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CHARACTERIZATION OF *bla*-TEM AND *bla*-SHV GENES FROM BACTERIAL ISOLATES OF VARIOUS ENVIRONMENTAL SOIL SAMPLES IN KAMPAR

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By

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ABSTRACT

CHARACTERIZATION OF *bla*-TEM and *bla*-SHV GENES FROM BACTERIAL ISOLATES OF VARIOUS ENVIRONMENTAL SOIL SAMPLES IN KAMPAR

The illegal disposal of municipal wastes that contain antibiotics or antimicrobial drugs can cause selection pressure to act on the soil microbial community, leading to the emergence of resistance toward currently available antibiotics. Beta-lactamases are a group of enzymes that are capable of hydrolyzing the beta-lactam antibiotics which could potentially evolve and lead to the formation of extended-spectrum beta-lactamases. Beta-lactamases are capable of hydrolyzing broad-spectrum antibiotics has posed challenges in disease control. The purpose of this study was to characterize and amplify the *bla*-TEM and *bla*-SHV homologous genes. Fourteen bacterial isolates were successfully isolated from various soil samples in Kampar by isolation carried out on LB media that was supplemented with ampicillin. Further, characterization of the bacterial isolates were performed using selected biochemical tests. PCR amplifications were performed using corresponding *bla*-TEM primers and *bla*-SHV primers on bacterial isolates designated as 3P, 7P, 3, 7 and 10 and they consist of DNA fragment corresponding to approximately 870 base pairs. BlastX alignment search was carried out. Subsequently, the nucleotide sequences were translated into protein sequences for multiple sequence alignment and phylogenetic tree analysis was done. Based on the result, 3P was identified as TEM class A beta-lactamase, while 7P and 10 were

classified as SHV class A beta-lactamase and LEN class A beta-lactamases respectively. On the other hand, translated gene fragment of 3 and 7 corresponded to broad spectrum OKP-B beta-lactamases. Based on phylogenetic analysis, bacterial isolate 3 and 7 belong to sister group. On the other hand, 7P and 10 also identified as sister group, but 10 was outgroup.

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DECLARATION

I hereby declare that this final year project report 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 other degree at UTAR or other institutions.

BU KAH YEE

APPROVAL SHEET

This project report entitled "**Characterization of *bla*-TEM and *bla*-SHV Genes from Bacterial Isolates of Various Environmental Soil Samples in Kampar**" was prepared by BU KAH YEE and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

Approved by:

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Date: 20-09-2023

PERMISSION SHEET

It is hereby certified that **BU KAH YEE** (ID No: **20ADB05923**) has completed this final year project entitled **"CHARACTERIZATION OF *bla*-TEM and *bla*-SHV GENES FROM BACTERIAL ISOLATES OF VARIOUS ENVIRONMENTAL SOIL SAMPLES IN KAMPAR"** supervised by Asst. Prof. Dr. Choo Quok Cheong (Supervisor) from the Department of Biological Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project dissertation in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

I hereby give permission to my supervisor to write and prepare manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date provided that my name is included as one of the authors for this article. Arrangement of the name depends on my supervisor.

Yours truly,

(BU KAH YEE)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

bla	Beta-Lactamase
BLAST	Basic Local Alignment Search Tools
bp	Base Pair
DNA	Deoxynucleic Acid
dNTPs	Deoxynucleotide Triphosphate
EMB	Eosin Methylene Blue
ESBL	Extended-Spectrum Beta-Lactamase
FYP	Final Year Project
H2O2	Hydrogen Peroxide
LB	Luria-Bertani
O-F	Oxidation-Fermentation
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SBLs	Serine-Beta-Lactamases
MBLs	Metallo-Beta-Lactamases

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

The soil environment is home to diverse population of microbes. These environments create the interaction between microbes, animals and plants as well as provide the well-being of human (Wall, Nielsen, and Six, 2015). The microorganisms that live in the soil consist of pathogenic and non-pathogenic microbes which interact with each other to provide disease control as microbes-predator interaction occurs (Glushakova, et al., 2022). Natural soil environment has been shown to provide the gene pool of antibiotics resistant genes as some of the soil microbes could produce small amount of antibiotics as a weapon to compete for nutrients and spaces toward other microbes for surviving in the soil (Allen, et at., 2009). Release of antibiotics in significant amount can cause selective pressure towards the bacteria community. As a result, the microorganisms that is adapted to the environment gradually developed antibiotic resistance (Chen, et al., 2017). Additionally, the extended-spectrum beta-lactamases gene could evolve from the microbial population because of mutation occurred in the antibiotic resistance genes (Rawat and Nair, 2010).

