI and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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DECLARATION

I, Joseph Yong Ming Wei hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any or other institutions.

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ABBREVIATIONS

ESBL	Extended Spectrum β-Lactamase
CLSI	Clinical and Laboratory Standards Institute
g	Gravity force
mm	Millimetre
µl	Microlitre
ml	Millilitre
ng	Nanogram
g	Gram
MIC	Minimal Inhibitory Concentration
FRIM	Forest Research Institute Malaysia
LB	Luria Bertani
EMB	Eosin Methylene Blue
MAC	MacConkey
MH	Mueller-Hinton
NSBL	Narrow Spectrum β-Lactamase
IRT	Inhibitor Resistant TEMs
CMT	Complex Mutant TEM
pAmpC	Plasmid-mediated AmpC
MDR	Multidrug-Resistant
HGT	Horizontal Gene Transfer
FQ	Fluoroquinolone
qRT PCR	Quantitative Real-Time PCR
MLST	Multilocus Sequence Typing
PFGE	Pulsed-Field Gel Electrophoresis
MBLs	Metallo-β-lactamases

CHAPTER 1

INTRODUCTION

Resistance of infectious microorganisms to antibiotics has become a global concern both in health care and agribusiness settings. Intensive applications and exploitation of antibiotics in the human health sector, veterinary, and agriculture are predominantly the contributing factor to this phenomenon (Alekshun and Levy, 2007). The use of antibiotics in human and animal health care has resulted in the widespread prevalence of antibiotic resistant bacteria not only in humans and animals, but also in the environment (Kümmerer, 2004; Baquero et al., 2008; Zhang et al., 2009; Wright, 2010). A particular type of antibiotic resistance that represents a major public health concern is the third generation cephalosporin resistance induced by extended spectrum β-lactamase (ESBL) production (Cantón et al., 2008).

Despite the effectiveness of β-lactam antibiotics in treating bacterial infections, they also represent the major source of antibiotic resistance amongst Gram-negative bacteria. Continuous exposure of some bacterial strains to β-lactams has induced the production of β-lactamases, which will eventually lead to the mutations of β-lactamase genes and extend their activity even against the newly developed β-lactam antibiotics (Paterson and Bonomo, 2005; Pitout and Laupland, 2008). ESBLs confer resistance to most of the β-lactam antibiotics,

including the third and fourth generation of cephalosporins, which poses extreme difficulties in treating infections caused by these bacteria. These strains include the multidrug ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (Alekshun and Levy, 2007). These pathogens are the leading cause of nosocomial infections throughout the world and most of them are multidrug-resistant isolates, which is one of the greatest challenges in clinical practice.

Although earlier prevalence of ESBL was primarily reported in hospital infections caused by *K. pneumoniae*, it is also often associated with community-acquired infections (Paterson and Bonomo, 2005; Livermore et al., 2007), as well as the commensal *Escherichia coli* strains isolated from humans and livestock (Mevius et al., 2012; Huijbers et al., 2013; Trott, 2013). The ensuing selective pressure leads to the emergence of ESBL genes, which could be spread into the environment through the food chain or contamination of water and animal waste (Pappas, 2011).

Use of cephalosporins for the treatment of mastitis is common, especially in dairy cows. ESBL-producing *E. coli* has been frequently reported in farm animals and many descriptions of faecal carriage of such organisms have been reported such as the food-producing animals of broiler poultry and pig farms (Costa et al., 2009). Interaction between animals and the food chain has resulted in zoonotic spread of antimicrobial resistance. However, the correlation

between the frequency of transmission of antibiotic-resistant bacteria in farm animals and the threat to public health are not known. Nevertheless, cultivated soils are frequently fertilised with agricultural or urban organic residues that may contain antibiotic-resistant microorganisms, which may act as environmental reservoirs of resistance genes (Moodley and Guardabassi, 2009).

Various questions remain regarding the role of the natural environment as a resistant gene bank and the degree of exchange of these resistant genes between indigenous bacteria and clinical isolates. Many contradicting reports of inconsistent statistics concerning the exact extent of antibiotic production by indigenous soil microorganisms have been reported (Gottlieb, 1976). Some reports indicated that the concentration of antibiotics produced in the soil is high, which inhibits the growth of the bacterial community within the same vicinity (Li and Alexander, 1990; Thomashow et al., 1990; Hansen et al., 2001; Anukool et al., 2004). These findings demonstrated that the development of antibiotic resistance mechanisms is an essential survival strategy that is selective enough via *in situ* conditions in order to counter antibiotic-producing bacteria.

In this study, one soil sample representing each of the ecology of cow and chicken farms was collected. The ESBL and multiple antibiotic-resistant microorganisms were identified and characterised using DNA sequencing methodology. Phylogeny of *bla*-TEM antibiotic resistance profiles was compared to the intrinsic resistance profiles of uncultured soil bacteria. This investigation may provide insights into the biodiversity, evolution and probable

mechanistic process of the *bla*-TEM genes within the selected cow and chicken soil samples from Tanjung Tualang and Sitiawan, respectively.

The general objective of this study was to use culture-dependent and culture-independent approaches to investigate the prevalence and diversity of β-lactamase genes from cow and chicken farm soil samples.

The specific objectives were:

a) To determine the probable presence of multiple antibiotic resistance among ESBL producers;
b) To determine the sequences of novel *bla*-TEM homologous genes;
c) To study the diversity of β-lactamase producing bacteria from cow and chicken farm soils;
d) To investigate the inferred evolutionary relationships among the *bla*-TEM translated gene sequences using phylogenetic trees.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotic Resistance in Soil Environment

Antibiotic resistance is considered as one of the most important threats to the global health, food security, and development of new medicine today. Emergence of antibiotic resistance was mainly due to the extensive use of antibiotics in human and animals. Selection for antibiotic resistance was common within an infected person treated with antibiotics. Nevertheless, different environments such as agricultural environments, waste water treatment systems, or within the natural environment may also encourage antibiotic selection by bacterial antibiotic producers (William et al., 2008; Thenmozhi et al., 2014).

Bacteria that were previously subjected to antibiotic treatment in a nosocomial environment, or the naturally occurring bacteria in the environment may have been one of the major contributing factors towards the origins of mobile antibiotic resistance genes. In many situations, resistance is commonly conferred via selection through the application of antibiotics and mutation. Nonetheless, antibiotic resistance can also occur by the acquisition of a novel gene through horizontal gene transfer (HGT) via conjugation, transformation,

or transduction. Soil is one of the common examples of environmental reservoirs for HGT. The interactions between the microorganisms that contain the drug resistance genes, clinical and veterinary antibiotics, and the chemical compounds with indigenous soil bacteria may have arisen through practices such as sewage sludge and animal slurry practice as shown in Figure 2.1 (William et al., 2008). A potential increase in antibiotic resistance selection in the soil and introduction of pathogens may have resulted from the antibiotics or the active intermediates from human and veterinary medicines in human and animal wastes, which ultimately retains their selective capabilities in the soil (Boxall et al., 2002).

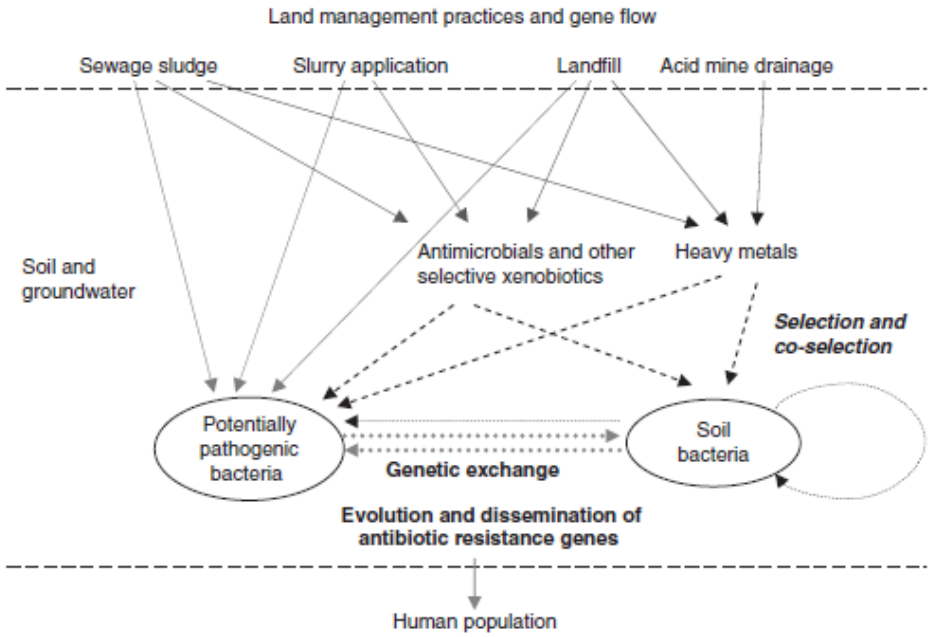

Figure 2.1 Acquisition and selective pressure of indigenous soil bacteria for antibiotic resistance via anthropogenic sources (William et al., 2008).

2.2 Antibiotics and Resistance Genes in Livestock Animals

When soils are treated with manure, the bacteria carrying resistance genes and the residues of antibiotics used in veterinary medicines are then introduced into the soil, subsequently reaching the food chain (Witte et al., 2000). According to the National Office of Animal Health, hundreds tonnes of antibiotics such as the tetracyclines, macrolides, beta-lactams, trimethoprim/sulphonamides, and fluoroquinolones are sold for use in food animals in the UK. Many synthetic veterinary antibiotics used cannot be broken down through normal processes and they could possibly remain for an extended period in the natural environment, adsorbed to soil particles, resulted in stockpiling of high concentration of antibiotic residues (Kummerer, 2004).

Faeces and urine containing mixture of the parent products and metabolites by grazing animals enter the farm environment directly. Interactions between these excreted products and reared animals could possibly took place through the application of slurry and manure (Hutchison et al., 2004). Several studies have reported the isolation of *E. coli* strains conferring CTXM-2 from cattle faeces in Japan (Shiraki et al., 2004) and ESBLs from healthy and sick food animals in Spain (Brinas et al., 2002). Various reports have demonstrated the correlation between the emergence of antibiotic resistance genes and the extended use of antibiotics in animals. Modern farming practice attempts to reduce the dependency on antibiotics, however, antibiotic resistant genes may be carried by a vast majority of unculturable bacteria, which makes the study on ecology of resistance genes extremely difficult. The dissemination of these resistant

bacteria from animal farms through various routes exerts pressure on the surrounding environment and even influences the living environment of human beings (Smalla et al., 2000).

In the past, the correlation of resistant genes between the clinical setting and environmental bacteria was a challenge to investigate. Nevertheless, detailed comparison and analysis on the prevalence of resistant genes in the environment and the health sector were made possible through various metagenomic techniques. For instance, the epidemiological studies of key resistant determinants in total microbial DNA were investigated using quantitative real-time PCR (qRT-PCR) (William et al., 2008). Analysis of the entire resistant gene pool or metagenome could be accomplished via construction of clone libraries and functional metagenomics. Various studies have shown that antibiotic-resistant bacteria in the environment could be transmitted to the community via several routes such as direct or indirect contact with animals, ingestion of contaminated food and water, transmission of airborne bacteria, as shown in Figure 2.2. In fact, the core roots of antibiotic resistance in clinical bacteria should be fully understood between clinical and non-clinical environments (William et al., 2008).

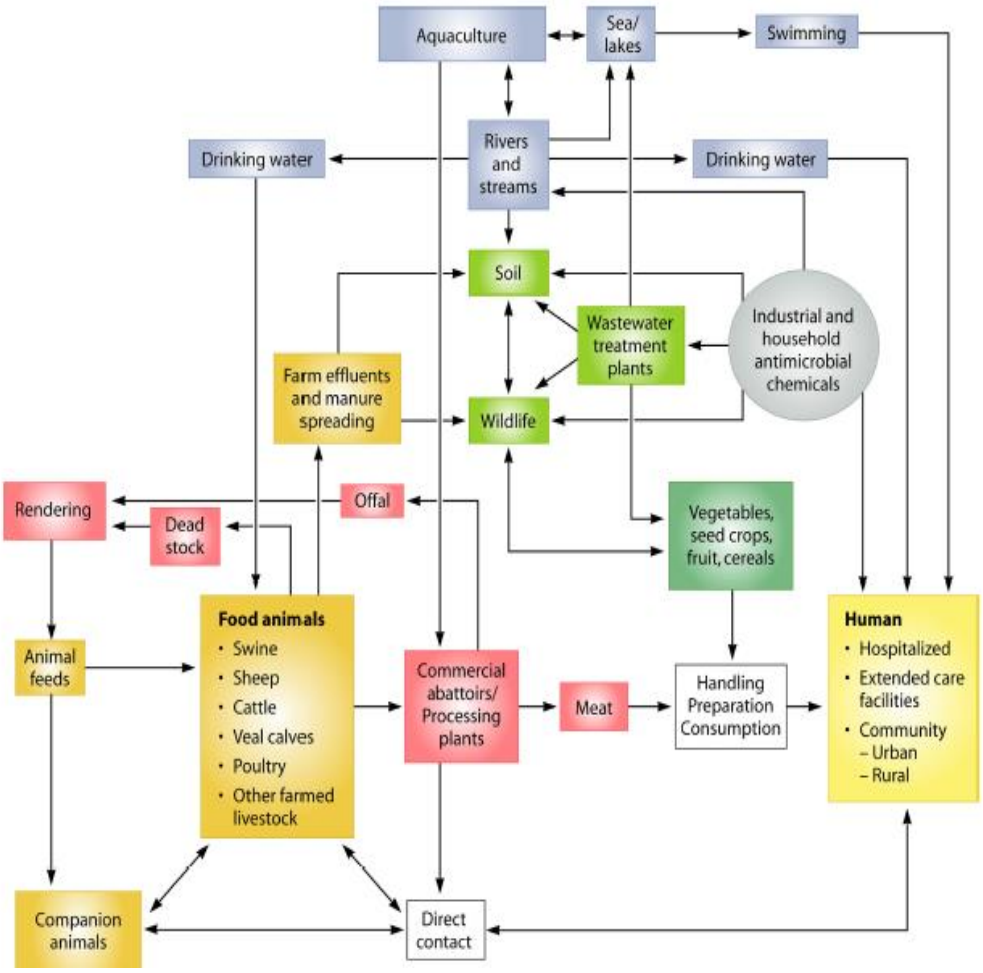

Figure 2.2 Dissemination of antibiotic resistant genes in various environments (Linton, 1977; Doyle, 2006).

2.3 Classification of Extended Spectrum β-Lactamases

ESBLs are β-lactamases conferring antibiotic resistance to penicillins, the first-, second-, and third-generation cephalosporins, and aztreonam through hydrolysis, and are inhibited by β-lactamase inhibitors (Bush et al., 1995). Beta-lactamases are often classified based on two classification schemes: the Bush-Jacoby-Medieros functional classification system and the Ambler molecular classification scheme, as shown in Table 2.1 (Ambler et al., 1991; Bush et al., 1995; Rasmussen and Bush, 1997). The Bush-Jacoby-Medeiros classification scheme classifies β-lactamases based on their functional similarities (substrate and inhibitor profile), thus making this scheme to be more applicable in the clinical settings. On the other hand, the Ambler scheme groups β-lactamases into four major classes (A, B, C, and D), as shown in Table 2.2. The roots of this classification scheme are based on protein homology (amino acid similarity), and not phenotypic characteristics.

2.3.1 Group 1 (Ambler Class C) β-Lactamases

Group 1 β-lactamases, also known as species-specific AmpC β-lactamases, are resistant to penicillins, cephamycins, the first-, second- and third- generation cephalosporins, and β-lactamase inhibitors such as clavulanic acid but are sensitive to cefepime and carbapenems (Sanders et al., 1996). The enzymes in this group are mostly found among members of the *Enterobacteriaceae* family (Jacoby, 2009). Even though the enzymes under this class are inducible, their expression of the enzymes in *Enterobacteriaceae* species remains low. Thus any exposure of bacteria to β-lactam antibiotics leads to an upsurge of different

levels of β-lactamase enzyme production. Numerous studies have also shown the shift of genes from chromosome to plasmid in some bacteria such as *Klebsiella* spp. and *E. coli* (Sanders and Sanders, 1992). These plasmid-mediated enzymes include those of the FOX, ACC, CMY, LAT, MIR, ACT, MOX and DHA families.

2.3.2 Group 2 (Ambler Class A) β-Lactamases

Beta-lactamases classified under Group 2 are harboured within plasmids. Thus, they could easily be transferred to other bacteria, causing rapid resistance to various antibiotics. Representative enzymes of this class are the plasmid-mediated broad-spectrum TEM and SHV (De Champs et al., 1991). TEM-1 was first discovered in 1965 in *E. coli* and *K. pneumoniae* which were subsequently disseminated to other bacteria which include *Vibrio, Haemophilus,* and *Neisseria* spp. SHV-1 was first identified in 1979 and is typically associated with *E. coli* and *Klebsiella* spp. (De Champs et al., 1991). Point mutations of amino acid sequence in the parental SHV-1, TEM-1, and TEM-2 enzymes have contributed to the ESBL, facilitating the hydrolysis of many oxyimino-cephalosporins. Group 2 enzymes hydrolyse ampicillin, the first-, second- and third- generation cephalosporins, and monobactams (Livermore, 1995). Actions of the ESBLs are mainly constrained by cephamycins, carbapenems and β-lactamase inhibitors.

2.3.3 Group 3 (Ambler Class B) β-Lactamases

Group 3 enzymes are metallo-β-lactamases (MBLs) that are structurally different from the other β-lactamases, with a zinc ion embedded in their active site that is capable of hydrolysing penicillins, cephalosporins and carbapenems (Burn-Buisson et al., 1987). These enzymes are inhibited by metal ion chelators, for instance, dipicolinic acid and EDTA (Laraki et al., 1999; Marchiaro et al., 2008), and are frequently found in Gram-positive bacteria, and occasionally in Gram-negative bacilli such as *Stenotrophomonas maltophilia* and *P. aeruginosa* (Livermore and Woodford, 2000).

2.3.4 Group 4 (Ambler Class D) β-Lactamases

Group 4 β-lactamases represent those unusual penicillinases that do not fit into the first three groups. Several of these enzymes exhibit high hydrolysis rates with carbenicillin and/or cloxacillin and some exhibit unusual reaction involving metal ion (Cosgrove et al., 2002). Little is known about this class of enzymes and whether these enzymes represent another molecular class of β-lactamases is not known.

Table 2.1 Classification schemes for bacterial β-lactamases (Bush and Jacoby, 2010).

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1) B (B3)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1 L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

Table 2.2 Major families of β-lactamases (Bush and Jacoby, 2010).