The increase in usage of antibiotics throughout the years in human health care and other applications led to the release, residues containing antibiotics or antimicrobial drugs as wastes that entered the environment in the form of pharmaceuticals,

antibiotics or antimicrobial medicines, faecal and other wastes via municipal solid wastes (Anand, et al., 2021). The development of urbanization would lead to increase of municipal solid waste disposed as human population increased. This would unintentionally affect soil diversity due to municipal solid waste disposable. Direct disposal of wastes especially containing antibiotics medicine without prior treatment would be released to the environment and this could potentially induce the formation of resistance genes among the bacterial communities (Anand, et al., 2021). When the selective pressure applied to bacteria is significant enough, it can induce the formation of antibiotics resistance gene as the bacteria tend to adapt to such harsh environment leading to the mutation formation of extended-spectrum beta-lactamases (ESBLs) (Gatica, et al., 2015). Antibiotics resistant genes could also be transferred to other pathogenic microbes through horizontal gene transfer (Höfle et al., 2020). This could pose a serious threat to human health as these soil microbes could cause infections.

The *Enterobacteriaceae* family comprised mostly of Gram-negative bacteria that are capable of producing the beta-lactamases (BLs) naturally in the soil environment. When the BLs producing bacteria from *Enterobacteriaceae* are exposed to multiple antibiotics, this could induce the emerging of broad-spectrum or extended-spectrum beta-lactamase (Mondragón-Quiguanas, et al., 2022). The emergence of the ESBLs originated from *bla*-TEM and *bla*-SHV which had mutation occurring in the gene sequences. The ESBLs share the same ability as beta-lactamases in hydrolyzing beta-lactam antibiotics like penicillins. In addition,

ESBL also can hydrolyze first, second and third generation of cephalosporins and aztreonam but can be inhibited by clavulanic acid. Since the last decade, the prevalence of ESBLs producing *Enterobacteriaceae* has been increasingly worldwide (Adekanmbi, Oluwaseyi and Oyelade, 2021). They pose danger to human health as most of these bacteria can potentially infect human and cause infectious diseases that are difficult to cure by current antibiotics as stronger antibiotics was needed to treat such infections (Będzichowska, et al., 2019).

This project focus on the characterization of ESBL gene from bacteria that isolated from soil closed to dumping sites in few areas that located at Kampar, Perak residential area.

The objectives of this study include the following:

- To screen for the presence of antibiotic resistance genes in the bacterial isolates from selected soil samples in Kampar.
- To characterize the bacteria using Gram staining and selected biochemical tests.
- To amplify and sequence *bla*-TEM and *bla*-SHV homologous genes.
- To characterize *bla*-TEM and *bla*-SHV homologous genes using bioinformatics analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Soil Microbes and Municipal Solid Wastes

Soil is a complex environment that contains broad diversity of biotic community which consist of prokaryotes and eukaryotes. The soil formation is due to the after effect of corrosion of rocks that gave rise of minerals while organic matter formed from fallen leaves, dead branches and others that decompose by microorganisms. Some of the microorganisms are able to produce antibiotics to compete for the confine spaces and nutrients in soil. These phenomena could create a natural antibiotics resistance gene pool for the microorganisms to survive in such environment (Allen, et at., 2009).

As urbanization develop, increasing human population led to significant increase of municipal solid wastes (Anand, et al., 2021). This problem is common especially around residential area where illegal dumping sites were located. Those municipal solid wastes consist of household wastes that mainly food, agriculture, expired medicinal, and others garbage wastes (Egbenyah, et al., 2021). They would be exposed to rain leading to leachate which could pollute the surrounding soils and contaminating ground water. Soils exposed to the leachate, especially came from medical waste such as expired medicine, pharmaceuticals and residual antimicrobial agents could affect the soil microbes (Anand, et al., 2021). Antibiotics

would create the selective pressure on the bacteria community which causes the emerge of multi-drugs resistant bacteria. As the bacteria would evolve and adapt to such environment, the microorganisms tend to mutate and induce the formation of resistance to antibiotics (Sitotaw, et al., 2021.).

Antibiotics resistant bacteria could pose a threat to human health. The opportunistic characteristic of soil pathogenic bacteria leading to serious infection or diseases. Besides that, the antibiotics resistant bacteria could disseminate the resistance genes to other bacteria through horizontal gene transfer. Thus, human pathogen that acquired such resistance genes can cause the infectious diseases which are hard to cure (Li, et al., 2020).