Enzyme family[a]	Functional group or subgroup	No. of enzymes[b,c]	Representative enzymes
CMY	1, 1e	50	CMY-1 to CMY-50
TEM	2b, 2be, 2br, 2ber	172	
	2b	12	TEM-1, TEM-2, TEM-13
	2be	79	TEM-3, TEM-10, TEM-26
	2br	36	TEM-30 (IRT-2), TEM-31 (IRT-1), TEM-163
	2ber	9	TEM-50 (CMT-1), TEM-158 (CMT-9)
SHV	2b, 2be, 2br	127	
	2b	30	SHV-1, SHV-11, SHV-89
	2be	37	SHV-2, SHV-3, SHV-115
	2br	5	SHV-10, SHV-72
CTX-M	2be	90	CTX-M-1, CTX-M-44 (Toho-1) to CTX-M-92
PER	2be	5	PER-1 to PER-5
VEB	2be	7	VEB-1 to VEB-7
GES	2f	15[d]	GES-2 to GES-7 (IBC-1) to GES-15
KPC	2f	9	KPC-2 to KPC-10
SME	2f	3	SME-1, SME-2, SME-3
OXA	2d, 2de, 2df	158	
	2d	5	OXA-1, OXA-2, OXA-10
	2de	9	OXA-11, OXA-14, OXA-15
	2df	48[e]	OXA-23 (ARI-1), OXA-51, OXA-58
IMP	3a	26	IMP-1 to IMP-26
VIM	3a	23	VIM-1 to VIM-23
IND	3a	8	IND-1, IND-2, IND-2a, IND-3 to IND-7

[a]Enzyme families include numbers that have been assigned based on primary amino acid structures (G. Jacoby and K. Bush, http://www.lahey.org/Studies/).
[b]Compiled through December 2009.
[c]The sum of the subgroups in each family does not always equal the total number of enzymes in each family, because some enzyme numbers have been withdrawn, and some enzymes have not been assigned a functional designation by the investigators who provided the amino acid sequence.
[d]GES-1, unlike other members of the GES family, has little detectable interaction with imipenem (Poirel et al., 2001).
[e]Nine clusters of OXA carbapenemases with their individual members have been designated by Queenan and Bush (2007).

2.4 Diversity of ESBLs

The genes for β-lactamases are the most progressively disseminated globally. Random mutations of these genes have given rise to the increasing extended spectrum of resistance in most bacteria (Gniadkowski, 2008). One of the highest representative of plasmid-encoded β-lactamases, TEM, conferred tremendous impact on other interrelated enzymes, demonstrating solid evidence of such adaptability. Beta-lactamase genes are frequently discovered in remote and desolate environments (Barlow and Hall, 2002; Baquero et al., 2008; Allen et al., 2009). This finding suggested that the novel β-lactamases underwent mutations and altered substrate profile within the natural environment.

2.4.1 TEM Type

The TEM enzyme was named after the patient (Temoneira) from which it was first isolated from *E. coli* (Datta and Kontomichalou, 1965). TEM-1 is the most commonly encountered β-lactamase in Gram-negative bacteria such as *E. coli* and *K. pneumoniae*. The production of TEM-1 in *E. coli* is responsible for up to 90% of ampicillin resistance. Clustering of amino acids of the ESBL phenotype around the active site of the enzyme changes its configuration, thus allowing binding of oxyimino-β-lactam substrates (Poirel et al., 2004). The susceptibility of the enzyme towards β-lactamase inhibitors is greatly enhanced through the opening of the active site to β-lactam substrates. Substitutions of a single amino acid at positions 104, 164, 238, and 240 produce different ESBL phenotypes, however, substitutions involving more than one amino acid typically produce

ESBLs with broader spectrum (Chaibi et al., 1999). Presently, over 200 TEM-type enzymes have been described (www.lahey.org/studies).

2.4.2 SHV Type

SHV-1, another β-lactamase commonly reported in *K. pneumoniae*, was named after the term "sulfhydryl reagent variable" (Matthew et al., 1979). SHV-1 shares 68 % of its amino acids with TEM-1 and has a similar overall structure (Bush, 2013). SHV type ESBLs exhibit amino acid substitutions at positions 238 and 240 that change the configuration of the enzyme around the active site. Several studies have reported that even a single amino acid substitution is enough to convey an extended spectrum phenotype (Philippon et al., 2016).

SHV β-lactamases can be divided into three subgroups on the basis of their molecular characteristics or functional properties. The first subgroup, designated as subgroup 2b, is able to hydrolyse penicillins and cephalosporins, but is greatly inhibited by clavulanic acid and tazobactam. Subgroup 2br comprises of broad-spectrum β-lactamases that are resistant towards clavulanic acid while subgroup 2be comprises of enzymes that are able to hydrolyse one or more oxyimino β-lactams (cefotaxime, ceftazidime, and aztreonam) (Tzouvelekis and Bonomo, 1999). To date, more than 100 SHV variants have been elucidated (www.lahey.org/studies). Among these, SHV-5 and SHV-12 are the predominant ESBL types and are most commonly found worldwide (Bush, 2013; Philippon et al., 2016).

2.4.3 CTX-M Type

CTX-M enzymes are well-known based on their superior hydrolytic activity against cefotaxime. CTX-M family is made up of non-homogeneous and convoluted group of enzymes (Bonnet, 2004), which only shares 40 % identity with TEM or SHV β-lactamases. Phylogenetic analysis suggests that CTX-M did not arise by mutations from previous plasmid-meditated enzymes but originated via the mobilisation of chromosomal *bla* genes from *Kluyvera* spp. (Canton, 2008). From an evolutionary point of view, CTX-M diverged by point mutations due to antibiotic selective pressure, which gave rise to new variants that could enhance the hydrolytic activity against ceftazidime (Bonnet, 2004; Poirel et al., 2008). Presently, over 100 CTX-M enzymes have been reported (www.lahey.org/studies).

2.4.4 Other ESBL Types

Despite the fact that majority of ESBLs were derived from TEM and SHV, distantly related β-lactamases such as PER, VEB, and GES β-lactamases have been discovered. They were reported infrequently and were usually found in members of *Enterobacteriaceae* at constricted environmental sites. The PER-type ESBLs constitute about 25–27 % homology with known TEM and SHV (Bauernfeind et al., 1996). PER-1 β-lactamases demonstrate effective penicillin and cephalosporin hydrolysation but are susceptible to clavulanic acid. They were discovered in bacterial isolates in Turkey, France, and Italy (Vahaboglu et al., 1997). VEB-1 β-lactamase is another enzyme that is to some extent related to PER-1 (Poirel et al., 1999). VEB-1 was first discovered from *E. coli* in

Vietnam, and was subsequently discovered in *P. aeruginosa* in Thailand (Naas et al., 1999), which were subsequently spread to other bacteria of Southeast Asia nations (Poirel et al., 1999). Another enzyme that constitutes the minority of the ESBLs is the GES-1–lactamase. GES-1 was discovered in a *K. pneumoniae* isolate in France (Poirel et al., 2000). GES-1 demonstrates enzymatic properties that resemble those of class A ESBLs, which have great hydrolytic activity against penicillins and extended spectrum cephalosporins, but are susceptible to cephamycins or carbapenems and inhibited by β-lactamase inhibitors (Castanheira et al., 2004).

2.5 Detection of ESBL

2.5.1 Phenotypic Detection of ESBL

ESBL detection tests should accurately discriminate between the bacteria producing these enzymes and those with other mechanisms of resistance to β-lactams. Two methods for phenotypic detection of ESBLs are proposed by the Clinical and Laboratory Standards Institute (CLSI) known as the disc diffusion method and the dilution antimicrobial susceptibility tests. Principally, the guidelines are based on the hydrolytic activity of ESBLs towards the third-generation cephalosporins but are inhibited by clavulanic acid. The common practice involves initial screening with cefpodoxime, cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by confirmatory tests with both cefotaxime and ceftazidime in combination with clavulanic acid. Disc diffusion test for antibiotic susceptibility test screens for production of ESBL enzymes by measuring the inhibition zone diameters (CLSI, 2006). The use of more than

one of these antibiotics for screening improves the sensitivity of detection and if any of the zone diameters shows ESBL production, phenotypic confirmatory tests should be performed to ascertain the results. The test concentration of 1 µg/ml for ceftazidime, aztreonam, cefotaxime, or ceftriaxone is used for the dilution antimicrobial susceptibility tests (CLSI, 2006). Any bacterial growth at this antibiotic concentration (cephalosporin MIC of ≥2 µg/ml) would indicate probable ESBL production and should be subjected to phenotypic confirmatory tests.

For the phenotypic confirmation of production of ESBLs, CLSI advocates the use of ceftazidime discs (30 µg) or cefotaxime (30 µg) with or without clavulanate (10 µg) with confluent bacterial growth on Mueller-Hinton agar (CLSI, 2006). The production of ESBL is confirmed when a difference of ≥5 mm between the zone diameters of cephalosporin with and without clavulanic acid is observed (CLSI, 2006). One concern about this method is the inability to detect CTX-M-producing organisms when ceftazidime alone is used (Brenwald et al., 2003). Therefore, both ceftazidime and cefotaxime with and without clavulanate should be used. Phenotypic confirmatory test for the detection of ESBL production can also be performed by the broth microdilution assay using ceftazidime and cefotaxime with and without clavulanic acid. A decrease in MIC of cephalosporin with or without clavulanic acid when twofold-serial-dilution is used would confirm the production of ESBL.

2.5.2 Genotypic Detection of ESBL

The high sensitivity and specificity of phenotypic confirmatory tests have made them a great confirmatory test for production of ESBLs (Wu et al., 2001). However, phenotypic confirmatory test is unable to distinguish among the specific enzymes responsible for ESBL production and may result in false positive or negative results (Wu et al., 2001). In contrast, genotypic detection uses molecular techniques for detection of the genes that are responsible for the production of ESBL. The molecular method that is widely used is the PCR amplification of the respective ESBL genes using a set of specific oligonucleotide primers, followed by sequencing. Multiplex PCR using different primer pairs of ESBL genes in a single reaction would be another option in facilitating the detection of ESBL. Other advanced molecular technology such as DNA probe hybridisation, ligase chain reaction and DNA microarray system are the other options in detecting the production of ESBL. Molecular techniques undoubtedly play an important role in screening, tracking and monitoring the transmission of ESBL-producing organisms without culturing (Pitout and Laupland 2008).

2.6 *Enterobacteriaceae* and ESBL

Transmission of ESBL-producing *Enterobacteriaceae* species poses challenges healthcare facilities worldwide on the implementation of effective infection control measures to limit further nosocomial spread. ESBL-producing *Enterobacteriaceae* was first described in 1983 (Bradford, 2001) in relation to hospital-acquired infections and had rapidly increased globally ever since. In the 2000s, the epidemiology of ESBL-producing organisms changed as *Escherichia coli* producing the CTX-M ESBL type was increasingly described as an important cause of community-acquired urinary tract infections worldwide (Pitout et al., 2005), supporting the hypothesis that in more recent years ESBL-producing *Enterobacteriaceae* had probably been transferred into hospitals rather than vice versa. Possible community sources may include foodstuffs (Kluytmans et al., 2013) and colonisation resulting from global travel, especially to the Indian subcontinent (Kuenzli et al., 2014). Furthermore, ESBL-producing *Enterobacteriaceae* had been recovered in water samples from the Swiss rivers and lakes (Zurfluh et al., 2013), possibly constituting an underappreciated exposure route for dissemination of antibiotic resistance.

Transmission of ESBL-producing *Enterobacteriaceae* is further complicated by ESBL genes being encoded on self-transmissible plasmids, which can be exchanged among similar and different species of *Enterobacteriaceae*. The contribution of this horizontal transfer of plasmids carrying ESBL genes between *Enterobacteriaceae* and the epidemiology of ESBL producers, however, remains elusive, as analyses of plasmids are challenging due to their

flexible architecture and the non-availability of suitable reference sequences, as they are far less conserved than bacterial chromosomes.

The members of the *Enterobacteriaceae* family are Gram-negative bacilli, and commonly reside within the gastrointestinal tract. One of *Enterobacteriaceae*'s most significant resistance mechanisms is the ability to reduce the effectiveness of modern extended spectrum cephalosporins by inactivating these compounds via hydrolysis of their β-lactam ring. Such resistance has led to the increase in different point mutation variants of classical ESBL (Bush and Jacoby, 2010). Options for treatment of infections include β-lactam antibiotics, β-lactam antibiotics in combination with β-lactamase inhibitors, tigecycline, aminoglycosides, and quinolones (Richards et al., 2000). Carbapenems are often used as the last resort to treat serious infections. The combination of outer membrane protein cleavage and β-lactamase production could induce resistance towards carbapenems. However, resistance towards carbapenems in *Enterobacteriaceae* species is very rare.

2.7 Metagenomic Analysis of Environmental Samples

Molecular biology and gene expression techniques have been extensively used in the identification of natural resistance genes in randomly recombined clones from bacterial DNA libraries of environmental samples (Riesenfeld et al., 2004; Allen et al., 2010). One of the most remarkable approaches is the advent and development of metagenomics. Metagenomics ignores the need to isolate or culture microorganisms to study the genomic content in a complex mixture of microorganisms. Direct isolation of DNA from environmental samples has demonstrated that this approach is effective in comparing and discovering the respective ecology, metabolic analysis of complex environmental microbial communities, and identification of novel biomolecules (Daniel, 2005; Ferrer et al., 2009; Simon and Daniel, 2010). The underlying problem is that it requires a heterologous host for expression of the cloned genes to be able to identify the functional resistance. Several worldwide sequencing approaches were done by Costa, et al. (2006) and Dantas et al. (2008) and the results indicate the spike in the number of resistance genes identified using other expression systems and hosts.

In recent years, the emergence of gene expression and production of microbial proteins have led to an increase in metagenomic analysis on a DNA approach. Understanding the functional dynamics of microbial communities has been made available via metatranscriptomic and metaproteomic approaches. There are over 200 different metagenomes that have been successfully sequenced from various environments, such as faeces, the human gut, oceans, and soil (Goll et

al., 2010). Collectively, these studies demonstrated the existence of numerous novel antibiotic resistance genes and the nature of ESBL mechanisms in the ecology.

The development of clinical drug resistance remains hypothetical, and the major metabolic functions of the resistance genes in microbial populations are still under investigation within environmental reservoirs such as soil. It should be noted that there is little or no evidence that any of the putative resistance genes identified in environmental studies have been mobilised into pathogenic bacteria and expressed as resistance phenotypes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Soil Sampling

Soil samples were collected from two different animal farms; cow and chicken farms located at Tanjung Tualang (4°17’54”N 101°3’6”E) and Sitiawan (4°12’60”N 100°41’59”E), respectively. Five samplings using random sampling technique were performed to make a composite sample in their respective sites. Each soil sample was obtained using a shovel at a depth of approximately 10 cm from the surface and stored in a sterile zip-lock bag with proper labelling. All equipment was cleaned with 75% ethanol and a final rinse with sterile distilled water after each use to avoid cross-contamination between samples. Soil samples were kept on ice and transported to the laboratory before proceeding to subsequent experimental process. The soil samples were also outsourced to the Forest Research Institute Malaysia (FRIM) for physiochemical analysis.

3.2 Isolation of Ampicillin-Resistant Bacteria

The composite sample from each farm was thoroughly mixed in the zip-lock bag. Approximately 5 g of the soil sample was weighed and suspended in 10 ml of 0.9% saline (NaCl). A ten-fold serial dilution was made for each mixture using saline. A volume of 0.1 ml for each dilution was spread onto Luria Bertani (LB) agar, eosin methylene blue (EMB) agar, and MacConkey (MAC) agar, each supplemented with 50 µg/mL ampicillin. The plates were then incubated overnight at 37 ℃. Bacterial colonies with different morphological characteristics were selected and sub-cultured onto LB agar plates to obtain pure culture. The pure culture was then subjected to biochemical analysis.

3.3 Gram Staining

A single bacterial colony was picked and suspended in a drop of sterile water on a glass slide. The suspension was spread thinly using the inoculating loop and fixed over a gentle flame for three times. The smear was allowed to air-dry before performing the staining procedures. Then, crystal violet was added over the smear and left for 1 minute. The excess stain was rinsed off over a gentle stream of water. Iodine solution was then added, left for one minute and the rinsing step was repeated. The smear was further decolourised with 95% alcohol for five seconds, followed by a gentle rinse of water. A counter stain, the safranin, was added onto the smear and left for 1 minute and the rinsing step was repeated. The stained smear was allowed to air-dry. The results of the stained smear were observed under oil immersion (1000 x) using a light microscope.

3.4 Identification of *Enterobacteriaceae* Species

3.4.1 Oxidase Test

A single colony of the bacterial isolate was smeared onto a filter paper soaked with the oxidase reagent (substrate tetramethyl-p-phenylenediamine dihydrochloride). A colour change (colourless to purple) within 10 seconds would indicate a positive reaction.

3.4.2 Catalase Test

A single colony of the bacterial isolate was mixed with a drop of 3 % hydrogen peroxide on a glass slide. Effervescence within 5-10 seconds would indicate a positive reaction whereas absence of bubbles would indicate a negative reaction.

3.4.3 Oxidation Fermentation Test

The oxidation fermentation (OF) test was performed by inoculating bacterial isolate into two tubes of OF basal medium containing filter-sterilised 10% (w/v) glucose. One of the two tubes was covered with mineral oil to prevent diffusion of oxygen into the medium and to create an anaerobic condition, whereas the other tube was left uncovered to create aerobic condition. All tubes were incubated for 24-48 hours at 37 ℃. If the inoculated organisms utilise the carbohydrate in both the open and sealed tubes, which would be indicated by change in the color of the medium from green to yellow, then the organism is

fermentative. If the inoculated organisms utilise carbohydrate only in the open tube (medium remained green), the organism is identified to be oxidative.

3.4.4 Bacterial Identification Using API 20 E System

3.4.4.1 Strip Preparation

A humid atmosphere was created by adding 5 ml of distilled water into the honey-combed wells of the tray. A strip was placed into the incubation box and the isolate information was recorded on the elongated flap of the tray.

3.4.4.2 Preparation of Inocula and Inoculation of Strip

A single well-isolated bacterial colony was picked using a pipette and emulsified in sterile 0.9 % saline before the test to achieve a 0.5 McFarland homogeneous bacterial suspension. For CIT, VP, and GEL, the respective tubes were filled (up to cupule) with the bacterial suspension whereas the remaining tubes were half filled. An anaerobic condition was created for tubes ADH, LDC, ODC, H_2S, and URE by overlaying with mineral oil upon addition of the bacterial suspension. The API 20 E strip was then incubated at 37 ℃ for 18-24 hours.

3.4.4.3 Reading and Interpretation of API 20 E Strip

The test strip was compared to the reading table provided by the manufacturer (BioMérieux). If the strip has less than three positive results, the strip would be incubated for another 24 hours at 37 ℃. If more than three tests (GLU+ etc.) showed positive results, all the spontaneous reactions were recorded on a result sheet and further tests requiring addition of reagents were performed. For the TDA test, a drop of TDA reagent was added into the tube. A reddish brown colour would indicate a positive reaction. Similarly, for the IND test, a drop of James reagent was added and a pink colour developed in the whole cupule would indicate a positive reaction. For the VP test, a drop of VP 1 reagent, followed by VP 2 reagent was added into the tube. After 10 minutes of standing at room temperature, formation of pink or red colour would indicate a positive reaction whereas a slightly pinkish colour would indicate a negative reaction. Lastly, identification was performed using identification software (apiweb) whereby a 7-digit profile number obtained for the 20 tests of the API 20 E strip was keyed in and compared to the database https://apiweb.biomerieux.com/servlet/Authenticate?action=prepareLogin).

3.5 Antibiotic Susceptibility Tests

3.5.1 Disc Diffusion Assay

In this study, the antibiotic disc diffusion assay was carried out to determine the resistance profile of the bacterial isolates towards 18 antibiotics. A 0.5 McFarland turbidity standard was prepared by adding 0.5 mL of 0.048 M $BaCl_2$ to 99.5 mL of 0.18 M H_2SO_4. About five to six bacterial colonies of the same isolate were picked and emulsified in 5 mL of saline solution and the turbidity of the resulting suspension was compared to that of the McFarland turbidity standard. This step was repeated until the turbidity of the swab matches the standard turbidity. A cotton swab was dipped into the bacterial suspension and swabbed evenly onto a Mueller-Hinton (MH) agar by rotating the agar plate 60° for three times. Antibiotic discs were then placed about 25 mm apart from each other using sterilised forceps (Table 3.1). The plates were then incubated at 37 ℃ for 24-28 hours. The diameter of the zone of inhibition was then measured to the nearest millimetre and compared to the standard in the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Table 3.1 List of antibiotics used in the disc diffusion assay and their respective disc content.