2.2 *Enterobacteriaceae*

Enterobacteriaceae can be found in soil, water, plants, and even in the upper and lower gastrointestinal tracts of humans and animals. Typically, *Enterobacteriaceae* are rod-shaped, Gram-negative, non-sporulating, oxidase-negative, nitrate-reducing, and facultative anaerobic microorganisms (Donnenberg, 2014). Members of the *Enterobacteriaceae* family are responsible for a wide variety of infections both in the community and in hospitals (Janda and Abbott, 2021). Examples of clinically significant pathogenic strains that are commonly involved in infections and diseases include *Escherichia coli*, *Klebsiella*, *Enterobacter*, and *Salmonella*. *Escherichia coli* and *Salmonella* are particularly prevalent species that contribute

to foodborne diseases due to their toxins being transmitted through food. For instance, *Escherichia coli* (O157:H7) can produce exotoxins like Shiga toxin, leading to severe diarrhoea (Rock and Donnenberg, 2014). Additionally, *Klebsiella pneumoniae* is implicated in respiratory diseases, such as pneumonia, as well as other nosocomial infections. The prevalence of antibiotic resistance in *Enterobacteriaceae* has been steadily increasing. One of the main reasons for the dissemination of antibiotic resistance genes is the horizontal gene transfer of beta-lactamase genes (Janda and Abbott, 2021). This issue has reached a concerning level globally due to the introduction of beta-lactamase genes into the community.

2.3 Classification of Beta-Lactamase

2.3.1Ambler classification

Beta-lactamase can be classified into 4 major classes which are A, B, C, D based on the similarity in the amino acids sequences but not the feature of phenotype when referring to the Ambler classification system. From there, the Class A, C and D were serine-beta-lactamase (SBLs) while for Class B, was classified as metallo-beta-lactamase (MBLs) (Hall and Barlow, 2005.). SBLs use a serine as an enzyme active centre had similarity on the tertiary structures, however the catalytic mechanism and the primary structures that different which to be classified into Class A, C and D (Sawa, Kooguchi and Moriyama, 2020). while MBLs were dissimilar from SBLs in-term of mechanistically and structurally as containing zinc ions to disrupt the beta-lactam ring (Bush and Jacoby, 2010).

2.3.2 Bush-Jacoby-Medeiros functional classification

Based on Bush-Jacoby-Medeiros functional classification beta-lactamases functional can be classified into group 1, 2 and 3 depending on the degradation of β-lactam substrates and the effects of inhibitors (Nagshetty, et al., 2021). Class A SBLs was belong to group 2 and can be further divided into few subgroups according to the Bush-Jacoby classification. The group 2 comprised of subgroups include 2a, 2b, 2be, 2br, 2c, 2ce and 2e (Bush, 2018). Functional subgroup 2a, is able to hydrolyse limited spectrum of penicillins. On the other hand, the subgroup 2b is able to degrade early cephalosporin and mostly plasmid-mediated were reported in 1970-1980 like TEM-1 and SHV-1. Bush-Jacoby functional subgroup 2be efficiently degrades cefotaxime but inhibited by clavulanic acid such as TEM-3 and SHV-2. For the subgroup 2br, they confer relatively resistance toward clavulanic acid and able to hydrolyse penicillins such as TEM-30 and SHV-10. TEM-50 has the ability to hydrolyse monobactams, sulbactam, tazobactam, extended-spectrum cephalosporins and displayed resistance toward clavulanic acid that belong to subgroup 2be.

Another subclass 2c could hydrolyse carbenicillin but are inhibited by clavulanic acid or tazobactam, subclass 2ce, extended spectrum carbenicillinase, and hydrolyses carbenicillin, cefepime, and cefpirome. Subclass 2ce, which includes CepA could hydrolyses extended spectrum cephalosporins and is inhibited by clavulanic acid or tazobactam but not aztreonam (Bush, 2018).

Class B MBLs possesses the metal Zn^{2+} at the active centre of beta-lactamase enzyme which is capable of hydrolysing carbapenems. They belong to group 3 under Bush-Jacoby classification (Boyd, et al., 2020). The active centre contains a metal ion that can be supressed by the chelating agent EDTA. This MBLs could classified into three subclasses such as B1, B2, and B3 based on the amino acid sequence. The presence of two zinc ions molecules in the enzyme active centre such as Zn1 and Zn2 lead to broader range of substrates degradation of the Bush-Jacoby functional subgroup 3a (Nagshetty, et al., 2021.). MBLs of subclass B2 having one Zn^{2+} at the active centre, are narrow spectrum when compared to B1 and B3. These MBLs are classified into Bush-Jacoby functional subgroup 3b (Bush, 2018).

Class C SBLs, a cephalosporinase derived from ampC that carried on genome of *Enterobacteria* genus of *Enterobacteriaceae*. They are sensitive to cephamycins such as cefoxitin and ceftazidime but resistance to clavulanic acid that belong to subgroup 1 (Philippon, et al., 2022). Bush-Jacoby functional subgroup 1e, ampC mutants have been linked to reduced sensitivity to imipenem, ceftazidime, and cefepime such as plasmid-encoded CMY-19, CMY-10, and CMY-37 mutants (Malcolm, 2020).

Class D SBLs, also known as oxacillinase, posed similarity to Class A and Class C beta-lactamases. They can hydrolyse cloxacillin and oxacillin that belonging to Bush-Jacoby functional subgroup 2d (Poirel, Naas and Nordmann, 2010). As for

the subgroup like 2de, they able to degrade extended spectrum cephaloporins but not carbepenems. Carbapenem-hydrolysing β-lactamases from Class D are OXA enzymes are able hydrolyse carbapenems and belong to Bush-Jacoby functional subgroup 2df. The overall level of amino acid sequences dissimilarity is about 16% between class D and class A or class C (Antunes and Fisher, 2014).

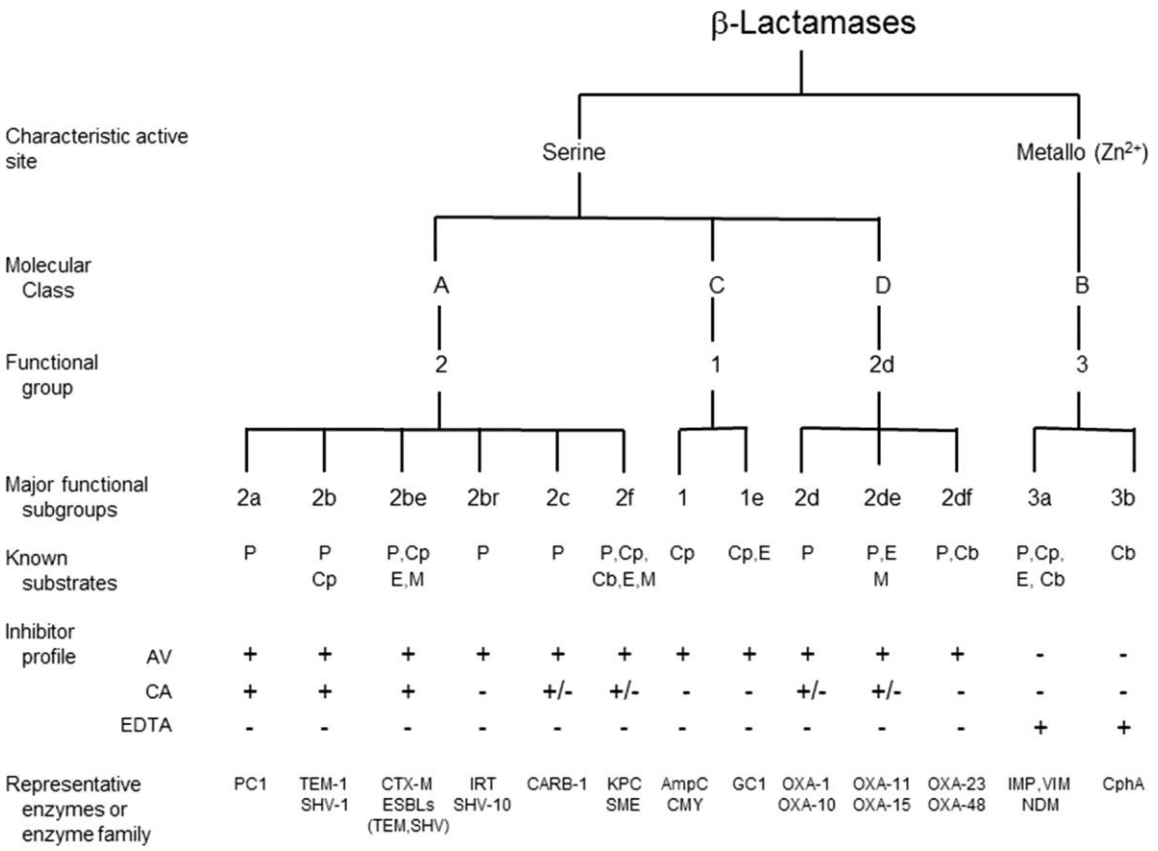

Figure 2.1: Molecular and functional relationships among β-lactamases (adapted from Bush, 2018.). AV, avibactam; CA, clavulanic acid; Cb, carbapenem; Cp, cephalosporin; E, expanded-spectrum cephalosporin; M, monobactam; P, penicillin.