Type of antibiotics	Antibiotic	Disc content (μg)
First-, second-, third-generation of β-lactamases	Aztreonam	30
	Cefepime	30
	Cefotaxime	30
	Cefoxitin	30
	Cefpodoxime	10
	Ceftazidime	30
	Ceftriaxone	30
	Cephalothin	30
	Ciprofloxacin	5
	Cefotaxime/clavulanate	30/10
	Ceftazidime/clavulanate	30/10
Penicillins	Ampicillin	10
	Oxacillin	10
Carbapenem	Imipenen	10
Quinolone	Norfloxacin	10
Aminoglycoside	Gentamicin	10
Penicillin combinations	Piperacillin/Tazobactam	85
Tetracycline	Tetracycline	30

3.5.2 Phenotypic Disc Test for ESBL Detection

The disc diffusion assay was used for the screening of ESBL production using aztreonam, cefotaxime, cefpodoxime, ceftriaxone, and ceftazidime, according to the CLSI guidelines. Bacterial isolates that were resistant to any of these five agents would indicate probable ESBL production and were subjected to further ESBL phenotypic confirmatory test by comparing the inhibition zone around the cephalosporin disc with and without clavulanate. An increase of inhibition zone diameter of ≥5 mm of antibiotic in combination with clavulanate would indicate ESBL production.

3.6 DNA Extraction

3.6.1 DNA Extraction from Bacterial Cultures

Total DNA was extracted by using Bio Basic EZ-10 Spin Column Genomic DNA Minipreps Kit according to the manufacturer's instructions. A volume of 5 ml of overnight broth culture was centrifuged at 6,000 x*g* for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl of TE buffer. Then, 400 µl of digestion solution was added to the suspension and mixed well. A volume of 3 µl of Proteinase K solution (0.0133 mg/µl) was added to the suspension and incubated at 55 ℃ for 5 minutes. After that, 260 µl of 100 % ethanol was added to the suspension. Then, the mixture was transferred to an EZ-10 spin column that was placed in a 2 ml collection tube and centrifuged at 8,000 x*g* for 2 minutes. The flow-through was discarded and 500 µl of wash solution was added to the column, which was then centrifuged at 8,000 x*g* for 2 minutes. The washing step was repeated and the column was centrifuged for an additional minute to remove any residual wash solution. Subsequently, the column was transferred to a new 1.5 ml microcentrifuge tube. Then, 50 µl of elution buffer was added to the centre part of the membrane in the column, which was incubated at room temperature for 30 minutes. Lastly, the column was centrifuged at 8,000 x*g* for 2 minutes to elute DNA from the membrane. The concentration and the purity of the purified DNA was measured and the purified DNA was stored at -20 ℃ until further use.

3.6.2 Total Microbial DNA Extraction from Soil Samples

Total microbial DNA was directly extracted from the soil samples by using MO BIO PowerSoil® DNA Isolation Kit according to manufacturer's protocol, with minor modification. An amount of 1 g of soil sample was added to the PowerBead Tube provided and gently vortexed to mix. Then, 60 µl of solution C1 was added to the tube and gently mixed. The PowerBead Tube was secured horizontally with tape and vortexed for 10 minutes. The tube was then centrifuged for 1 minute at 10,000 x*g* and the supernatant was transferred to a 2 ml collection tube.

Next, 250 µl of solution C2 was added to the collection tube. The tube was vortexed for 5 seconds and incubated at 4 ℃ for 5 minutes. The tube was then centrifuged for 1 minute at 10,000 x*g*. Then, not more than 600 µl of the supernatant was carefully transferred to a new, 2 ml collection tube. A volume of 200 µl of solution C3 was added to the tube and vortexed, followed by incubation at 4 ℃ for 5 minutes. This was followed by centrifugation for 1 minute at 10,000 x*g*. Not more than 750 µl of the resulting supernatant was then carefully transferred to a new 2 ml collection tube. Next, 1.2 mL of solution C4 was added to the supernatant and the tube was vortexed for 5 seconds.

Subsequently, 675 µl of the mixture was loaded to a spin filter and centrifuged at 10,000 x*g* for 1 minute. The flow-through was discarded and the remaining of the mixture was loaded to the filter and centrifugation was repeated. After that, 500 µl of solution C5 was added to the spin filter and the tube was centrifuged at 10,000 x*g* for 30 seconds. The flow-through was discarded, followed by centrifugation at 10,000 x*g* for 1 minute. The spin filter was then transferred to a new 2 ml collection tube. Then, 100 µl of solution C6 was added to the centre of the white filter membrane and incubated for 10 minutes at room temperature. Lastly, the tube was centrifuged at 10,000 x*g* for 1 minute. The concentration and the purity of the total DNA recovered were measured and stored at -20 ℃.

3.7 Polymerase Chain Reaction

3.7.1 16S rDNA PCR Assay

The culture DNA extracts were subjected to PCR amplification targeting the 16S rRNA gene. The primers used were as follows: 16S_F (5'-AGAGTTTGATYMTGGCTCAG -3') and 16S_R (5'-TRACGGSCRGTGTGTA -3') (Jiang et al., 2006). All PCR amplifications were performed in reaction volume of 25 µL. The reaction mixture contained 1X PCR buffer (Fermentas), 10 ng of DNA, 200 µM dNTP mix (Promega), 0.2 µM of forward and reverse primers, 2 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Fermentas). The optimised PCR was performed as follows: denaturation at 94 ℃ for 10 minutes, 30 cycles with denaturation at 94 ℃ for 1

minute, annealing at 55 ℃ for 1 minute, extension at 72 ℃ for 1 minute, and final extension at 72 ℃ for 10 minutes. After the amplification process, the PCR products were analysed on 1.5 % (w/v) agarose gel.

3.7.2 PCR Amplification of ESBL Genes

The purified bacterial culture DNA and total microbial DNA were subjected to PCR amplification of nine ESBL genes (TEM, SHV, CTXM, OXA, PER, VEB, CMY, ACC, and DHA). The primers used for the ESBL genes are listed in Table 3.2. All PCR amplifications were performed in reaction volume of 25 µL. The reaction mixture contained 1X PCR buffer (Fermentas), 10 ng of DNA, 200 µM dNTP mix (Promega), 0.2 µM of forward and reverse primers, 2 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Fermentas). The optimised PCR was performed as follows: denaturation at 94 ℃ for 10 minutes, 30 cycles with denaturation at 94 ℃ for 1 minute, annealing at 50 ℃ for 1 minute, extension at 72 ℃ for 1 minute, and final extension at 72 ℃ for 10 minutes. After the amplification process, the PCR products were analysed on 1.5 % (w/v) agarose gel.

Table 3.2 Oligonucleotides of β-lactamase genes.

No.	Target Gene	Primer	Primer Sequence (5' to 3')	Size (bp)	Reference
1	*bla*-TEM	TEM_F	ATG AGT ATT CAA CAT TTC CG	~ 800	(Rasheed et al., 1997)
		TEM_R	CTG ACA GTT ACC AAT GCT TA		
2	*bla*-SHV	SHV_F	TGG TTA TGC GTT ATA TTC GCC	~ 800	(Kiratisin et al., 2008)
		SHV_R	GGT TAG CGT TGC CAG TGC T		
3	*bla*-CTXM	MA1	ATG TGC AGY ACC AGT AAR GTK ATG GC	593	(Mulvey et al., 2003)
		MA2	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		
4	*bla*-OXA	OXA_F	ATG AAA AAC ACA ATA CAT ATC AAC TTC GC	820	(Kiiru et al., 2012)
		OXA_R	GTG TGT TTA GAA TGG TGA TCG CAT T		
5	*bla*-PER	PER_F	ATG AAT GTC ATT ATA AAA GC	925	(Nordmann and Naas, 1994)
		PER_R	AAT TTG GGC TTA GGG CAG AA		
6	*bla*-VEB	VEB_F	ATT TAA CCA GAT AGG ACT ACA	1000	(Zafaralla and Mobashery, 1992)
		VEB_R	CGG TTT GGG CTA TGG GCA G		
7	*bla*-CMY	CMY_F	ATG ATG AAA AAA TCG TTA TGC	1200	(Koeck et al., 1997)
		CMY_R	TTG CAG CTT TTC AAG AAT GCG C		
8	*bla*-ACC	ACC_F	AGC CTC AGC AGC CGG TTA C	818	(Bauernfeind et al., 1999)
		ACC_R	GAA GCC GTT AGT TGA TCC GG		
9	*bla*-DHA	DHA_F	TGA TGG CAC AGC AGG ATA TTC	997	(Koh et al., 2007)
		DHA_R	GCT TTG ACT CTT TCG GTA TTC G		

3.7.2.1 Primary PCR Amplification of *bla*-TEM Gene

Purified bacterial culture DNA was subjected to PCR amplification of *bla*-TEM gene. The primers used are as follows: TEM_F (5'-ATGAGTATTCAACATTTCCG -3') and TEM_R (5'-CTGACAGTTACCAATGCTTA -3'). All PCR was performed in the reaction volume of 25 µl. The reaction mixture contained 1X PCR buffer (Fermentas), 10 ng of DNA, 200 µM dNTP mix (Promega), 0.2 µM of forward and reverse primers, 2 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Fermentas). The optimised PCR was performed as follows: denaturation at 94 ℃ for 10 minutes, 30 cycles with denaturation at 94 ℃ for 1 minute, annealing at 50 ℃ for 1 minute, extension at 72 ℃ for 1 minute, and final extension at 72 ℃ for 10 minutes. After the amplification process, the PCR products were analysed on 1.5 % (w/v) agarose gel.

3.7.2.2 Nested PCR Amplification of *bla*-TEM Gene

Primary PCR products were subjected to nested PCR to reduce non-specific binding. Three successive 10-fold serial dilution was performed on the primary PCR products before proceeding to nested PCR amplification. The primers used are as follows: TEM_F2 (5'- CTGAAGATCAGTTGGGTGCAC -3') and TEM_R2 (5'- CTCAGCGATCTGTCTATTTCG -3'). All PCR was performed in the reaction volume of 25 µl. The reaction mixture contained 1X PCR buffer (Fermentas), 10 ng of DNA, 200 µM dNTP mix (Promega), 0.2 µM of forward and reverse primers, 2 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Fermentas). The optimised PCR was performed as follows: denaturation at 94

℃ for 10 minutes, 30 cycles with denaturation at 94 ℃ for 1 minute, annealing at 55 ℃ for 1 minute, extension at 72 ℃ for 1 minute, and final extension at 72 ℃ for 10 minutes. After the amplification process, the PCR products were analysed on 1.5 % (w/v) agarose gel.

3.8 PCR Product Purification

PCR purification was performed by using Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit according to the manufacturer's protocol with minor modifications. A volume of 70 µl PCR product was mixed with 140 µl of buffer NTI. The mixture was loaded in a column placed into a collection tube and centrifuged for 30 seconds at 11,000 x*g*. The flow-through was discarded and 700 µl of wash buffer NT3 was added to the column, followed by centrifugation as before. The washing step was repeated and the column was centrifuged for an additional 1 minute to remove any residual NT3 buffer. A volume of 50 µl of buffer NE was added to the column and incubated for 15 minutes at room temperature to maximise the recovery yield. Lastly, the column was centrifuged for 1 minute at 11,000 x*g*. The purified PCR products were recovered and subsequently stored at -20 ℃ until use.

3.9 Cloning

3.9.1 Ligation of PCR Product into pGEM-T Easy Vector

Ligation of purified PCR product into the pGEM-T Easy Vector cloning vector was performed as described by the manufacturer (Promega). A ligation reaction was set up as follows: 5 µl of 2X Rapid Ligation Buffer, 1 µl of pGEM-T® vector (50 ng), 1 µl of T4 ligase (3 U/µL), and 3 µl of PCR product. The mixture was incubated overnight at 4 ℃ to maximise the efficiency of ligation. The ligated product was subsequently transformed into competent *E. coli* strain JM109 cells.

3.9.2 Transformation into *E. coli* JM109

3.9.2.1 Preparation of Competent Cells

A single colony of *E. coli* JM109 was inoculated to 10 ml of LB broth and incubated at 37 ℃ overnight with agitation at 200 rpm. A volume of 1 ml of overnight culture was transferred to 25 ml of LB broth and agitated at 200 rpm at 37˚C until it reached the OD_{600} of 0.4-0.5. The cells were then pelleted by centrifugation at 5,000 x*g* for 10 minutes at 4 ℃. After resuspension in 2 ml of ice cold 1 M calcium chloride, the competent cells were incubated on ice for at least 2 hours before the bacterial transformation process.

3.9.2.2 Transformation of Ligated PCR Product into *E. coli* JM109

A volume of 3 µl of the ligation mixture was gently mixed with 200 µl of competent cells in a pre-chilled 1.5 ml microcentrifuge tube and incubated on ice for an hour. The mixture was then subjected to heat-shock at 42 ℃ for exactly 90 seconds and incubated on ice for 5 minutes. Immediately, 800 µl of LB broth was added to the tube, followed by incubation at 37 ℃ for 45 minutes with agitation at 80 rpm. The tube content was then centrifuged at 7,000 x*g* for 10 minutes. Subsequently, the cells were resuspended in 100 µl of LB broth and plated onto LB agar containing 50 µg/mL ampicillin, 100 mM IPTG and 50 mg/mL X-Gal. A positive control was performed by transforming the competent cells with pUC19 plasmid whereas a negative control was prepared without any pUC19 plasmid. The LB agar plates were then incubated overnight at 37 ˚C. White bacterial colonies were subsequently sub-cultured onto new LB agar plates containing 50 µg/mL ampicillin, 100 mM IPTG and 50 mg/mL X-Gal to avoid false positive results.

3.9.3 Colony PCR

Recombinant colonies containing the desired DNA inserts were PCR amplified using the pair of primers (e.g. TEM_F2 and TEM_R2 primers) targeting the desired *bla*-TEM gene. Briefly, a colony was picked using a white pipette tip, and suspended in a 25-µL PCR mixture containing the following components: 1X PCR buffer (Fermentas), 0.2 mM dNTP mix, 0.2 mM of TEM-F2 and TEM-R2 primers, 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (Fermentas). The optimised PCR was performed as follows: denaturation at 94 ℃ for 10 minutes,

30 cycles with denaturation at 94 ℃ for 1 minute, annealing at 55 ℃ for 1 minute, extension at 72 ℃ for 1 minute, and final extension at 72 ℃ for 10 minutes. After the amplification process, the PCR products were analysed on 1.5 % (w/v) agarose gel.

3.10 Purification of Recombinant Plasmids

The purification of recombinant plasmids was performed using the Wizard® Plus SV Minipreps DNA Purification System kit (Promega). A single colony was inoculated to 5 ml of LB broth containing 50 µg/mL ampicillin and incubated at 37 ℃ with agitation at 200 rpm overnight. Approximately 5 ml of the resulting culture was harvested by centrifugation at 10,000 x*g* for 5 minutes. The supernatant was discarded and the tube was inverted and blotted on a paper towel to remove excess medium. A volume of 250 µl of Cell Resuspension Solution was added to the tube and the supernatant was completely resuspended by vortexing. Then, 250 µl of Cell Lysis Solution was added to the mixture and mixed by inverting the tube four times until the cell suspension turned clear. Then, 10 µl of Alkaline Protease Solution (250 µg) was added to the mixture and mixed by inverting the tube four times and incubated for 5 minutes at room temperature. Next, 350 µl of Neutralization Solution was added to the mixture and immediately mixed by inverting the tube a few times. The bacterial lysate was then centrifuged at 14,000 x*g* for 10 minutes.

The resulting supernatant was transferred to a spin column and centrifuged at 14,000 x*g* for 1 minute. After discarding the flow-through, 750 µl of Column Wash Solution was added and the tube centrifuged at 14,000 x*g* for 10 minutes. The flow-through was discarded and the washing step was repeated. The tube was then centrifuged for an additional two minutes to remove any remaining residues. The column was then transferred to a new 1.5 ml microcentrifuge tube. Lastly, 100 µl of nuclease-free water was added to the column, which was incubated for 5 minutes before eluting the DNA by centrifugation at 14,000 xg for 1 minute. The purified plasmid DNA was stored at -20 ℃.

3.11 DNA Sequencing and Multiple Sequence Alignment

The purified recombinant plasmids were outsourced to First BASE Laboratories and myTACG for DNA sequencing. The *bla*-TEM clone sequences were examined and edited manually using the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The partial nucleotide sequences were aligned using the BlastX software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the translated nucleotide query sequences against existing sequences in the National Center for Biotechnology Information (NCBI) protein database. All verified *bla*-TEM homologous gene sequences were translated into their respective polypeptide sequences using ExPASy translate tool (https://web.expasy.org/translate/). Multiple sequence alignment of the translated polypeptide sequences was performed using the Clustal Omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

3.12 *bla*-TEM Variant Analysis

The translated amino acid sequences were compared with the amino acid sequences of TEM-1 provided by the Lahey Clinic database (http://www.lahey.org/Studies/temtable.asp) at their respective amino acid positions.

3.13 Phylogenetic Analysis

Construction of phylogenetic tree based on neighbour-joining method was performed. The GenBank accession numbers for *bla*-TEM representatives were provided by Lahey Clinic database (http://www.lahey.org/Studies/temtable.asp) and the respective *bla*-TEM phylogenies were retrieved from the GenBank database hosted on NCBI. The construction of phylogenetic tree was performed using the Mega 6 software (http://www.megasoftware.net/). The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (500 replicates) is indicated above the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. GenBank accession numbers for the recombinant clones are as follows: KP057231-KP057240 and KU745464-KU745525.

CHAPTER 4

RESULTS

4.1 Soil Analysis

The composite soil samples collected from the cow and chicken farms were analysed based on several physiochemical properties that include dry pH, nitrogen, organic carbon, coarse sand, fine sand, silt and clay contents. The results are shown in Table 4.1. Generally, most of the physiochemical properties such as dry pH, nitrogen content, organic carbon content, silt content, and clay content performed on the cow farm soil were higher as compared to the chicken farm soil. On the other hand, the chicken farm soil had higher coarse sand content as compared to the cow farm soil. Both samples had the same fine sand content.

Table 4.1 Physiochemical properties of soil samples from cow and chicken farms.

Soil Sample	Dry pH	Nitrogen, N (%)	Organic Carbon, OC (%)	Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)
Cow farm soil	7.04	0.74	5.95	51	21	13	19
Chicken farm soil	5.53	0.13	1.25	69	21	2	12

4.2 Isolation of Ampicillin-Resistant Bacteria

Several growth media, LB, MAC, and EMB, were used for the isolation of different types of bacteria. Ampicillin was added to the media to isolate ampicillin-resistant bacterial strains. Colony morphologies of the bacterial isolates were assessed based on the colour, shape, elevation, edge, opacity, and the surface were shown in Table 4.2. For the cow farm soil, four different colony morphologies were obtained on the MAC agar, while two were obtained on the LB agar, and three on the EMB agar each. As for the chicken farm soil, two different colony morphologies were obtained on the LB, MAC, and EMB agar each.

Table 4.2 Characterisation of bacterial isolates from soil samples of cow and chicken farms.