2.4 Extended-Spectrum Beta-Lactamase

Extended-Spectrum Beta-Lactamases can defined as (ESBLs) which was characterized in early 1980. They are derived from TEM and SHV genes due to mutation event (Palzkill, 2018.). These mutations led to the acquisition of resistance to a wider range of beta-lactam antibiotics (Brandt, et al., 2017). ESBLs are able hydrolyse penicillins, extended spectrum cephalosporins and inhibited by clavulanic acid (Castanheira, Simner and Bradford, 2021). ESBLs are mainly plasmid mediated and capable of transferring the resistance genes from bacteria to another. The prevalence of ESBLs worldwide causes numerous infections that threaten the community were mostly conferred by microorganisms within the *Enterobacteriaceae* family (Shaikh, et al., 2015).

2.5 Type of Beta-Lactamases

2.5.1 TEM (Temoniera)- Type Beta-Lactamase

The *bla*-TEM are the most commonly encountered enzymes that showed resistances towards the beta-lactam antibiotics. The first report of TEM-1 was traced to Patient, Temoniera, where it was isolated. The *bla*-TEM confers resistance to penicillins and the first generation of cephalosporins (Smet, et al., 2010).

The widespread of the TEM-type beta-lactamase in the worldwide resulted from uncontrolled usage of beta-lactam antibiotics, leading to the formation of mutant derivatives by point mutations. The mutation caused alteration of amino acid sequences. As a consequence, TEM-derivatives having the extended-spectrum

enzymes properties, confer resistance toward extended spectrum penicillins, cephalosporins and monobactams (Taşli and Bahar, 2005).

2.5.2 SHV (Sulf-Hydryl variable)-Type Beta-Lactamase

SHV-types are commonly found in *Klebsiella pneumonia* especially in clinical isolates. SHV-type beta-lactamase could classified into plasmid-mediated and integrated into the bacteria chromosome (Smet, et al., 2010). The *bla*-SHV, *bla*-LEN and *bla*-OKP were evolved from a common ancestor over millions year. Both *bla*-LEN and *bla*-OKP were chromosomally encoded beta-lactamase belong to class A (Tärnberg, Nilsson and Monstein, 2009.). It's commonly found in *klebsiella pneumoniae.* The nucleotide sequences encoding LEN beta-lactamase is more closely related to nucleotide sequence of SHV-1 beta-lactamase when compared to *bla*-OKP. The similarity between the amino acid sequences of SHV-1 and LEN-1 was of 88.9%. The *bla*-OKP was more heterogeneous than the *bla*-SHV and *bla*-LEN (Hæggman, et al., 2004). The OKP-A or OKP-B and LEN-1 variants are clearly related to each other, sharing at least 78% similarity in amino acid sequences. Similarity between the amino acid sequences of SHV-1 and TEM-1 was only a 63.7% (Fevre, et al., 2005).

2.6 Antimicrobial Agents and Antibiotics

Antimicrobial agents comprised of a large variety of chemicals compounds or physical agents with the properties to kill or inhibit the microbe's growth. These

agents are generally chosen for the treatment of infectious diseases caused by bacteria within the community (Leekha, Terrell and Edson, 2011). Antibiotics are vital antimicrobial tools to combat again infectious diseases. In 1928, Alexander Fleming isolated penicillin that was produced by *Penicillium notatum* (Lobanovska and Pilla, 2017).

Penicillin was the first antibiotic that kill and inhibit the growth of *S. aureus.* There are many derivatives of penicillins are available today that are used to treat infection that caused by Gram-positive bacteria and Gram-negative bacteria. They include amoxicillin, ampicillin, Penicillin G, Penicillin V, and others (Lobanovska and Pilla, 2017).

Antibiotics can be categorized into two group which are designated as bactericidal and bacteriostatic. Bacteriostatic antibiotics only inhibit the growth and do not kill the bacteria while bactericidal tend to kill bacteria. The bacteriostatic drugs include tetracycline and chloramphenicol. Some antibiotics could exhibit both bactericidal and bacteriostatic properties depending on the dosage concentration used and the state of intruding bacteria (Reygaert, 2018).

2.7 Beta-Lactam Drugs and Inhibitors

Beta-lactam drugs have a common feature which consist of a three carbon and a nitrogen to form a four membered cyclic ring known as beta-lactam ring. For antibiotics that consist of beta-lactam ring, they are classified as beta-lactam

antibiotics. The beta-lactam ring has an important role on the mechanisms of action toward inhibiting bacterial cell wall biosynthesis and mode of actions that has a lethal effect on bacteria (Bush and Bradford, 2016). Cephalosporin is a subclass of antibiotics consisting of beta-lactam ring (Abraham, 1971). It can be classified into five generation which each generation having different effectiveness against the bacteria (Bush and Bradford, 2016). The first to fifth generation of cephalosporin was tabulated in Table 1.1.