Soil Sample	Isolate	Growth Medium	Gram Negative / Gram Positive	Physical Morphology						Cellular Morphology
				Colour	Shape	Elevation	Edge	Opacity	Surface	
Cow farm	M1	MAC	Gram Negative	Purple	Circular	Raised	Entire	Opaque	Smooth and glistening	Bacilli
	M2	MAC	Gram Negative	Purple	Circular	Convex	Entire	Opaque	Smooth and glistening	Bacilli
	M3	MAC	Gram Negative	Purple	Circular	Convex	Entire	Translucent	Smooth and glistening	Bacilli
	M4	MAC	Gram Negative	Pink	Circular	Convex	Entire	Opaque	Smooth and glistening	Bacilli
	L6	LB	Gram Negative	White	Circular	Convex	Entire	Opaque	Smooth and glistening	Bacilli
	L7	LB	Gram Negative	White yellowish	Circular	Convex	Entire	Opaque	Smooth and glistening	Bacilli
	E11	EMB	Gram Negative	Dark blue black	Circular	Convex	Entire	Opaque	Smooth and glistening	Bacilli
	E12	EMB	Gram Negative	Purple	Circular	Convex	Entire	Translucent	Smooth and glistening	Bacilli
	E14	EMB	Gram Negative	Purple	Irregular	Flat	Entire	Translucent	Smooth and glistening	Bacilli
Chicken farm	LB1	LB	Gram Negative	White	Circular	Pulvinate	Entire	Translucent	Smooth and glistening	Bacilli
	LB2	LB	Gram Negative	White	Filamentous	Flat	Filamentous	Opaque	Dry, powdery	Bacilli
	MAC1	MAC	Gram Negative	Purple	Circular	Convex	Entire	Translucent	Smooth and glistening	Bacilli
	MAC2	MAC	Gram Negative	Colourless	Circular	Convex	Entire	Translucent	Smooth and glistening	Bacilli
	EMB1	EMB	Gram Negative	Pale purple	Circular	Convex	Entire	Translucent	Smooth and glistening	Bacilli
	EMB2	EMB	Gram Negative	Metallic Green Sheen	Circular	Convex	Entire	Opaque	Smooth and glistening	Bacilli

4.3 Preliminary Screening for *Enterobacteriaceae* Species

4.3.1 Gram Staining

Gram staining was performed on all the bacterial isolates to classify them into Gram-positive and Gram-negative bacteria. As shown in Table 4.2, all of the isolates obtained from both soil samples were Gram-negative bacteria and were of bacillus shape.

4.3.2 Oxidase Test

Oxidase test is an important differential test to identify bacteria that produce cytochrome c oxidase, a common enzyme lacking in *Enterobacteriaceae*. As shown in Table 4.3, negative reactions were observed for all bacterial isolates from the cow and chicken farm soil samples except for isolates LB1, LB2, MAC2, and EMB1. A negative reaction would indicate a member of the family *Enterobacteriaceae* whereas a positive reaction would indicate a member of the family *Pseudomadaceae*.

Table 4.3 Observations and reactions in biochemical tests of bacterial isolates from cow and chicken farm soil samples.

Soil Sample	Isolate	Biochemical Test					
		Oxidase Test		Catalase Test		OF Test	
		Observation	Reaction	Observation	Reaction	Observation	Reaction
Cow Farm	M1	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	M2	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	M3	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	M4	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	L6	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	L7	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	E11	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	E12	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	E14	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
Chicken Farm	LB1	Colour change from colourless to purple	Positive	Moderate evolution of bubble	Positive	Colour changed from green to yellow in tube without mineral oil and no colour change occur in oil-covered tube	Oxidative
	LB2	Colour change from colourless to purple	Positive	Moderate evolution of bubble	Positive	Colour changed from green to yellow in tube without mineral oil and no colour change occur in oil-covered tube	Oxidative
	MAC1	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative
	MAC2	Colour change from colourless to purple	Positive	Moderate evolution of bubble	Positive	Colour changed from green to yellow in tube without mineral oil and no colour change occur in oil-covered tube	Oxidative
	EMB1	Colour change from colourless to purple	Positive	Moderate evolution of bubble	Positive	Colour changed from green to yellow in tube without mineral oil and no colour change occur in oil-covered tube	Oxidative
	EMB2	No change in colour	Negative	Moderate evolution of bubble	Positive	Colour changed from green to yellow in both tubes	Fermentative

4.3.3 Catalase Test

The catalase test is commonly used for classification of *Enterobacteriaceae*. Members of *Enterobacteriaceae* are generally catalase-positive when tested with hydrogen peroxide. As shown in Table 4.3, all the bacterial isolates from the cow and chicken farm soil samples showed positive reactions as indicated by rapid evolution of bubbles.

4.3.4 Oxidation Fermentation Test

The oxidation fermentation (OF) test is used to differentiate microorganisms that utilise carbohydrates (glucose) aerobically (oxidation) from those that utilise carbohydrates (glucose) anaerobically (fermentation). As shown in Table 4.3, bacterial isolates from the cow farm soil were fermentative, which would indicate a member of the family *Enterobacteriaaceae.* For the chicken farm soil sample, four bacterial isolates (designated as LB1, LB2, MAC2, and EMB1) were oxidative, which would indicate a member of the family *Pseudomadaceae*, while another two isolates, MAC1 and EMB2, were fermentative.

4.4 Antibiotic Susceptibility Tests

The diameters of the inhibition zone of antibiotics of bacterial isolates were recorded in Table 4.4. The susceptibilities of the isolates were designated as resistant (R), intermediate (I) or susceptible (S). A more comprehensive summary of the results is tabulated in Table 4.5. All 15 bacterial isolates obtained from both farm soil showed resistance towards at least three β-lactam antibiotics, and the highest number of antibiotic resistance of thirteen was seen in isolates LB1 and EMB1.

For phenotypic screening of ESBLs, bacterial isolates M2, E11, E12, E14, LB1, LB2, MAC1, MAC2, EMB1, and EMB2 were resistant to at least one out of the five test antibiotics. Isolates LB2, MAC2 and EMB1 were resistant to all five ESBL screening antibiotics. Eleven isolates (M2, M4, L6, E11, E12, E14, LB1, LB2, MAC1, EMB1, and EMB2) showed an increase in their zone diameter of 5 mm to at least one of the antibiotics in combination with clavulanic acid (CTX/CLA or CAZ/CLA) versus its zone when tested in the absence of clavulanic acid, which indicated that these bacteria were potential ESBL producers. Of particular interest are bacterial isolates E14, LB1, LB2, MAC1, and EMB1 whereby they showed an increase in zone diameter for both antibiotics supplemented with clavulanic acid.

Table 4.4 Diameter of inhibition zones of bacterial isolates from cow farm and chicken farm soil samples in the Kirby-Bauer assay.

Bacterial isolate	Antibiotic/inhibition zone diameter (mm)																	
	β-lactam & other classes of antibiotics											ESBL Phenotypic screening test					ESBL confirmation test	
	AMP	FEP	FOX	KF	CIP	CN	IPM	NOR	OXA	TZP	TET	ATM	CRO	CTX	CAZ	CPD	CTX/ CLA	CAZ/ CLA
M1	0 (R)	30 (I)	23 (S)	13 (R)	28 (S)	13 (R)	27 (S)	28 (S)	0 (R)	24 (S)	13 (R)	33 (S)	27 (I)	25 (I)	27 (S)	23 (S)	29	30
M2	0 (R)	29 (I)	23 (S)	14 (R)	27 (S)	15 (R)	25 (S)	28 (S)	0 (R)	25 (S)	13 (R)	31 (S)	27 (I)	23 (R)	23 (I)	23 (S)	29	25
M3	0 (R)	31 (I)	23 (S)	14 (R)	29 (S)	17 (I)	26 (S)	29 (S)	0 (R)	28 (S)	14 (R)	30 (S)	27 (I)	28 (I)	25 (I)	23 (S)	29	28
M4	0 (R)	29 (I)	24 (S)	15 (I)	31 (S)	7 (R)	28 (S)	29 (S)	0 (R)	23 (S)	13 (R)	28 (S)	28 (S)	27 (I)	25 (I)	22 (S)	33	28
L6	0 (R)	29 (I)	24 (S)	20 (S)	13 (R)	18 (I)	25 (S)	12 (R)	0 (R)	26 (S)	14 (R)	34 (S)	29 (S)	28 (S)	26 (I)	30 (S)	34	27
L7	0 (R)	30 (I)	22 (I)	13 (R)	28 (S)	14 (R)	25 (S)	26 (S)	0 (R)	24 (S)	13 (R)	31 (S)	28 (S)	29 (I)	26 (I)	23 (S)	29	29
E11	0 (R)	29 (I)	0 (R)	0 (R)	13 (R)	14 (R)	25 (S)	13 (R)	0 (R)	21 (I)	0 (R)	26 (I)	26 (I)	23 (R)	21 (R)	17 (R)	28	23
E12	0 (R)	24 (R)	18 (I)	17 (I)	26 (S)	15 (R)	23 (S)	24 (I)	0 (R)	19 (R)	26 (S)	26 (I)	23 (R)	26 (I)	21 (R)	21 (S)	28	25
E14	0 (R)	30 (I)	23 (S)	13 (R)	28 (S)	14 (R)	24 (S)	25 (I)	0 (R)	23 (S)	13 (R)	30 (S)	26 (I)	13 (R)	17 (R)	23 (S)	28	25
LB1	0 (R)	19 (R)	0 (R)	0 (R)	24 (S)	18 (I)	37 (S)	21 (I)	0 (R)	18 (R)	21 (I)	9 (R)	15 (R)	6 (R)	6 (R)	0 (R)	12	17
LB2	0 (R)	0 (R)	11 (R)	11 (R)	25 (S)	22 (S)	32 (S)	23 (I)	0 (R)	23 (S)	20 (I)	0 (R)	6 (R)	0 (R)	0 (R)	0 (R)	23	21
MAC1	0 (R)	24 (R)	19 (R)	19 (S)	25 (S)	15 (R)	22 (S)	23 (I)	0 (R)	18 (R)	24 (S)	24 (I)	22 (R)	15 (R)	2 (R)	26 (S)	24	19
MAC2	0 (R)	23 (R)	0 (R)	0 (R)	28 (S)	19 (I)	35 (S)	26 (S)	0 (R)	19 (R)	24 (S)	14 (R)	18 (R)	15 (R)	18 (R)	0 (R)	15	18
EMB1	0 (R)	22 (R)	0 (R)	0 (R)	27 (S)	19 (I)	34 (S)	26 (S)	0 (R)	19 (R)	20 (I)	12 (R)	16 (R)	15 (R)	18 (R)	0 (R)	31	25
EMB2	0 (R)	29 (I)	23 (S)	15 (I)	18 (I)	14 (R)	26 (S)	18 (R)	0 (R)	25 (S)	10 (R)	21 (R)	33 (S)	13 (R)	17 (R)	20 (S)	23	21

*AMP=Ampicillin, ATM=Aztreonam, FEP=Cefepime, CTX=Cefotaxime, FOX=Cefoxitin, CPD=Cefpodoxime, CAZ=Ceftazidime, CRO=Ceftriaxone, KF=Cephalothin, CIP=Ciprofloxacin, CN=Gentamicin, IPM=Imipenen, NOR=Norfloxacin, OXA=Oxacillin, TZP=Piperacillin/Tazobactam, TET=Tetracycline, R=Resistant, I=Intermediate, S=Susceptible

Table 4.5 Characterisation of bacterial isolates according to phenotypic resistance patterns.

Antimicrobial agents that bacterial isolates were resistant																		
Penicillins & 1st generation cephalosporins				2nd generation cephalosporins or cephamycins	3rd generation cephalosporins				4th generation cephalosporins	Antibiotics with β-lactamase inhibitors (≥ 5 mm increase in the zone diameter)		Carbapenem & monobactam		Multidrug-resistant antibiotics				Bacterial isolate	Most probable phenotype
AMP	OXA	KF	TZP	FOX	CTX	CPD	CAZ	CRO	FEP	CTX/CLA	CAZ/CLA	IPM	ATM	CIP	CN	NOR	TET		
✓	✓	✓													✓		✓	M1	[b]IRT
✓	✓	✓			✓					✓					✓		✓	M2	[a]NSBL
✓	✓	✓															✓	M3	[b]IRT
✓	✓									✓					✓		✓	M4	[a]NSBL
✓	✓									✓				✓		✓	✓	L6	[a]NSBL
✓	✓	✓													✓		✓	L7	[b]IRT
✓	✓	✓		✓	✓	✓	✓			✓				✓	✓	✓	✓	E11	[c]ESBL
✓	✓		✓				✓	✓	✓	✓					✓			E12	[d]CMT
✓	✓	✓			✓		✓			✓	✓				✓		✓	E14	[c]ESBL
✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓					LB1	[e]pAmpC
✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓		✓					LB2	[e]pAmpC
✓	✓		✓	✓	✓		✓	✓	✓	✓	✓				✓			MAC1	[e]pAmpC
✓	✓	✓	✓	✓	✓	✓	✓	✓	✓				✓					MAC2	[e]pAmpC
✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		✓					EMB1	[e]pAmpC
✓	✓				✓		✓			✓			✓		✓	✓	✓	EMB2	[c]ESBL

[a]NSBL isolates developed penicillin resistance but were susceptible to other classes of β-lactam antibiotics.
[b]IRT isolates degrade penicillins, not inhibited by β-lactamase inhibitors but were susceptible to other classes of β-lactam antibiotics.
[c]ESBL carriers were resistant to penicillins, first and most third generation cephalosporins and monobactam, developed intermediate resistance to fourth generation cephalosporins and were susceptible to cephamycins, carbapenems and β-lactamase inhibitors.
[d]CMT were resistant to most β-lactam antibiotics and β-lactamase inhibitors but were susceptible to cephamycin and carbapenems.
[e]pAmpC isolates were resistant to all generations of β-lactam antibiotics, but were susceptible to carbapenem
*AMP=Ampicillin, ATM=Aztreonam, FEP=Cefepime, CTX=Cefotaxime, FOX=Cefoxitin, CPD=Cefpodoxime, CAZ=Ceftazidime, CRO=Ceftriaxone, KF=Cephalothin, CIP=Ciprofloxacin, CN=Gentamicin, IPM=Imipenen, NOR=Norfloxacin, OXA=Oxacillin, TZP=Piperacillin/Tazobactam, TET=Tetracycline, R=Resistant, I=Intermediate, S=Susceptible, NSBL=Narrow spectrum β-lactamase, IRT=Inhibitor resistant TEMs, CMT=Complex mutant TEM, pAmpC=plasmid-mediated AmpC

Based on phenotypic resistance patterns from the 15 bacterial isolates, five different β-lactamase phenotypes were obtained. NSBL-producing bacterial isolates (M2, M4, and L6) were resistant to penicillins but were susceptible to other classes of β-lactam antibiotics. Isolates M1, M3 and L7 belong to IRT phenotypes due to their ability to degrade penicillins, not inhibited by β-lactamase inhibitors and susceptible to other classes of β-lactam antibiotics. The ESBL producers (E11, E14, and EMB2) were resistant to penicillins, the first and most of the third-generation cephalosporins and monobactam. They showed intermediate resistance to fourth generation cephalosporins but susceptible to carbapenem and β-lactamase inhibitors. CMT phenotype was observed for isolate E12, which was resistant to most of the β-lactam antibiotics and β-lactamase inhibitors but was susceptible to cephamycin and carbapenem. Lastly, pAmpC phenotype was observed for isolates LB1, LB2, MAC1, MAC2, and EMB1 as they were resistant to all generations of β-lactam antibiotics, but were susceptible to carbapenem. Also, most of the isolates were resistant to at least one of the non β-lactam antibiotics except for isolates LB1, LB2, MAC2, and EMB1. Besides, eight of the bacterial isolates namely M1, M2, M4, L6, L7, E11, E14 and EMB2 were considered MDR as they are resistant to at least two or more MDR antibiotics tested.

4.5 API 20 E Bacterial Identification

The possible bacterial species isolated from the cow and chicken farm soils were identified using the API 20 E bacterial identification kit (Table 4.6). The isolates from the cow farm soil sample belonged to the *Enterobacteriaceae* family, comprising the genera *Citrobacter*, *Pantoea*, *Escherichia*, *Klebsiella*, and *Kluyvera*. As for the chicken farm soil sample, the isolates consisted of *Klebsiella*, *Escherichia*, and *Pseudomonas* species.

Table 4.6 Probable species of bacterial isolates from cow and chicken farm soil samples identified by API 20 E test.

Soil sample	Isolate	Family	Possible species	% ID
Cow farm	M1	*Enterobacteriaceae*	*Citrobacter koseri/farmeri*	68.7
	M2	*Enterobacteriaceae*	*Pantoea* spp. 4	74.3
	M3	*Enterobacteriaceae*	*Pantoea* spp. 4	74.3
	M4	*Enterobacteriaceae*	*Escherichia coli* 1	99.9
	L6	*Enterobacteriaceae*	*Klebsiella pneumoniae* ssp.	97.5
	L7	*Enterobacteriaceae*	*Pantoea* spp. 4	78.8
	E11	*Enterobacteriaceae*	*Kluyvera* spp.	74.6
	E12	*Enterobacteriaceae*	*Klebsiella pneumoniae* ssp.	97.5
	E14	*Enterobacteriaceae*	*Pantoea* spp. 4	75.1
Chicken farm	LB1	*Pseudomonadaceae*	*Pseudomonas oryzihabitans*	93.1
	LB2	*Pseudomonadaceae*	*Pseudomonas fluorescens/putida*	69.8
	MAC1	*Enterobacteriaceae*	*Klebsiella oxytoca*	97.3
	MAC2	*Pseudomonadaceae*	*Pseudomonas luteola*	78.8
	EMB1	*Pseudomonadaceae*	*Pseudomonas luteola*	92.1
	EMB2	*Enterobacteriaceae*	*Escherichia coli* 1	99.9

4.6 DNA Extraction

4.6.1 Total DNA Extracted from Bacterial Isolates from Cow and Chicken Farm Soil Samples

As shown in Figure 4.1, several bands corresponding to 1,500 bp, 2,000 bp and more than 10,000 bp were seen in the agarose gel for most of the bacterial isolates, indicating the presence of high molecular weight DNA and different possible plasmid DNA conformations. DNA concentration and purity of bacterial isolates were recorded in Table 4.7.

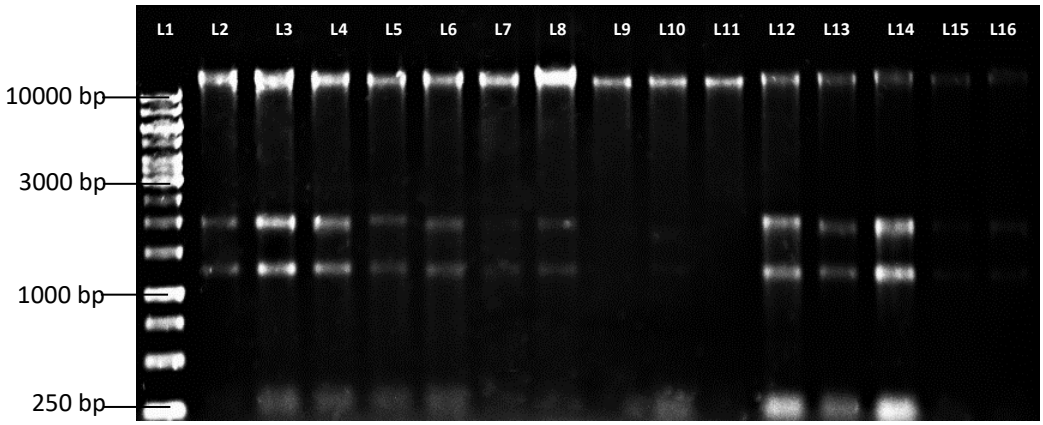

Figure 4.1 Total DNA extracted from the bacterial isolates from cow and chicken farm soil samples in 1 % agarose gel. Lane 1: 1 kb DNA ladder (1 µg); Lane 2: M1; Lane 3: M2; Lane 4: M3, Lane 5: M4; Lane 6: L6; Lane 7: L7; Lane 8: E11; Lane 9: E12; Lane 10: E14; Lane 11: LB1; Lane 12: LB2; Lane 13: MAC1; Lane 14: MAC2; Lane 15: EMB1; Lane 16: EMB2.

Table 4.7 DNA concentration and purity of bacterial isolates.

Bacterial isolate	DNA Concentration (µg/µl)	DNA purity (A_{260}/A_{280})
M1	126.9	1.78
M2	131.2	1.81
M3	127.8	1.82
M4	124.5	1.82
L6	129.4	1.86
L7	141.6	1.88
E11	112.8	1.77
E12	136.3	1.83
E14	126.4	1.88
LB1	133.7	1.79
LB2	122.7	1.84
MAC1	135.1	1.85
MAC2	128.9	1.82
EMB1	136.3	1.83
EMB2	113.7	1.79

4.6.2 Total DNA Extracted Directly from Cow and Chicken Farm Soil Samples

DNA fragment above 10,000 bp was seen in both samples as shown in Figure 4.2, indicating the presence of high molecular weight DNA, representing genomic DNA. DNA concentration and purity of total microbial DNA from cow and chicken farm soil samples were recorded in Table 4.8.

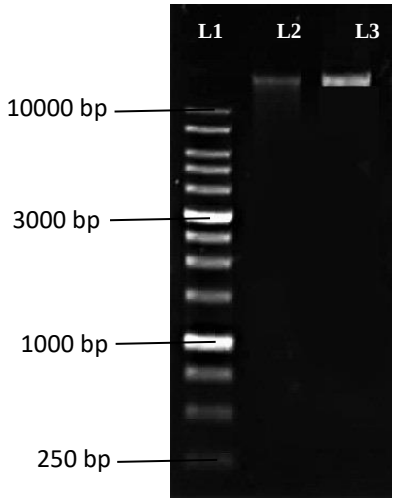

Figure 4.2 Total DNA extracted from cow and chicken farm soil samples in 1 % agarose gel. Lane 1: 1 kb DNA ladder (1 µg); Lane 2: Total DNA of cow farm soil; Lane 3: Total DNA of chicken farm soil.

Table 4.8 Total DNA concentration and purity of cow and chicken farm soil samples.

Soil Sample	Total Microbial DNA Concentration (µg/µl)	DNA purity (A_{260}/A_{280})
Cow farm	47.7	1.88
Chicken farm	45.3	1.85

4.7 PCR Amplification

4.7.1 PCR Amplification of 16S rRNA Gene

A distinct band was observed at approximately 1,500 bp for all the bacterial isolates, corresponding to the expected size of the 16S rRNA gene (Figure 4.3). The concentration and purity of the PCR products were in the range of 300-500 ng/µl and 1.8-2.0, respectively.

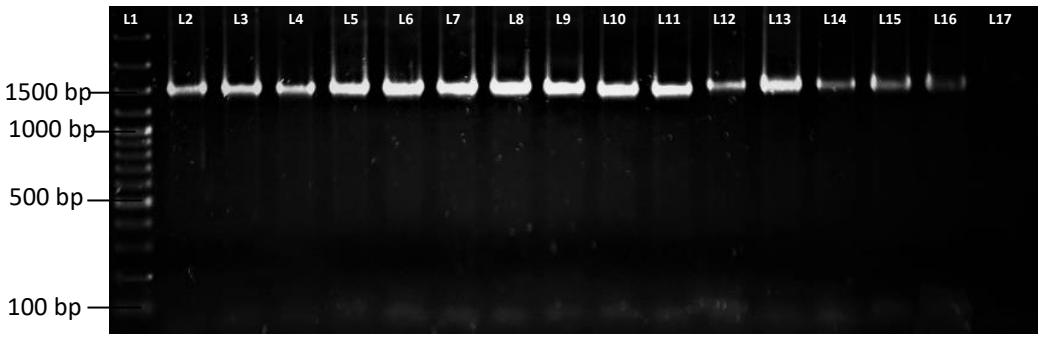

Figure 4.3 PCR product of 16S rRNA gene of bacterial isolates from cow farm soil and chicken farm soil in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: M1; Lane 3: M2; Lane 4: M3, Lane 5: M4; Lane 6: L6; Lane 7: L7; Lane 8: E11; Lane 9: E12; Lane 10: E14; Lane 11: LB1; Lane 12: LB2; Lane 13: MAC1; Lane 14: MAC2; Lane 15: EMB1; Lane 16: EMB2; Lane 17: Negative control.

4.7.2 Primary and Nested PCR Amplification of *bla*-TEM Gene

4.7.2.1 PCR Amplification of *bla*-TEM Gene from Bacterial Culture DNA

A distinct band of approximately 800 bp was seen for all the bacterial isolates, corresponding to the expected size of the targeted *bla*-TEM region (Figure 4.4). The concentration and purity of the primary PCR products were in the range of 300-500 ng/µl and 1.8-2.0, respectively.

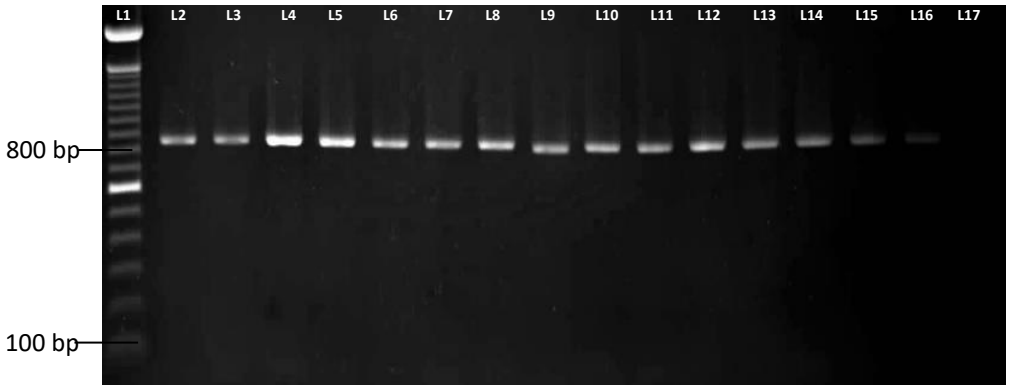

Figure 4.4 Primary PCR product of *bla*-TEM gene of bacterial isolates from cow farm soil and chicken farm soil in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: M1; Lane 3: M2; Lane 4: M3, Lane 5: M4; Lane 6: L6; Lane 7: L7; Lane 8: E11; Lane 9: E12; Lane 10: E14; Lane 11: LB1; Lane 12: LB2; Lane 13: MAC1; Lane 14: MAC2; Lane 15: EMB1; Lane 16: EMB2; Lane 17: Negative control.

A distinct band at approximately 700 bp was seen for all the bacterial isolates, corresponding to the expected size of the targeted *bla*-TEM region (Figure 4.5). The concentration and purity of the nested PCR products were in the range of 300-500 ng/µl and 1.8-2.0, respectively.

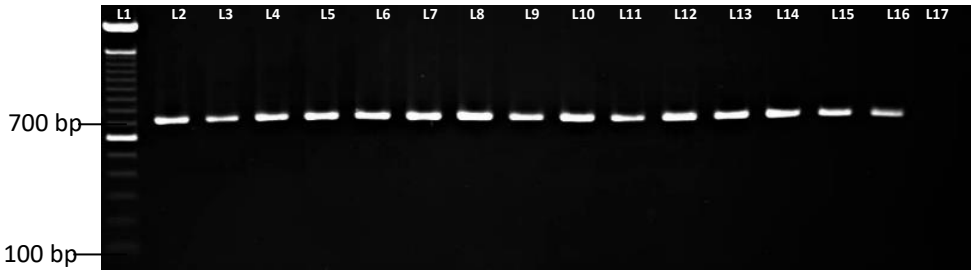

Figure 4.5 Nested PCR product of *bla*-TEM gene of bacterial isolates from cow farm soil and chicken farm soil in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: M1; Lane 3: M2; Lane 4: M3, Lane 5: M4; Lane 6: L6; Lane 7: L7; Lane 8: E11; Lane 9: E12; Lane 10: E14; Lane 11: LB1; Lane 12: LB2; Lane 13: MAC1; Lane 14: MAC2; Lane 15: EMB1; Lane 16: EMB2; Lane 17: Negative control.

4.7.2.2 Primary and Nested PCR Amplification of *bla*-TEM Gene from Total Microbial DNA Extracted Directly from Soil Samples

A faint band at approximately 800 bp was seen in both soil samples, corresponding to the expected size of the targeted *bla*-TEM region (Figure 4.6). The concentration and purity of the primary PCR products were in the range of 300-500 ng/µl and 1.8-2.0, respectively.

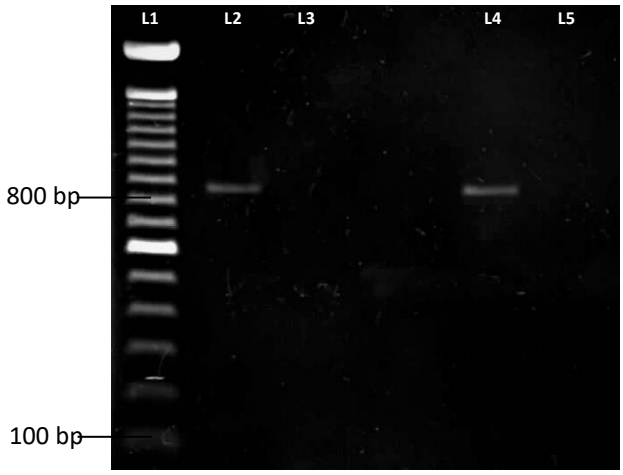

Figure 4.6 Primary PCR product of *bla*-TEM gene of total microbial DNA from cow and chicken farm soils in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: Cow farm soil; Lane 3: Negative control; Lane 4: Chicken farm soil; Lane 5: Negative control.

A distinct band at approximately 700 bp was seen for both soil samples, corresponding to the expected size of the targeted *bla*-TEM region (Figure 4.7). The concentration and purity of the nested PCR products were in the range of 300-500 ng/µl and 1.8-2.0, respectively.

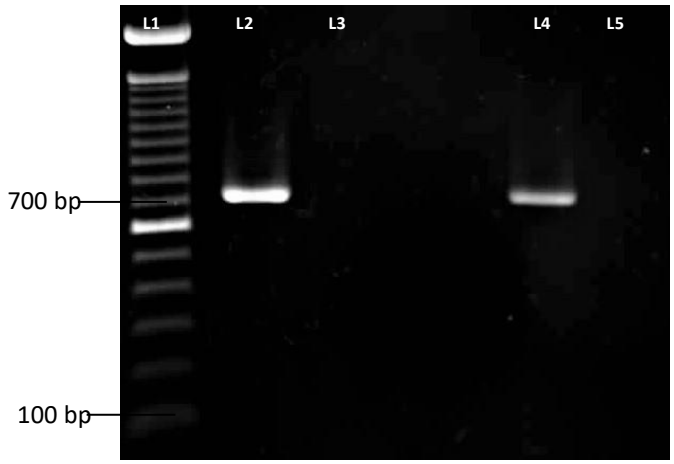

Figure 4.7 Nested PCR product of *bla*-TEM gene of total microbial DNA from cow and chicken farm soils in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: Cow farm soil; Lane 3: Negative control; Lane 4: Chicken farm soil; Lane 5: Negative control.

4.7.3 PCR Amplification of other ESBL Genes

PCR amplification of the other eight ESBL genes, namely SHV, CTXM, OXA, PER, VEB, CMY, ACC, and DHA, showed negative results except for SHV. However, due to time and budget constraints, SHV was not characterised in this study.

4.8 Colony PCR of Positive Recombinants Carrying *bla*-TEM Gene from Cow and Chicken Farm Soils

A distinct DNA fragment of approximately 700 bp was seen in 122 of the recombinants from the cow and chicken farm soils (Figures 4.8 and 4.9), indicating the presence of the desired insert DNA. Absence of the desired band might indicate a false positive result and they were excluded from subsequent analysis. The concentration and purity of the PCR products were in the range of 300-500 ng/µl and 1.8-2.0, respectively.

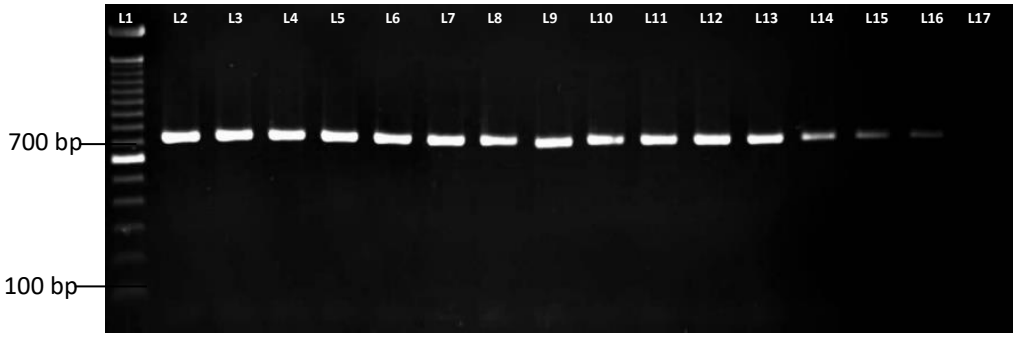

Figure 4.8 Colony PCR product of positive recombinants from cow farm soil in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: pCF1; Lane 3: pCF2; Lane 4: pCF3, Lane 5: pCF4; Lane 6: pCF5; Lane 7: pCF6; Lane 8: pCF7; Lane 9: pCF8; Lane 10: pCF9; Lane 11: pCF10; Lane 12: pCF11; Lane 13: pCF12; Lane 14: pCF13; Lane 15: pCF 14; Lane 16: pCF15; Lane 17: Negative control.

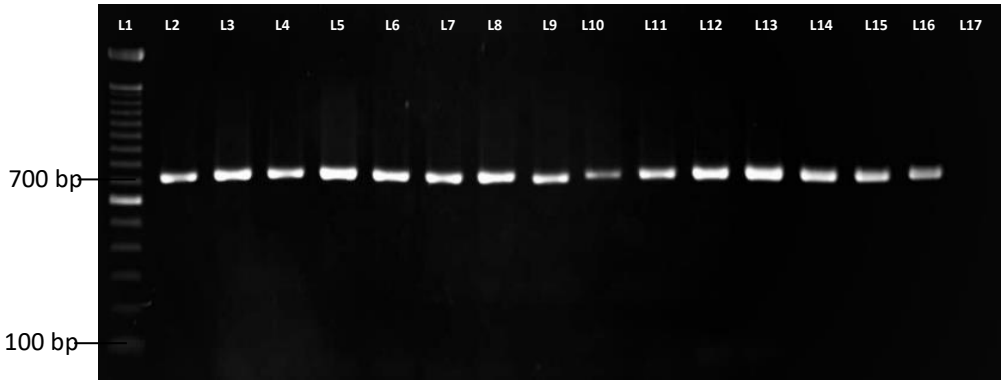

Figure 4.9 Colony PCR product of positive recombinants from chicken farm soil in 1.5 % agarose gel. Lane 1: 100 bp DNA ladder (0.2 µg); Lane 2: pCH1; Lane 3: pCH2; Lane 4: pCH3, Lane 5: pCH4; Lane 6: pCH5; Lane 7: pCH6; Lane 8: pCH7; Lane 9: pCH8; Lane 10: pCH9; Lane 11: pCH10; Lane 12: pCH11; Lane 13: pCH12; Lane 14: pCH13; Lane 15: pCH14; Lane 16: pCH15; Lane 17: Negative control.

4.9 Plasmid Purification of Cow and Chicken Soil Positive Recombinants

A distinct band of approximately 3000 bp was seen in all of the recombinants, indicating the presence of the purified recombinant plasmid (Figures 4.10 and 4.11).

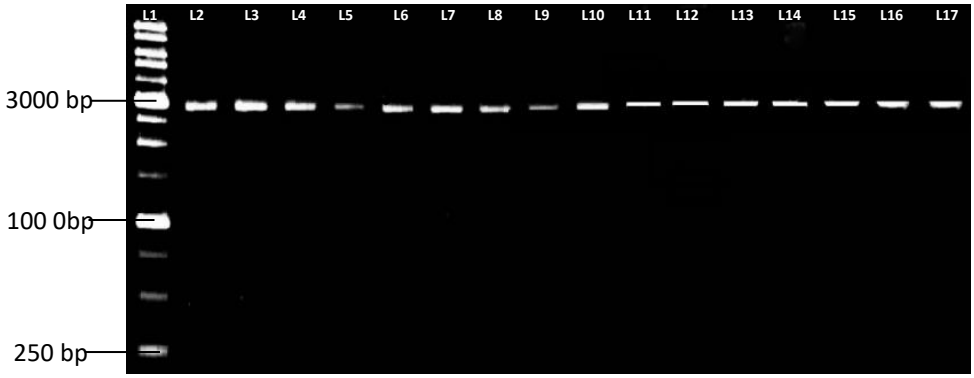

Figure 4.10 Purified plasmid of positive recombinants from cow farm soil in 1 % agarose gel. Lane 1: 1 kb DNA ladder (1 µg); Lane 2: pCF1; Lane 3: pCF2; Lane 4: pCF3, Lane 5: pCF4; Lane 6: pCF5; Lane 7: pCF6; Lane 8: pCF7; Lane 9: pCF8; Lane 10: pCF9; Lane 11: pCF10; Lane 12: pCF11; Lane 13: pCF12; Lane 14: pCF13; Lane 15: pCF14; Lane 16: pCF15; Lane 17: pCF16.

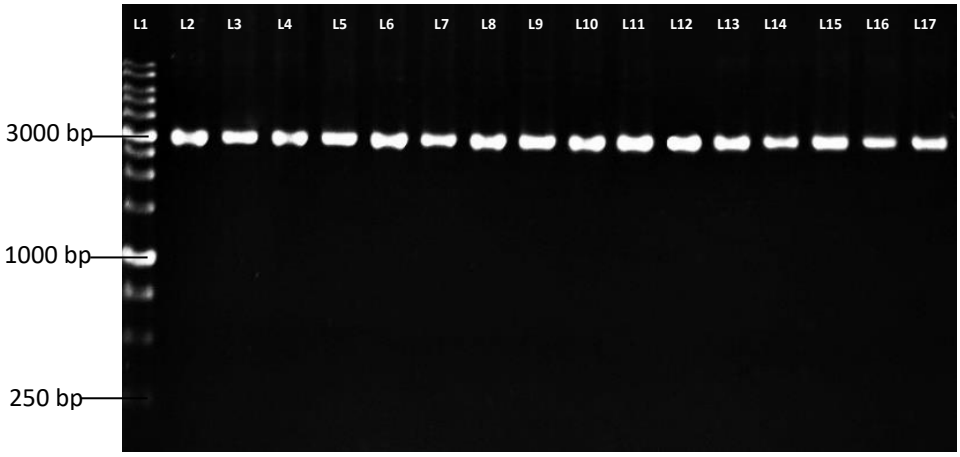

Figure 4.11 Purified plasmid of positive recombinants from chicken farm soil in 1 % agarose gel. Lane 1: 1 kb DNA ladder (1 µg); Lane 2: pCH1; Lane 3: pCH2; Lane 4: pCH3, Lane 5: pCH4; Lane 6: pCH5; Lane 7: pCH6; Lane 8: pCH7; Lane 9: pCH8; Lane 10: pCH9; Lane 11: pCH10; Lane 12: pCH11; Lane 13: pCH12; Lane 14: pCH13; Lane 15: pCH14; Lane 16: pCH15; Lane 17: pCH16.