Beta-lactamase inhibitors are the drugs with the capability to inhibit serine beta-lactamase (SBLs). They exert high affinity toward the active site of beta-lactamase enzyme, permanently causing enzyme inactivation. These inhibitors are usually use collaboratively with beta-lactam antibiotics to kill bacteria more effectively and preventing the antimicrobial resistance. For example, Avibactam and relebactam work by the early mechanism, bind to the active site of beta-lactamases and prevent them from degrading the beta-lactam antibiotics (Li, et al., 2022).

For sulbactam, tazobactam, and clavulanic acid work by the latter mechanism, indirect inhibit the enzyme's activity by formation of irreversible complexes with the beta-lactamase. This helps protect the beta-lactam antibiotics from degradation and allows them to remain effective against the bacterial infection. Inhibitors for MBLs are mainly acting on the active site metal chelating and metal deprivation while SBLs are mainly acting on either covalently or non-covalently interaction on

the active site. MBL inhibitors like bicyclic boronates, substituted pyrroles, thiols, and succinate derivatives have been reported as MBL inhibitors in the scientific literature (Farley, et al., 2021).

Table 2.1: Five generation of cephalosporin.

Cephalosporin Generation	Examples
First	Cefadroxil, Cefazolin, Cephalexin and Cefezedone.
Second	Cefaclor, Cefotetan, Cefoxitin, Cefprozil and Cefuroxime.
Third	Cefotaxime, Cefdinir, Cefditoren, Cefixime Cefoperazone, Cefpodoxime, Ceftazidime, Ceftibuten and Ceftriaxone.
Fourth	Cefepime and Cefquinone.
Fifth	Ceftaroline, Ceftobiprole and Ceftolozane.

2.8 Mechanism of Action of Antibiotics

Depending on the type of antibiotics, antibiotics have different mode of actions. Their mode is fully dependent on the structure and affinity of binding to the target sites within the bacterial cell. There are five basic mechanisms of antibiotics action. They are inhibition of cell wall synthesis, inhibition of protein synthesis, alteration of cell membranes, inhibition of nuclei acids synthesis and anti-metabolite activity (Kırmusaoğlu, Gareayaghi and S. Kocazeybek, 2019).

The first mode of action is disruption of the synthesis of peptidoglycans to build cell wall. The bacterial cell wall is mainly made from basic building block of peptidoglycans. These beta-lactam antibiotics act as substrate that react and bind to Penicillin-binding proteins (PBPs) with high affinity, causing the transpeptidation to be blocked, then inactivating transpeptidase domain of PBPs. Thus, would subsequently kill the microorganisms as the peptidoglycan biosynthesis had been inhibited (Kapoor, Saigal and Elongavan, 2017).

The second mode of action is the inhibition of protein synthesis. Protein synthesis is a complex process that require multiple steps of processing involving many enzymes. Most antibiotics can interfere with the 70S bacteria ribosome 30S or 50S subunit which led to blockage of synthesis of the bacteria proteins. For example, tetracyclines can block the aminoacyl site of 30S ribosome and prevent the binding of aminoacyl-tRNA (Wivagg, et al., 2014).

The third mode of action is interruption of cell membrane function. The cell membrane plays an important role as selective membrane that regulate the movement of substances in and out of the bacterial cell. When exposed to antibiotics, interference toward the interaction of lipophilic moiety with the bacteria membrane occurred. This would lead to destruction of bacteria membrane structure, leading to functional impairment which subsequently kill the bacteria (Rice, 2012).

The fourth mode of action is inhibition of nucleic acids synthesis. Certain antibiotics can inhibit the topoisomerase enzyme involved in DNA replication and RNA synthesis by inhibiting RNA polymerase in bacterial cell. This inhibition will lead to bacterial cell death (Kırmusaoğlu, Gareayaghi and S. Kocazeybek, 2019). Lastly, the metabolic pathways are inhibited whereby the antibiotics could serve as bacterial metabolism inhibitors that target the amino acids and nucleic acids synthesis pathway. For example, the biosynthesis of TH4 (Tetrahydro-folic acid) serve as an important key coenzyme for the biosynthesis of nucleic acids and certain amino acids using the precursor para-aminobenzoic acid (PABA). When the bacterial metabolism was inhibited, it causes interferences of the TH4 synthesis that subsequently led to cell death (Kırmusaoğlu, Gareayaghi and Kocazeybek, 2019).