4.10 DNA Sequence Analysis

4.10.1 BLASTn Analysis of 16S rDNA

The 16S rDNA sequences were aligned and compared against the nucleotide sequence available in the database using BLASTn. Bacteria corresponding to the highest query hits were chosen. BLASTn analysis showed that bacterial isolates M1, M2, M3, L7 and E14 from the cow farm soil ecotype exhibit high similarity towards *Enterobacter cloacae*; isolates M4 and E11 towards *E. coli*; and isolates L6 and E12 towards *K. pneumoniae* as shown in Table 4.9. For the chicken farm soil ecotype, bacterial isolates LB1, MAC2 and EMB1 show high similarity towards *Pseudomonas putida*; isolate LB2 towards *Bacillus cereus*; isolate MAC1 towards *Raoultella ornithinolytica*; and isolate EMB2 towards *E. coli*.

Table 4.9 BLASTn analysis of 16S rDNA of bacterial isolates from cow and chicken farm soil samples.

Soil sample	Isolate	Percentage of similarity (Identity)	Highest hit	Closest known bacteria species
Cow farm	M1	99	15	*Enterobacter cloacae*
	M2	99	11	*Enterobacter cloacae*
	M3	99	11	*Enterobacter cloacae*
	M4	99	71	*Escherichia coli*
	L6	99	84	*Klebsiella pneumoniae*
	L7	99	11	*Enterobacter cloacae*
	E11	99	67	*Escherichia coli*
	E12	99	89	*Klebsiella pneumoniae*
	E14	99	11	*Enterobacter cloacae*
Chicken farm	LB1	99	58	*Pseudomonas putida*
	LB2	98	53	*Bacillus cereus*
	MAC1	99	50	*Raoultella ornithinolytica*
	MAC2	99	50	*Pseudomonas putida*
	EMB1	99	48	*Pseudomonas putida*
	EMB2	99	28	*Escherichia coli*

4.10.2 BLASTx Analysis of *bla*-TEM Homologous Genes of Culture-dependent Bacterial Isolates

The translated sequences of *bla*-TEM homologous genes were aligned and compared against the protein sequences available in the database using BLASTx software. Each translated gene fragment with the highest identity (and lowest E-value) was chosen and tabulated in Table 4.10. As shown, most (n=8) of the translated nucleotide sequences obtained from the cow farm soil sample are similar to TEM-1 with identities of 100%, except for that of E12, which shows 100% identity with TEM-116. On the contrary, most (n=5) translated nucleotide sequences obtained from the chicken farm soil sample were identical to TEM-116 except for EMB2, which showed 100% identity with TEM-1.

Table 4.10 BLASTx analysis of *bla*-TEM homologous genes of bacteria isolated from cow and chicken farm soil samples.

Soil sample	Most similar gene fragment	Percentage of similarity (Identity)	Closest known microorganism	Recombinant clones	Accession no.
Cow farm	TEM-1	100	*Escherichia coli*	M1, M2, M3, M4, L6, L7, E11, E14	AKE33362.1
	TEM-116	100	*Aeromonas hydrophila*	E12	ACN82383.1
Chicken farm	TEM-1	100	*Escherichia coli*	EMB2	AKE33362.1
	TEM-116	100	*Aeromonas hydrophila*	EMB1, LB1, LB2, MAC1, MAC2	ACN82383.1

4.10.3 BLASTx Analysis of Recombinant Plasmids Containing *bla*-TEM Homologous Genes from Cow and Chicken Farm Soil Samples

A total of 72 positive recombinant clones were obtained for the cow farm soil ecotype (Table 4.11). BLASTx analysis of the recombinant sequences showed that most of the translated sequences (n=70) were similar to TEM-1 and only two showed similarities to TEM-116. Of the 70 sequences that were similar to TEM-1, 28 sequences were identical to TEM-1 while 42 sequences were 99 % similar to TEM-1. Of the two sequences that were similar to TEM-116, one was identical to TEM-116 while the other showed 99 % similarity to TEM-116. Similarly, 37 out of 50 positive recombinant clones for the chicken farm soil ecotype demonstrated high similarities to TEM-1 with identities of 99-100% (Table 4.12). Of the 37 sequences that were similar to TEM-1, 11 sequences were identical to TEM-1 while the remaining 26 sequences were 99 % similar to TEM-1. Conversely, of the 13 sequences that were similar to TEM-116, four sequences were identical to TEM-116 while the remaining nine sequences were 99 % similar to TEM-116.

Table 4.11 BLASTx analysis for 72 translated DNA fragments within the recombinant plasmids for the cow farm soil ecotype.

Most similar gene fragment	Percentage of similarity (Identity)	Closest known bacteria species	Recombinant clones	Accession no.
TEM-1	100	*Escherichia coli*	pCF1, pCF5, pCF7, pCF16, pCF20, pCF21, pCF22, pCF25, pCF29, pCF31, pCF32, pCF37, pCF38, pCF39, pCF41, pCF42, pCF45, pCF46, pCF47, pCF48, pCF51, pCF53, pCF54, pCF56, pCF62, pCF63, pCF66, pCF69	AKE33362.1
	99	*Escherichia coli*	pCF2, pCF3, pCF4, pCF6, pCF8, pCF9, pCF10, pCF11, pCF12, pCF13, pCF14, pCF15, pCF17, pCF18, pCF23, pCF24, pCF26, pCF27, pCF28, pCF30, pCF34, pCF36, pCF40, pCF43, pCF44, pCF49, pCF50, pCF52, pCF55, pCF57, pCF58, pCF59, pCF60, pCF61, pCF64, pCF65, pCF67, pCF71, pCF72, pCF73, pCF74, pCF76	
TEM-116	100	*Aeromonas hydrophila*	pCF75	ACN82383.1
	99	*Aeromonas hydrophila*	pCF77	

Table 4.12 BLASTx analysis for 50 translated DNA fragment within the recombinant plasmids for the chicken farm soil sample.

Most similar gene fragment	Percentage of similarity (Identity)	Closest known bacteria species	Recombinant clones	Accession no.
TEM-1	100	*Escherichia coli*	pCH15, pCH19, pCH27, pCH29, pCH37, pCH38, pCH40, pCH41, pCH44, pCH46, pCH47	AKE33362.1
	99	*Escherichia coli*	pCH11, pCH12, pCH13, pCH14, pCH16, pCH17, pCH18, pCH21, pCH22, pCH23, pCH24, pCH25, pCH28, pCH30, pCH31, pCH32, pCH33, pCH35, pCH36, pCH39, pCH42, pCH43, pCH45, pCH48, pCH49, pCH50	
TEM-116	100	*Aeromonas hydrophila*	pCH5, pCH7, pCH8, pCH9,	ACN82383.1
	99	*Aeromonas hydrophila*	pCH1, pCH2, pCH3, pCH4, pCH6, pCH10, pCH20, pCH26, pCH34	

4.11 *bla*-TEM Variant Analysis

As shown in Table 4.13, a total of 122 translated sequences were obtained in which 39 were identical to TEM-1, five were identical to TEM-116, seven resemble TEM-176 and five resemble TEM-215. The remaining 66 translated sequences have at least one amino acid substitution at different position that are not previously reported (www.lahey.org/studies).

Table 4.13 Comparison of amino acid residue substitutions of soil recombinants against *bla*-TEM-1.

Position	**44**	**45**	**47**	**48**	**51**	**52**	**53**	**72**	**74**	**77**	**82**	**83**	**84**	**86**	**88**	**90**	**96**	**99**	**100**	**109**	**111**	**116**	**117**	**121**
bla-TEM-1	V	G	I	E	L	N	S	F	V	C	S	R	V	A	Q	Q	H	Q	N	T	K	G	M	E
Soil recombinants	A (1)	R P V (4)	T (1)	G (2)	P (2)	D (1)	G (1)	S (1)	I (2)	R (1)	P (1)	H (3)	I (10)	T (1)	H (1)	R (1)	R (2)	P (1)	D (1)	A (1)	R (2)	D (1)	V (1)	G (1)
Position	**122**	**124**	**127**	**136**	**138**	**143**	**148**	**150**	**151**	**153**	**156**	**160**	**172**	**177**	**178**	**182**	**184**	**188**	**189**	**192**	**201**	**204**	**219**	**222**
bla-TEM-1	L	S	I	N	L	G	L	A	F	H	G	T	A	E	R	M	A	T	T	K	L	R	P	R
Soil recombinants	S (1)	R G (2)	T (1)	S (1)	R (2)	E (1)	P (2)	T (1)	S (1)	R (6)	V (1)	S (1)	V (1)	G (1)	H C (3)	T (3)	V (12)	A (1)	A (1)	I (3)	P (1)	Q (1)	L (1)	X (1)
Position	**223**	**224**	**225**	**233**	**235**	**246**	**247**	**248**	**251**	**252**	**259**	**260**	**262**	**266**	**269**	**272**								
bla-TEM-1	S	A	L	D	S	I	A	A	P	D	V	V	Y	S	T	E								
Soil recombinants	A (1)	V G (17)	F (2)	V (1)	P (1)	F (1)	V (1)	G (1)	S (2)	G (2)	A (1)	A (1)	H (2)	G (1)	A (1)	V (1)								

4.12 Phylogenetic Analysis of *bla*-TEM Homologous Gene Sequences

4.12.1 Cow Farm Soil Ecotype

The phylogenetic tree was constructed based on neighbour-joining method (Saitou and Nei, 1987). A total of 72 *bla*-TEM translated gene sequences and 30 known *bla*-TEM representatives retrieved from the protein database were used for the construction of the tree (Figures 4.12 to 4.16). The tree was collapsed into five subgroups (CFA-1 to CFA-5) for further analysis. Generally, most of the *bla*-TEM representatives and the translated sequences clustered among themselves suggesting the existence of a highly divergent *bla*-TEM variants. A total of 243 amino acid positions were obtained in the final dataset.

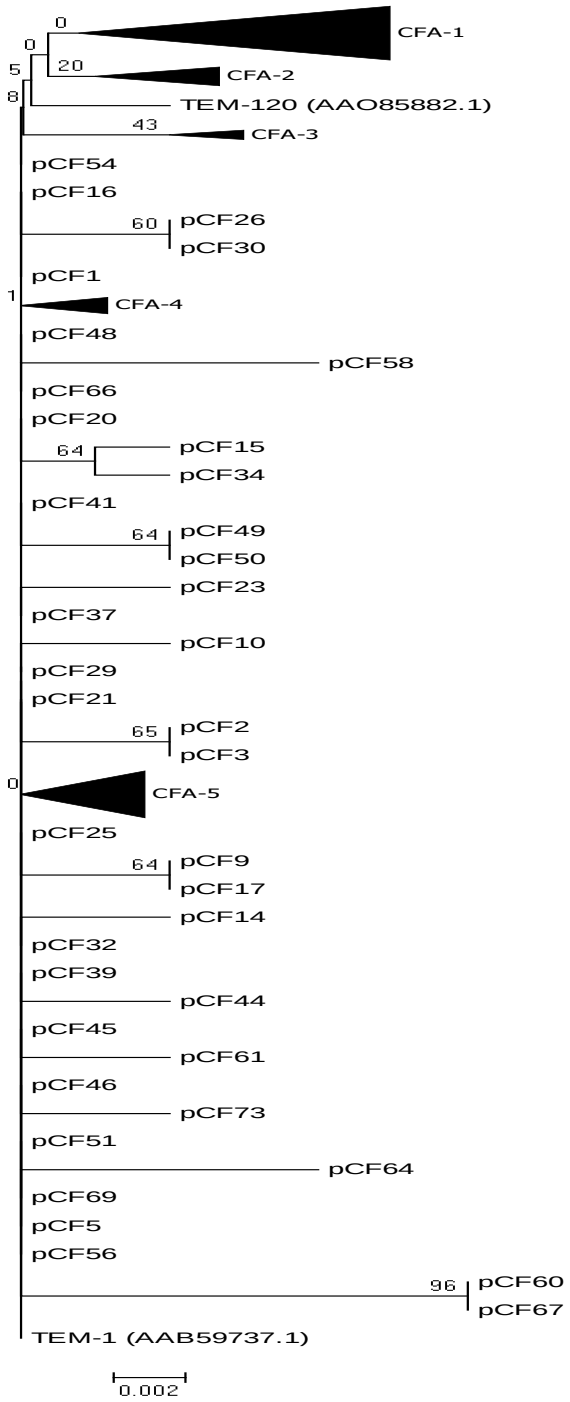

Figure 4.12 Overview of the *bla*-TEM phylogeny for the cow farm soil ecotype. Positive recombinants were designated as pCF while TEM representatives with accession number in parenthesis were retrieved from the protein database.

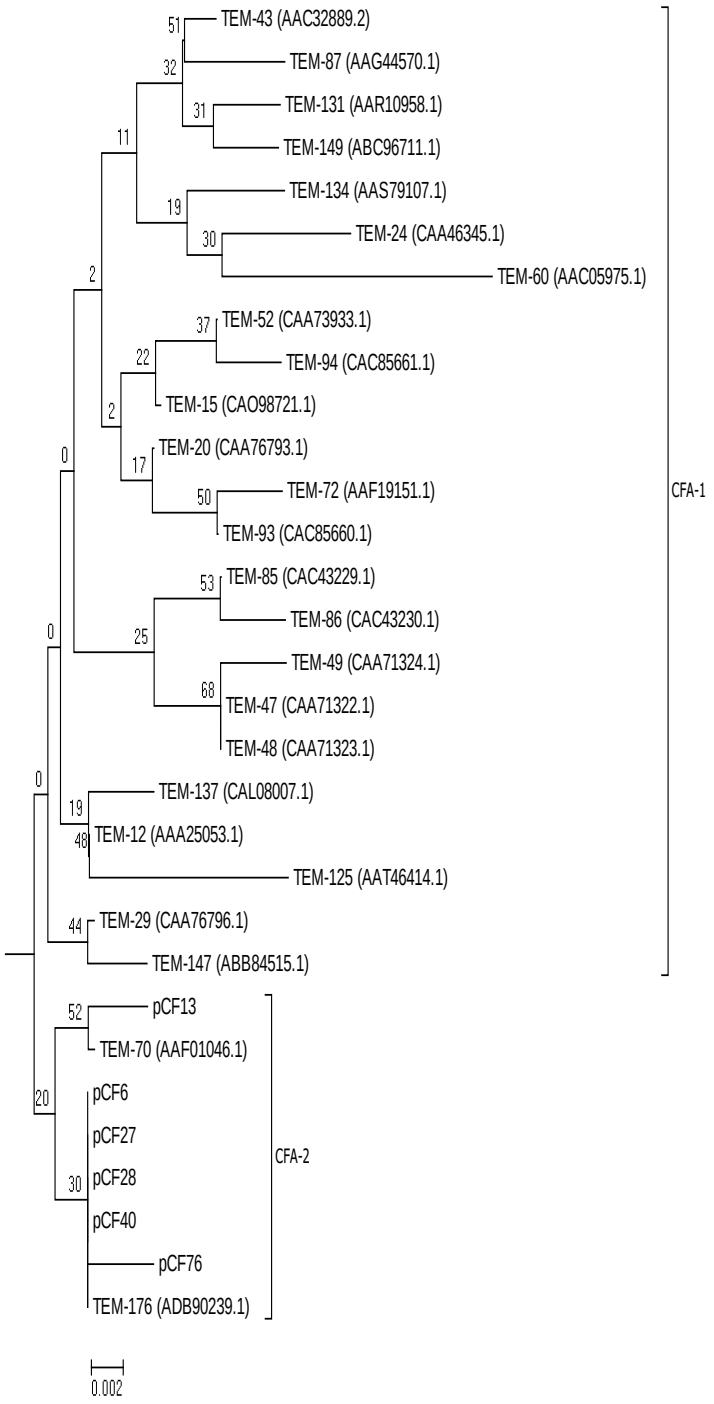

Figure 4.13 Phylogeny of Subgroups CFA-1 and CFA-2. These subgroups comprise of six translated gene sequences, and 25 known *bla*-TEM representatives.

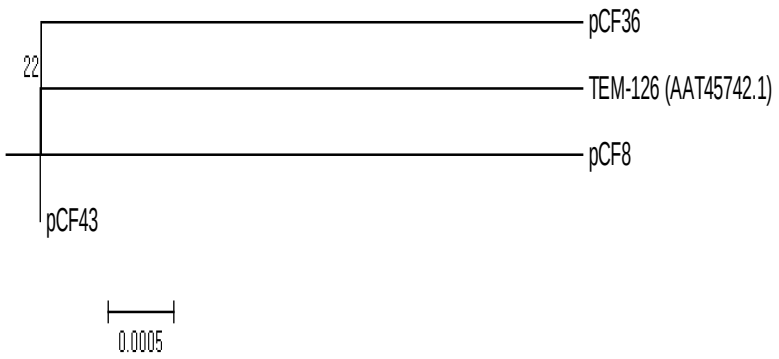

Figure 4.14 Phylogeny of Subgroup CFA-3. This subgroup comprises of three translated gene sequences and one *bla*-TEM representative.

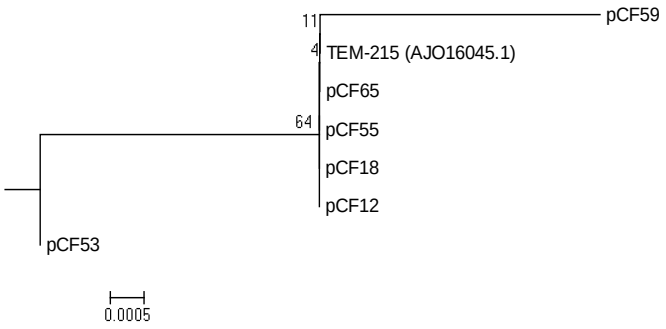

Figure 4.15 Phylogeny of Subgroup CFA-4. This subgroup comprises of six translated gene sequences and one *bla*-TEM representative.

Figure 4.16 Phylogeny of Subgroup CFA-5. This subgroup comprises of 18 translated gene sequences and 2 *bla*-TEM representatives.

4.12.2 Chicken Farm Soil Ecotype

The phylogenetic tree was constructed based on neighbour-joining method (Saitou and Nei, 1987). A total of 50 *bla*-TEM translated homologous gene sequences and 30 known *bla*-TEM representatives were retrieved from the Genbank database and used for the construction of the tree (Figures 4.17 to 4.19). The tree was collapsed into three subgroups (CHA-1 to CHA-3) for further analysis. Generally, most of the *bla*-TEM representatives and the translated sequences clustered among themselves suggesting the existence of a highly divergent *bla*-TEM variants. A total of 242 amino acid positions were obtained in the final dataset.

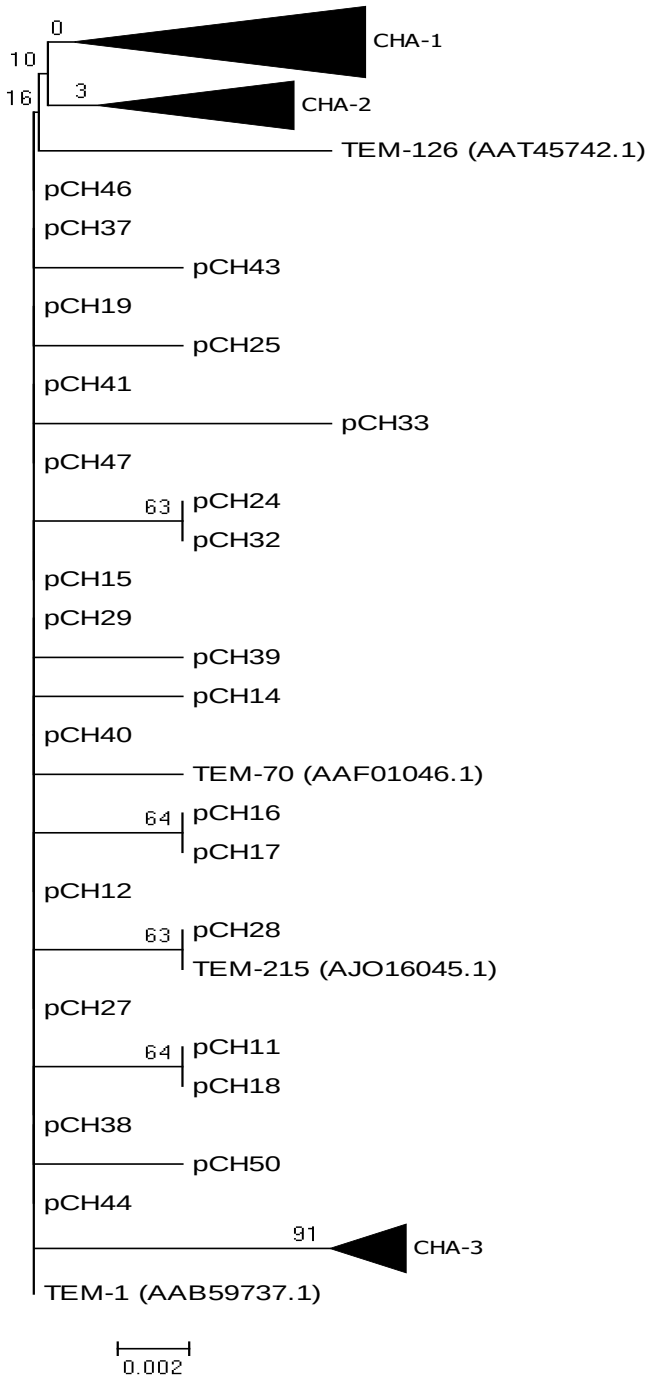

Figure 4.17 Overview of *bla*-TEM phylogeny for the chicken farm soil ecotype. Positive recombinants were designated as pCH while TEM representatives with accession number in parenthesis were retrieved from the protein database.

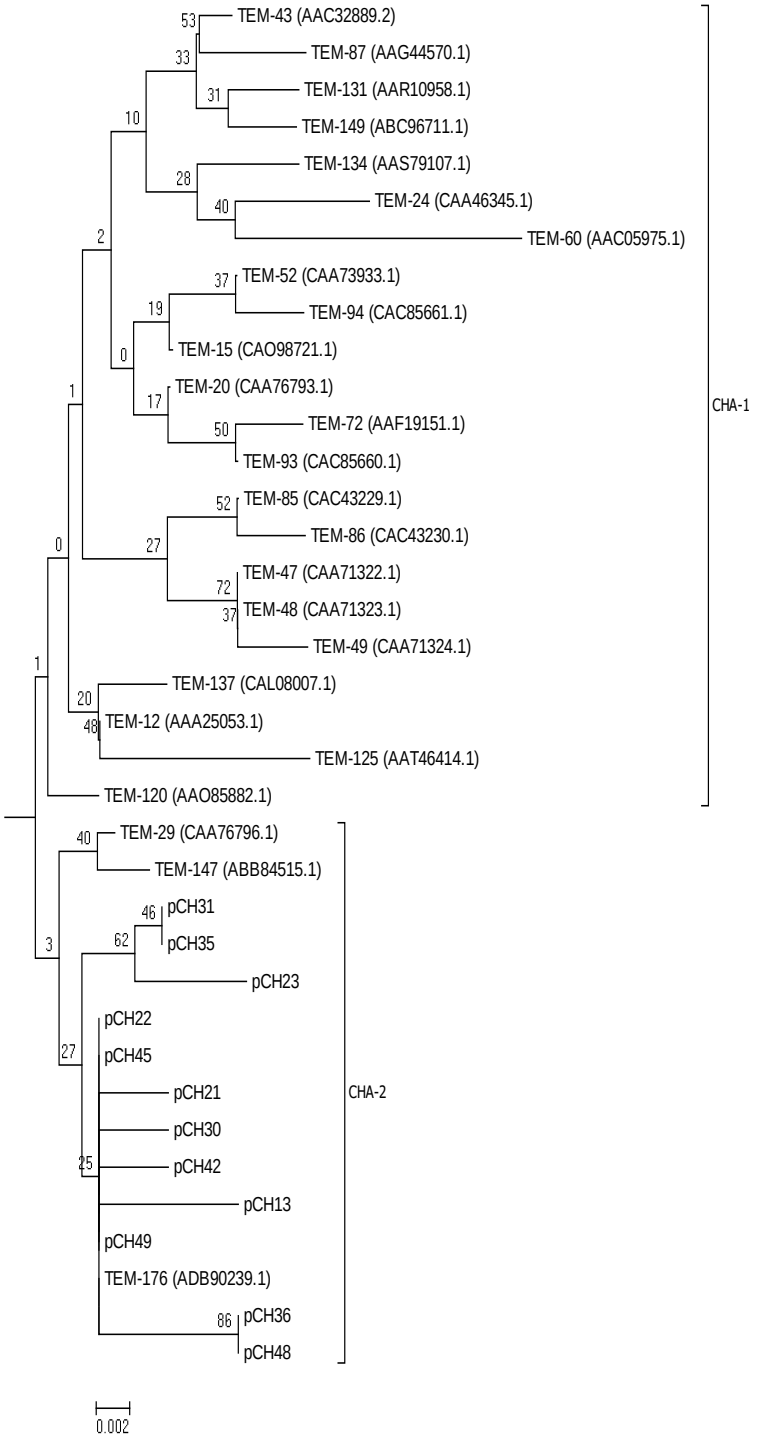

Figure 4.18 Phylogeny of Subgroups CHA-1 and CHA-2. These subgroups comprise of 12 translated gene sequences and 25 known *bla*-TEM representatives.

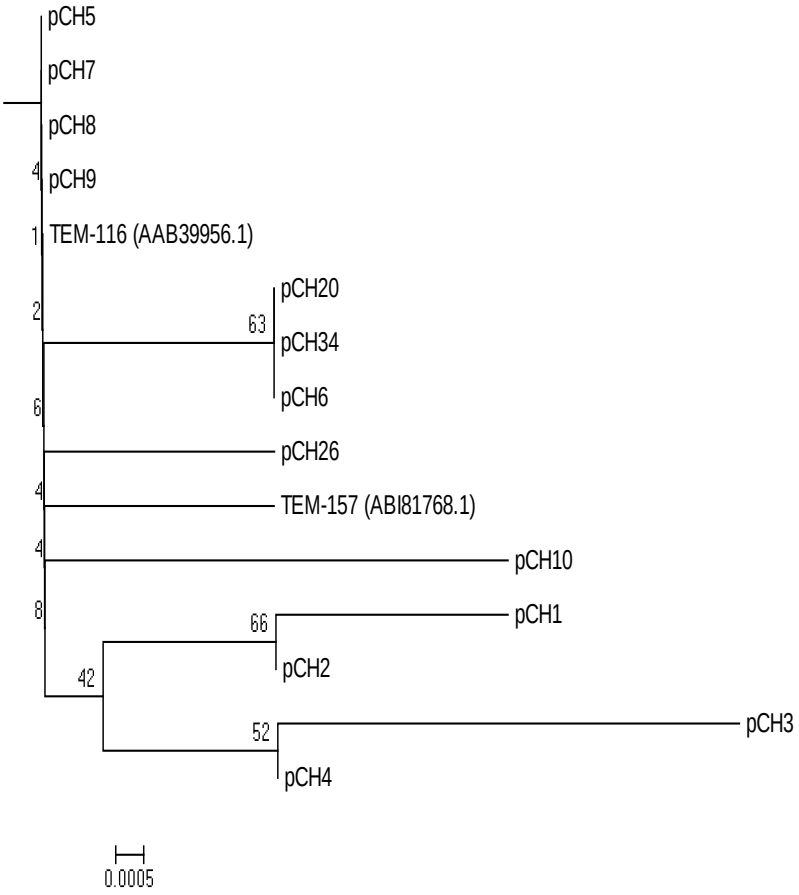

Figure 4.19 Phylogeny of Subgroup CHA-3. This subgroup comprises of 13 translated gene sequences and two known *bla*-TEM representatives.

CHAPTER 5

DISCUSSION

5.1 Soil Ecotypes in Animal Farm Settings

In recent years, the dissemination of ESBL-producing bacteria has become an evolving issue that poses huge impact not only to the health care sector but also in food safety and environmental integrity (Geser et al., 2012). Overuse of antibiotics in health care and agriculture has amplified the prevalence of acquired antibiotic resistance in bacteria, especially in the environmental settings (Davies and Davies, 2010). Leverstein et al. (2011) demonstrated that the usage of antimicrobial drug in livestock sector to prevent, control, and treat infections and to improve growth and feed efficiency, has contributed significantly to this antibiotic resistance crisis.

In this study, bacteria isolated from cow and chicken farm soils were investigated. As shown in Table 4.2, a total of fifteen morphologically different bacterial isolates were obtained from the cow and chicken farm soil samples. In cattle, antimicrobials such as amoxicillin, gentamicin, penicillin, quinolones, erythromycin, tylosin, tilmicosin, novobiocin, and tetracycline are widely used for the treatment and prevention of bovine pneumonia, diarrhoea, and shipping fever (McEwen and Fedorka-Cray, 2002). For the treatment of more severe

diseases such as pneumonia, the second-, third-, and fourth-generation cephalosporins are being used as the last resort. In poultry, antibiotics used for therapeutic reasons are usually administered through water whereas for growth-promoting purposes, antibiotics are added in the feed (Hofacre et al., 2013). The most commonly used antibiotics are penicillins, quinolones, tetracyclines, macrolides, aminoglycosides, the sulphonamide/trimethoprim combination, polymyxins, and tiamulin (Marshall and Levy, 2011).

Geser et al. (2012) reported that the dominant species of bacteria that are generally found in various animal farms are *Kluyvera* spp., *Serratia marcescens, Pseudomonas cepacia, Staphylococcus faecalis, Gemella haemolysins, Leuconostoc, Staphylococcus aureus, Micrococus varians, Escherichia, Salmonella, Klebsiella, Enterobacter, Streptococcus* and *Staphylococcus*. The results (Tables 4.6 and Table 4.9) obtained in this study are mostly comparable with the research done by Geser et al. (2012) in which the bacteria isolated in this study mostly belonged to those dominant bacteria. Similar study was done by Tymczyna et al. (1999) in which they investigated the groundwater and soil samples taken from the surrounding of a swine farm and revealed the presence of *E. coli*, *Streptococcus, Clostridium perfringens, Pseudomonas* spp., *Bacillus subtilis* and *Proteus* spp. *E. coli, Citrobacter, Enterobacter, Klebsiella*, and *Pseudomonas* spp are considered as opportunistic pathogens that can cause mastitis, enteritis, pneumonia, and urinary tract infections in dairy cattle (Aslan et al., 2002).

The difference in the diversity of bacteria isolated from each farm could be affected by the physiochemical properties of the soil samples. A study conducted by Noah and Robert (2005) suggested that among the physiochemical properties of soil, soil pH is the best indicator of bacterial growth and diversity. Soil with lower acidity generally have higher bacterial growth as compared to higher acidity soil (Noah and Robert, 2005). This theory is in agreement with the results obtained in this study as shown in Table 4.1 whereby the cow farm soil, which has a pH value of 7.04, was able to isolate more morphologically different bacteria as compared to the chicken farm soil, which has a pH value of 5.53. The higher nitrogen and organic carbon content in the cow farm soil was probably due to the accumulation of organic matter, leaf matter and manure, and they play an important role in providing favourable environment for microbes to grow. Possible explanation is that soil microorganisms process plant deposits and residues into soil organic matter, a direct and stable reservoir of carbon and nitrogen that consists of living and dead organic materials subject to rapid biological decomposition. Similar results with such positive correlation of organic carbon to nitrogen and phosphorus have been reported by Rezende et al. (2004) and Verma and Shweta (2011).

5.2 Bacterial Identification Using API 20 E and 16S rDNA Sequencing

In this study, the accuracy and reliability of bacterial identification were compared using the phenotypic identification method (API 20 E) and a molecular identification method (16S rDNA sequencing). Significant differences between these two identification methods were observed as shown in Tables 4.6 and 4.9 whereby the majority (8 out of 15) of the bacterial isolates could not be accurately identified. The main problem of the API 20 E system is that the existing databases are limited and results seem reliable only when species assignment is reported as excellent or very good identification (Bosshard et al., 2006). This is evident for bacterial isolates M4, L6, E12 and EMB2 whereby their identification was reported as excellent and the results were equivalent to that of 16S rDNA sequencing. Also, the API 20 E test was able to identify isolates LB1, LB2, MAC2 and EMB1 up to the family level correctly but not to the genus level. Similar finding were reported by O'Hara et al. (1992). The studies by Smith et al. (1972) and Washington et al. (1972) demonstrated incorrect identification of *Enterobacteriaecae* by API 20E due to anomalous reactions by API and/or atypical strains. Another common limitation of the API 20 E system is that the results obtained depend on visual observation of colour change, which is sometimes difficult to be noted (for example, the difference between light red and rose). These might be the sources of error which may lead to ambiguous or false results.

For further verification of the identity of the bacterial isolates, all the bacterial isolates identified using biochemical reactions were analysed using the molecular approach. Molecular method is based on the amplification of 16S rRNA gene and it has distinctive benefits over the phenotypic identification method by API 20 E. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences that are useful for identification of bacteria (Kolbert and Persing, 1999; Filipe. et al., 2010). Besides, public databases such as GenBank, EMBL and DDBJ covering the whole spectrum of phylogenetic diversity are readily available. Furthermore, novel or undescribed species can be assigned to a group of related bacteria and the results are in general unambiguous and not dependent on strain variation or individual interpretation. However, the quality of public databases, such as GenBank, is critical. Sequences are deposited independent of their quality, e.g., regardless of the correct assignment, the length of the sequence, or the number of ambiguous nucleotides. Of particular concern is that sequences in public databases may be assigned to a designation which is possibly no longer valid due to taxonomic changes or which has never been officially published before. Despite all these minor limitations, 16S rDNA sequencing does not lead to false identification, and with some knowledge about taxonomy, a sequence can unambiguously be assigned. As a result, 16S rDNA sequencing has become prevalent in medical microbiology as a rapid and cheap alternative to phenotypic methods of bacterial identification (Jill, 2004).

Although both identification has its pros and cons, 16S rDNA sequencing is more labourious than phenotypic identification methods. Despite its drawbacks, the API 20 E test can still be used for preliminary identification. However, if identification is of low quality and the result seems to be not reliable, then the isolate should be subjected to 16S rDNA sequencing if accurate identification is of concern.

5.3 Antibiotic Susceptibility Patterns and ESBL Detection

All the 15 bacterial isolates from the cow and chicken farm soil samples showed great resistance towards the 16 antibiotics tested in this study. Based on the results shown in Tables 4.4 and 4.5, three of the isolates were characterised as NSBL whereby they were resistant to penicillins but were susceptible to other classes of β-lactam antibiotics due to production of TEM-1 or SHV-1 enzymes (Jacoby and Medeiros, 1991). However, overproduction of these narrow-spectrum enzymes may in turn confer resistance to other classes of of β-lactam antibiotics (Lartigue et al., 2002; Nelson et al., 2003; Tristram et al., 2005; Beceiro et al., 2011). Point mutations of TEM and SHV enzymes may also produce inhibitor-resistant enzymes such as the IRT (Canton and Coque, 2006). This can be observed in three of the isolates whereby they were resistant to penicillins and susceptible to other β-lactam antibiotics, but were not impeded by β-lactamase inhibitors such as clavulanic acid (Knox, 1995).

ESBLs may also derive from TEM-1 and SHV-1 and exhibit broad resistance towards different generations of cephalosporins but remain susceptible to β-lactamase inhibitors (Sirot et al., 1987). This phenotype was seen in three of the isolates and is consistent with other studies on the prevalence of ESBL in animal farms (Blanc et al., 2006; Wittum et al., 2010; Horton et al., 2011). Complex mutant TEM (CMT) phenotype was also observed in one out of 15 bacterial isolates in this study. CMT may derive from TEM-1 or TEM-2 and under selective pressure, this type of enzymes confers resistance to most β-lactam antibiotics and β-lactamase inhibitors but susceptibility to cephamycins and carbapenems remains. However, information on CMT is limited and low prevalence of CMT isolates was also reported by Henquell et al., (1995).

In addition, the prevalence of isolates carrying pAmpC β-lactamase has been reported worldwide in extended spectrum cephalosporin-resistant Gram-negative bacteria. pAmpC β-lactamase hydrolyses broad and extended spectrum cephalosporins but is not inhibited by β-lactamase inhibitors such as clavulanic acid (Thenmozhi et al., 2014). This phenotype was observed in bacterial isolates LB1, LB2, MAC1, MAC2, and EMB1. A notable finding in this study is that none of the bacterial isolates was resistant to imipenem, which is a carbapenem antibiotic. Carbapenems have been shown to be the stable antimicrobial agents and are less affected by many common antibiotic resistance mechanisms than other β-lactam antibiotics (Paterson, 2009). The persistent exposure of bacterial strains to a multitude of β-lactams has induced dynamic and continuous production and mutation of β-lactamases in these bacteria, expanding their activity even against the newly developed β-lactam antibiotics. Since the

effectiveness of non β-lactam agents is not affected by β-lactamases, they have always been considered alternative antibiotics against diseases caused by β-lactamases (Lili et al., 2015).

Beta-lactam antibiotics act by binding to cell wall synthesis enzymes known as penicillin-binding proteins (PBPs), thereby inhibiting peptidoglycan synthesis (Ghuysen, 1991). Inhibition of PBPs weakens the cell wall, resulting in inhibition of cell growth and frequently cell death. The three mechanisms of β-lactam resistance are reduced access to the PBPs, reduced PBP binding affinity, and destruction of the antibiotic through the expression of β-lactamase (enzymes that bind and hydrolyse β-lactams). In Gram-positive bacteria, antibiotics have free access to the bacterial cytoplasmic membrane, where the PBPs are located. In the Gram-negative bacteria, they have developed mechanisms to prevent the antibiotic from reaching its intracellular target by decreasing the uptake of the antimicrobial molecule. The β-lactam molecules are sufficiently excluded from this periplasmic space by either reduced entry or increased efflux. If β-lactamase molecules are severely concentrated, even a relatively weak β-lactamase can confer high levels of resistance (Livermore, 1992).

As the usage of β-lactam antibiotics is not closely monitored, prescriptions are mostly issued without reliable susceptibility data, and this could lead to antibiotics being severely misused. According to the Institute of Medicine (1999) in the US, the use of antibiotics in food animals was mainly utilised for

therapeutic, prophylactic, and sub-therapeutic purposes. Antibiotics are often used to treat a single animal with clinical disease or a large group of animals. Nevertheless, these uses are frequently indistinct; definitions of each type of use vary, and the approaches are often applied concurrently in livestock populations (Institute of Medicine, 1999).

Most antibiotics that were administered in livestock required veterinary prescriptions, although individual treatment decisions are often made by unqualified farm workers in accordance with guidelines provided by veterinarians (Sawant et al., 2005; Raymond et al., 2006). This may partially explain the presence of complex phenotypes such as ESBL, CMT, and pAmpC found in this study. Comparison with other studies would not necessarily give the same resistance patterns, as each farm has a different management regime such as housing of the cattle, the type of cattle, their nutrition and veterinary treatments, disposal of antibiotic-contaminated mastitic milk, use of disinfectants and other antimicrobials, and slurry handling. Hence, rational use of antibiotics and the establishment of effective management of food producing animal are necessary.