2.9 Resistance Mechanism to Beta-Lactam Antibiotics

The most common mechanism is the hydrolysis to inactivate the beta-lactam antibiotics to an acid. For example, penicillin can be hydrolysed by the enzymatic reaction of beta-lactamase by converting penicillin to penicilloic acid. Other resistance mechanisms toward beta-lactam antibiotics are a cause of a concern to public health as such resistance mechanisms increasingly worldwide (Kırmusaoğlu, Gareayaghi and Kocazeybek, 2019). Besides producing beta-lactamases, bacteria can alter the effective concentration of the antibiotic through multidrug efflux pumps. This efflux pumps have the function of preventing the transportation of the antibiotics out to the extracellular environment before targeting the penicillin binding protein (PBPs). The modification of the transpeptidase activity can also

evade the binding of beta-lactam antibiotics via target site modification. This mechanism is performed via point mutations causing the alteration of the diacylation rate. This enables the PBPs to regain back its functionality in a short period by ejecting the beta-lactam substrate (Worthington and Melander, 2013). Alternatively, the alteration of the PBPs by target site target site through point mutation can also acquire new PBP that have low affinity towards beta-lactam antibiotics. Finally, the alteration of the permeability of the hydrophobic bilayer within the outer membrane can render the ability of beta-lactam antibiotics being diffused through the lipid bilayer (Wivagg, Bhattacharyya and Hung, 2014.).

2.10 BLs Detection Method

2.10.1 Conventional Detection Methods

ESBLs detection methods are important to detect the presence of ESBLs producing bacteria. This would ultimately act as a prevention measurement in reducing the possibility outbreak of ESBLs related infections.

Double disk diffusion and combination disk method are most commonly used for the confirmatory of test for ESBLs. The specific zone of inhibition produced by the bacterial are based on the antibiotics disks that placed on the agar plate. Both methods utilize the clear zone formation depending on the antibiotics used (Garrec, et al., 2011.). The combination disk test is performed with or without the presence of clavulanic acid, ceftazidime and cefotaxime on Muller-Hinton Agar (MHA). The inoculation done by using of bacterial suspension with concentration of 0.5

McFarland turbidity followed by incubated overnight incubation at 37ºC. For the combination disk test, they comprised of antibiotics disks of ceftazdime, cefepime and cefotaxime which were placed 20 mm from amocillin-clavulanate disk incubated at 37ºC for 16-18 hours. The positive ESBL producing bacteria will produce a zone of inhibition after the incubation period (Dejenie, et al., 2019; Akpaka, et al., 2021).

The E-Test strips can also be used to detect the presence of ESBL. The two end sites of the strip contain the beta-lactam antibiotics while the other site containing clavulanic acid (Akpaka, et al., 2021). With the MIC was determined according to the manufacturer's guidelines. The sensitivity and specificity pf the test was reported up to 95%. Lastly, the Vitek test and minimum inhibitory concentration test was used by observing the growth of the bacteria in the well containing different types of antibiotics such as ceftazidime and clavulanic acid (Drieux, et al., 2008; Rawat and Nair, 2010).

2.10.2 Molecular Detection Methods

Polymerase chain reaction with specific primers targeting beta-lactamase genes could be carried out as a detection method. This method is easy to perform and able to detect *bla*-TEM and *bla*-SHV type genes. Rather than normal PCR, multiplex PCR can also be used for the detection of multiple beta-lactamase genes using different primers that specifically target each of the beta-lactamase genes (Lemlem, et al., 2023).

DNA probes is another molecular detection that was used in early detection of the beta-lactamase genes. The disadvantages of this methodology used in distinguish between the ESBL and non-ESBL was inaccurate. Oligo-typing is another method used to identify the variants within ESBLs due to single point mutation, but it is labour intensive and unable to detect new variant (Ejaz, et al., 2013). PCR-RFLP that can also be used to beta-lactamase genes. This method is carried out with the addition of Restricted Fragment Length Polymorphism (RFLP) analysis and PCR. This strategy is capable of detecting specific nucleotides changes and relatively easy to perform (Ejaz, et al., 2013; Abdelmoktader, 2019).

CHAPTER 3
MATERIALS AND METHODS

3.1 Bacterial Samples

Bacterial samples were obtained from different location within vicinity of garbage dumping sites near residential areas at Kampar, Perak. The samples were collected at the depth of 10-15 cm from the soil surface.

3.2 Materials and Apparatus

This project utilized chemical materials and apparatus from the Department of Biological Science, UTAR. All the chemical materials and apparatus were available at Biotechnology FYP Laboratory, Microbiology Laboratory and Molecular Biology Laboratory. The chemical materials and apparatus utilized are shown in Table 3.1 and Table 3.2 respectively.

Table 3.1: List of chemical materials and their Manufacturers.