5.4 Multidrug-Resistant *Enterobacteriaceae*

MDR *Enterobacteriaceae* has been frequently reported in different parts of the world as an emerging treatment problem (Kamlesh et al., 2015). MDR refers to the ability of bacteria to resist different classes of antibiotics that are structurally different and have different molecular targets (Nikaido, 2009). Isolates showing resistance to two or more classes of antibiotics are designated as MDR bacteria. In this study, three classes of non β-lactam agents (aminoglycosides, fluoroquinolones and tetracycline) were tested against the isolates. Eight bacterial isolates designated as M1, M2, M4, L6, L7, E11, E14 and EMB2 were considered MDR (Table 4.5). Aminoglycosides are very important group of antibiotics with activity against many Gram-negative rods. The most common mechanism of aminoglycoside resistance is the enzymatic modification of antibiotic molecule (Gonzalez and Spencer, 1998). In this study, nine of the bacterial isolates were resistant to gentamicin, indicating the possible presence of genes encoding aminoglycoside-modifying enzymes such as acetyltransferases, nucleotidyltransferases and phosphotransferases (Table 4.5) (Ramirez and Tolmasky, 2010).

In order to preserve carbapenems, which act as last resort antimicrobials towards various infections, any antibiotic options that may be available to treat infections caused by β-lactamases should be considered. As non β-lactam agents are not affected by β-lactamases, fluoroquinolones have been considered as alternatives for infections caused by β-lactamases. In the present study, two of the bacterial isolates were resistant to ciprofloxacin and three were resistant to

norfloxacin (Table 4.5). Although the resistance to fluoroquinolones was low in this study, it is inevitable that fluoroquinolone-resistant bacteria are present in animal farms. Several studies have shown that ciprofloxacin-resistant *Enterobacteriaceae* were present in manure and soil (Leal et al., 2012; Moraru et al., 2012; Pourcher et al., 2014). Continuous selective pressure exerted by β-lactam compounds is a risk factor for selection of resistant ESBL-producing strains (Rodríguez-Baño, 2004; Helfand and Bonomo, 2005). The associated co-resistance of these isolates to unrelated antimicrobials, such as aminoglycosides, tetracyclines, chloramphenicol, trimethoprim, sulphonamides, and quinolones, may play an important role in the spread and preservation of these isolates. High rate of tetracycline resistance was observed in the present study whereby nine of the bacterial isolates were resistant. In this scenario, alternative antimicrobial compounds are needed to cover infections in which these isolates are increasingly involved.

The resistance patterns varied significantly between the two farms, but were highly related among isolates within the same farm. As shown in Table 4.5, bacterial isolates from the cow farm showed higher resistance towards the MDR antibiotics as compared to those from the chicken farm and vice versa for β-lactam antibiotics. Despite the role of natural selective pressure and indigenous antibiotic producers in the soil, this finding also provides an insight on the use of various antibiotics in different aspects within these farms and the acquisition of resistance genes. From the survey done by Mainda et al., (2015), prevention and treatment of cattle diseases were the main motives for using

antibiotics in the farms. The most common disease reported in animal farms is mastitis, followed by diarrhoea and skin diseases. Mastitis is mostly treated with an intra-mammary infusion while treatments for diarrhoea are based on the injectable sulpha-based antibiotics such as sulphadimidine, sulphazine and trimethoprim sulphate. Nevertheless, tetracyclines and penicillins are the two main antibiotics that are used in various combinations interchangeably across all diseases (Mainda et al., 2015). In contrast, the bacterial isolates from the chicken farm conferred lower resistance towards the MDR antibiotics tested. A major reason for this is that penicillin and tetracycline are rarely used for stimulating growth in poultry production and these antibiotics are not approved by Food and Drug Administration (FDA) for use in combination with monensin. Some penicillins are used as the diluent for Marek's disease vaccine and tetracyclines are used for treatment of diseases and for improving suboptimal performance of birds (Van den Bogaard et al., 2001).

5.5 Prevalence and Polymorphism of ESBL Genes in Soil Bacteria from Cow and Chicken Farms

5.5.1 Culture-dependent Approach

In this study, the prevalence and diversity of β-lactamase genes were characterised using nine sets of primers targeting the conserved regions of the β-lactamase genes. Among the nine β-lactamase genes (TEM, SHV, ACC, CF, DHA, MA, OXA, PER, and VEB) tested, the resistance genes were only detected in *bla*-TEM and *bla*-SHV gene families. The distribution of TEM variants between the two farms showed opposing results. According to the BLASTx analysis results, about 89% of the bacterial isolates from the cow farm showed 100 % identity to TEM-1 gene variant and the remaining 11 % belonged to TEM-116 gene variant with 100 % identity (Table 4.10). On the other hand, about 83 % of the bacterial isolates from the chicken farm showed 100 % identity to TEM-116 gene variant and 17 % belonged to TEM-1 gene variant (Table 4.10). The distributions of the TEM variants correlate with the antibiotic resistance patterns whereby bacterial isolates belonging to TEM-116 gene variant conferred higher resistance towards different classes of antibiotics (penicillins, cephalosporins, fluoroquinolones, aminoglycosides, carbapenem, and monobactam) (Table 4.5). This further validated the studies done by various researchers whereby the mutations on gene encoding TEM-1 could give rise to the production of broad-spectrum enzymes that confer resistance to other classes of β-lactam antibiotics (Lartigue et al., 2002; Nelson et al., 2003; Tristram et al., 2005; Beceiro et al., 2011). In addition to those encoded by the TEM-116 gene, the bacteria may also have other antibiotic resistance

mechanisms or the expression in soil bacteria increased the antibiotic resistance spectrum to third-generation cephalosporins (Sandrine et al., 2008).

5.5.2 Culture-independent Approach

Among the nine ESBL genes tested, two types of β-lactamase genes (*bla*-TEM and *bla*-SHV) were present using the culture-independent approach, indicating the possibility of restricted soil diversity in this study as compared with clinical settings, which are commonly encountered with a more diverse types of β-lactamase genes. However, a high percentage of variants of each β-lactamase gene present was observed using the culture-independent approach as shown in Tables 4.11 and 4.12. This can be seen for the cow farm soil sample whereby about 58 % of the recombinants were 99 % similar to TEM-1 and 1 % of the recombinants were 99 % similar to TEM-116. Recombinants that were not 100 % identical to the respective gene fragments were considered as different variants of the TEM family. Similarly, a high degree of variants was also seen for the chicken farm soil sample whereby about 52 % of the recombinants were 99 % similar to TEM-1 and 18 % were 99 % similar to TEM-116. These results suggest particularly high polymorphisms within *bla*-TEM genes, further confirming the importance of using the culture-independent approach to avoid biases related to cultivation and selection methods (Jacoby, 2006).

5.5.3 Culture-dependent versus Culture-independent Approaches

The diversity of *bla*-TEM gene variants was not significantly different between the two soil samples (cow and chicken) when comparisons were made using bacterial genes from the culture-dependent approach. In contrast, significant differences were demonstrated when *bla*-TEM genes of microorganisms were characterised using the culture-independent approach, in which high degree of *bla*-TEM gene variants was observed (Tables 4.11 and 4.12). As cultivable bacteria represent only less than 1 % of the total bacteria, culture-independent strategy is strongly recommended in order to further investigate the diversity of *bla*-TEM genes in soil bacterial community. A total of 78 different *bla*-TEM gene variants which were not previously described were characterised in this study, indicating a particularly high polymorphism level in this family of genes. Correlation between point mutations and polymorphisms could elucidate the likelihood that soil bacteria may act as the reservoir of antibiotic resistance genes (Riesenfeld et al., 2004).

5.6 *bla*-TEM Variant Analysis

To further investigate and validate the diversity of *bla*-TEM variants in this study, the translated *bla*-TEM gene sequences obtained were compared to that of known TEM β-lactamases (Table 4.13). Among the commonly reported *bla*-TEM variants, TEM-1 β-lactamase was the first enzyme discovered, and is commonly detected in clinical isolates (Bastarrachea, 1998). When point mutations occur in the *bla* genes, β-lactamases may develop broader resistance

towards other generations of cephalosporins, with one to four amino acid substitutions (Jacoby and Medeiros, 1991; Bastarrachea, 1998).

In the present study, a sum of 122 translated sequences (72 for cow farm and 50 for chicken farm) were compared to TEM-1 by using the amino acid residue numeration in the Lahey database (www.lahey.org/studies.) A total of 243 unique amino acid sequences were obtained and the translated sequences were ensured to be free from sequencing error, presence of internal stop codon, and events of insertion or deletion of the reading frame. All of the translated sequences corresponded to the forward and reverse overlapping fragments, thus, eliminating any possibility of sequencing errors.

Among the 122 translated sequences, 39 were identical to TEM-1 and five were identical to TEM-116. As shown in Table 4.13, the remaining 78 translated sequences demonstrated 64 different amino acid substitutions with up to three amino acid residue modifications. Seven translated sequences (pCF6, pCF27, pCF28, pCF40, pCH22, pCH45, and pCH49) resemble TEM-176, whereby a change of amino acid from alanine (A) to valine (V) at position 224 was observed. Five translated sequences (pCF12, pCF18, pCF55, pCF65, and pCH28) resemble TEM-215, whereby a change of amino acid from histidine (H) to arginine (R) at position 153 was observed. Besides, nine translated sequences (pCF77, pCH1, pCH2, pCH3, pCH4, pCH6, pCH10, pCH20, and pCH26) resemble TEM-116, which has an amino acid change at position 84 (valine to isoleucine) and 184 (alanine to valine). In addition to the changes, they also

showed other amino acid substitutions in positions that were not reported previously. Nevertheless, the remaining 57 translated sequences have at least one amino acid substitution at different positions that does not correlate with any of the reported substitution in the existing database (www.lahey.org/studies).

These substitutions may represent novel *bla*-TEM variants that have arisen through several mutational events. These undescribed substitutions may also be due to the downside of PCR-based selection of *bla*-TEM genes that does not exclude non-functional or imperfect genes such as pseudogenes (Sandrine et al., 2008). These data suggest that the possibilities of mutational events in enhancing or maintaining the *bla*-TEM enzyme activity are not limited and soil bacteria apparently have access to wide range of adaptive alleles and may act as a reservoir of resistance genes.

5.7 Phylogenetic Analysis of *bla*-TEM Homologous Gene Sequences

Phylogenetic analysis was carried out to investigate the inferred evolutionary relationships among the translated *bla*-TEM gene sequences. Generally, a ladder-type phylogenetic tree was observed for both soil samples, suggesting that consistency with a group of highly divergent variants (Figure 4.12 and 4.17). For cow farm soil sample, the phylogeny of Group CFA-1 comprises most of the known *bla*-TEM representatives (n=23) and is located far away from the root (TEM-1) in the tree (Figure 4.13). This observation seems to suggest that

they diverged long ago and are distantly related to the ancestral gene of TEM-1. A speciation event had occurred between TEM-70 and TEM-176 as shown in Group CFA-2 (Figure 4.13) suggesting that they arose from common ancestry. The translated gene sequence for pCF13 clustered with TEM-70 while sequence pCF76 clustered with TEM-176. This result is consistent with the mutagenesis analysis (Table 4.13) where the two protein sequences showed additional amino acid substitution at different position to that of the TEM representatives (pCF13 positions 204 and 224; pCF76 positions 156 and 224). Similar event could be observed in Group CFA-3 whereby sequences for pCF8, pCF36, and pCF43 clustered with TEM-126 (Figure 4.14). Group CFA-4 with sequences for pCF12, pCF18, pCF55, pCF59, and pCF65 clustered with TEM-215 (Figure 4.15). Similarly, Group CFA-5 with sequences for pCF75 and pCF77 clustered with TEM-116 (Figure 4.16). A polytomy pattern was also observed in these groups. This assumption details the idea that multiple new lineages have arisen from a single originating population at the same time, or near enough in time to be indistinguishable from such an event.

A total of 25 translated gene sequences did not cluster with any of the TEM representatives (Figures 4.12 to 4.16). This observation suggests that multiple rapid speciation events may have occurred at the same time. In this case, all the daughter lineages are equally closely related to one another. This is an indication that novel *bla*-TEM variants arose from the culture-independent community within the same soil ecotype. The remaining gene sequences (n=28) did not show any speciation event and were identical to TEM representative TEM-1.

The phylogenetic tree for *bla*-TEM protein sequences for the chicken farm soil ecotype resembles similar phylogenetic patterns that of the cow farm (Figure 4.17). The phylogeny of Group CHA-1 comprises most of known *bla*-TEM representatives (n=22) and is located far away from the root (TEM-1) in the tree (Figure 4.18). Nine translated gene sequences clustered with TEM-176 in Group CHA-2 signifying their relatedness. Gene sequence for pCH23, pCH31, and pCH35 resembles the sister group of TEM-176, suggesting that they may have arisen from a common ancestor. Thirteen translated gene sequences clustered with TEM-116 and TEM-157 in Group CHA-3 (Figure 4.19). This result is in line with the result obtained in alignment analysis in which these gene sequences are 99-100 % similar to TEM-116. This result seems to suggest that multiple rapid speciation events have occurred in the thirteen gene sequences. The remaining gene sequences (n=11) did not show any speciation event that results in identical sequence to TEM-1.

Potential pitfalls of using molecular data in constructing phylogenetic trees have been reported. Difficulties in inferring positional homology and low likelihood of recovering the correct phylogeny given certain patterns in the timing of speciation events are the common problems reported (Nadler, 1995). However, advancement in molecular technology and phylogenetic methods for the past decade has changed this conventional approach significantly. Phylogeny analyses using molecular approaches have revealed that the large availability of characters for comparison in inferring a relationship and utility of molecular data for modelling patterns of nucleotide substitution are now recognised as an important approach in forming the basic framework for comparative study of a

wide spectrum of subjects (Wilson, 1985; Penny et al., 1992). Considering the low probability of mismatches among *bla*-TEM genes, the low bootstrap values, and the consistency of the results in phylogenetic analysis with other alignment and mutagenesis analysis in this study, phylogenetic trees should be viewed as a provisional hypothesis that may vary depending on the acquisition of other improved or reliable data and analytical procedures.

5.8 Future Studies and Implications

Due to time and resource constraints, this study only focused on the *bla*-TEM genes. Future studies will involve the characterisation of *bla*-SHV genes and screening of other complex ESBL genes such as GES, BES, TLA, and SFO. These studies are crucial in generating a more comprehensive depiction of ESBLs in the soil environment. Besides, isoelectric focusing (IEF) technique can also be applied to determine the isoelectric point (pI) of the variants. Comparison to other known databases can be used to further characterise the variants of the *bla*-TEM gene (Sharma et al., 2010). Comprehensive studies on gene expression using advanced technology such as reverse transcription quantitative PCR (RT-qPCR) and hybridisation microarray would offer tremendous data in predicting gene function and expression levels (Anna et al., 2010). Identification of bacterial isolates can also be improved by using multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) (Lucia et al., 2005). Besides, the number of soil samples tested in each animal farm should also be increased in order to make this study more comprehensive. Since minimal comparable studies have been performed in Malaysia, collection

and study of diverse soil samples from different animal farms across various regions should be taken into consideration in order to make a conclusive statement regarding the diversity of ESBL genes in the soil ecotypes.

CHAPTER 6

CONCLUSIONS

The evolution of antibiotic-resistant bacteria is one of the most significant problems in modern medicine and poses a serious threat to human health. Fifteen bacterial isolates from the cow and chicken farm soil samples showed high spectrum of resistance towards the 16 antibiotics tested. Three bacterial isolates were characterised as NSBL, IRT and ESBL phenotypes, respectively; one isolate represents CMT phenotype; and five isolates represent pAmpC phenotype. The antibiotic resistance profiles between the two farms and among the isolates of the same farm provide an insight on the usage of various antibiotics in different aspects within these farms and the probable acquisition of resistance genes. Bacterial identification using API 20 E test and 16S rDNA sequencing has its own pros and cons respectively but both suggest that the isolates in this study belonged to the families *Enterobacteriaceae* and *Pseudomonadaceae*.

Significant differences were demonstrated when the *bla*-TEM genes of microorganisms were characterised using the culture-independent approach in which high degree of *bla*-TEM gene variants was observed. A total of 78 different *bla*-TEM gene variants with 64 different amino acid substitutions with up to three amino acid residue modifications were obtained, indicating a

particularly high polymorphism level in this family of genes. These substitutions may represent novel *bla*-TEM variants that have arisen through several mutational events.

Phylogenetic analysis and *bla*-TEM variant analysis demonstrated the presence of a group of diverse *bla*-TEM variants. It is important that extensive studies of microbial ESBL and other antibiotic resistance in natural environments such as soil are encouraged extensively as the knowledge gained will contribute to understanding the ecology of resistance genes in infectious diseases and in their natural microbial settings. Resistance genes reside in environmental reservoirs pose serious threat to human health if they were to migrate to clinical settings, and ultimately transferred to pathogenic microorganisms. These results can be used to enhance the understanding of the emergence and dissemination of novel antibiotic resistance from natural reservoirs to clinical settings, which may aid the development of inhibitors of resistance mechanisms and resistant bacteria.

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APPENDIX A

Preparation of Culture Media

Luria Bertani (LB) agar

Tryptone 10 g

Yeast extract 5 g

Sodium chloride 5 g

Agar 10 g

Distilled water to bring final volume to 1 litre

Eosin Methylene Blue Agar

Peptone 10 g

Lactose 5 g

Sucrose 5 g

Dipotassium phosphate 2 g

Agar 13.5 g

Eosin Y 0.4 g

Methylene blue 0.065 g

Distilled water to bring final volume to 1 litre

MacConkey Agar

Peptone 17.0 g

Proteose peptone 3.0 g

Lactose 10.0 g

NaCl 5.0 g

Crystal violet 1.0 mg

Neutral red 30.0 mg

Bile salts 1.5 g

Agar 13.5 g

Distilled water Add to make 1 litre

APPENDIX B

List of Apparatus and Consumables

Table B List of apparatus and consumables and the respective brand or manufacturer.

No	Apparatus and Consumables	Brand/Manufacturer
1	Autoclave machine	HIRAYAMA
2	Centrifuge machine, microcentrifuge, Nanodrop 1000	Thermo Electron Corporation
3	Electronic balance	Adventurer™ Pro
4	Incubator, Water bath	Memmert
5	Incubator shaker	Hettich Zentrifugen
6	Laminar flow hood	Isocide™
7	PCR machine	Biometra
8	Spectrophotometre	BIO-RAD SmartSpec™
9	UV transilluminator	UVP
10	1kb DNA ladder, Agarose powder	Vivantis
11	TrackIt™ 100 bp DNA Ladder	Invitrogen™
12	PCR reagents	Invitrogen™
13	Absolute ethanol	Corpens Scientific
14	Ampicillin	Bio Basic Inc
15	LB agar, LB broth, MAC agar, EMB agar	MERCK, Bacto Laboratories
16	D-glucose	Rdeh
17	pGEM-T ligation set	Promega
18	PowerSoil™ DNA Kit	MO BIO Laboratories Inc
19	Wizard® Plus SV Minipreps DNA Purification System kit	Promega
20	NucleoSpin™ Gel and PCR Clean-up Kit	Macherey-Nagel™
21	*E. coil* JM109	Promega
21	pUC19	Yanisch-Perron, Vieira and Messing

APPENDIX C

Analysis of *bla*-TEM Variants

Table C Comparison of amino acid of *bla*-TEM recombinants to the known amino acid residue numeration of *bla*-TEM-1.