Chemical Materials	Manufacturers
Absolute Ethanol	Chem Soln.
Acetone	QRec
2X *Taq* Master Mix	Vazyme
Agarose powder	1st Base
bla-TEM and *bla*-SHV forward and reverse primers	IDT
Crytal violet	Chem Soln.
DNA staining dye	Bio Labs
Eosin Methylene Blue (EMB) agar, Luria-Bertani (LB) agar	Conda
Oxidation-Fermentation (OF) Basal Medium	Liofilchem
Glacial Acetic Acid	Liofilchem
Gram's Staining Kit	Systerm
Mineral Oil	Systerm
Sodium Chloride	EMSURE
Oxidase Reagent	Remel
100 bp DNA Ladder	SMOBIO
Glucose	Fisher Scientific
Hydrogen Peroxide 30%	Merk
Simmons Citrate Agar	Conda
Lugol's reagent (Iodine)	Merk
Tris Base Powder	SIGMA

Table 3.2: Chemical media and reagents preparation.

Chemical Media	Media Type	Preparation technique
Agar supplemented with 50 µg/mL of Ampicillin	Molten agar	A volume of 400 µL of ampicillin stock solution (50 mg/mL) added into 400 mL of molten agar which cooled to around 45ºC. Then media was well mixed.
Ampicillin Stock (50 mg/mL)	Antibiotic	Ampicillin powder that weighed 0.25g and dissolved in 5 mL of sterile distilled water. Thus was followed by filter-sterilized with 0.22 µm syringe filter.
Eosin Methylene Blue (EMB) Agar	Molten agar	A weight of 36.0 g of was dissolved and top up to 1 L with distilled water.
Glucose (1%)	Solution	Glucose powder was weighed 1.0 g was mixed in 100 mL of media.
Luria-Bertani (LB) Agar	Molten agar	LB agar powder was weighed 35.0 g was dissolved and top up to 1 L with distilled water.
Oxidation-Fermentation (O-F) Basal Medium	Semi-solid agar	Oxidation-Fermentation (O-F) Basal Medium was weighed 9.8 g the dissolved and top up to 1 L with distilled water.
S.I.M. Medium	Semi-solid agar	S.I.M. Medium was weighed 30.0 g the dissolved and top up to 1 L with distilled water.
Simmons Citrate Agar	Semi-solid agar	Simmons Citrate Agar was weighed 24.3 g the dissolved and top up to 1 L with distilled water.

3.3 Sterilization

Chemical medias, reagents, glasswares, micropipette tips and others was sterilized in autoclave machine at 121ºC for a duration of 15 minutes. After autoclaving, pipette tips and glassware were kept in a 70ºC oven for drying purpose. For media that is heat sensitive such as ampicillin and glucose, sterilization was achieved with filter-sterilization by using 0.22 µm syringe filter.

3.4 Cultivation of Bacterial Isolates from Soil Samples

A weight of 1.0 g soil sample was suspended in 9.0 mL of sterile distilled water. Serial dilution was carried out on the soil mixture until the concentration of soil mixture reached 10^{-5} g/mL. A volume of 100 µL from each of the dilution was plated onto the Luria-Bertani (LB) agar plates supplemented with 50 µg/mL of ampicillin. The agar plates were incubated overnight at 30ºC. After incubation, bacterial colonies with different morphologies were selected and streaked on Luria-Bertani agar supplemented with 50 µg/mL of ampicillin. The bacterial isolates were maintained by sub-culturing every two weeks.

3.5 Preliminary Characterization of Bacterial Isolates.

The bacterial isolates were characterized by EMB agar, Gram staining method, and several biochemical test which including Oxidase, Catalase, Oxidation-Fermentation, Sulphur Indole Motility (S.I.M) and Simmon citrate test. Fresh

cultures of the bacterial isolates were used in the test to ensure the accuracy and validity of the tests.

3.5.1 Gram staining

Freshly sub-cultured bacterial isolates were tested with Gram stains to differentiate Gram-positive bacteria and Gram-negative bacteria. First, a single pure bacterial colony was inoculated onto a microscopic slide that pre-dropped with a drop of sterile distilled water and spread evenly to get a thin smear with wire loop. The smear on the microscopic slide is then heat fixed. A few drops of crystal violet were dropped on the smear by a dropper to stain the bacteria for 30 seconds. It was then flushed the crystal violet stain gently with distilled water. The excess water on the slides was dried with a clean paper towel. A few drops of iodine solution were dropped on the smear to stain for 30 seconds. The iodine solution was then washed off gently by using the distilled water. The excess water on the microscope slides was dried and then decolorized by decolorizing agent (1 part ethanol : 1 part acetone) for around 10 seconds. Then washed off with distilled water and dried with a clean paper towel. Safranin solutions were dropped onto the smear for around 30 seconds. Excess safranin was washed off with distilled water, and the slides were let dry before observation under the microscope.

3.5.2 Selective and Differential Medium: Eosin Methylene Blue (EMB) agar

Single pure colony from each bacterial isolates streaked onto the Eosin Methylene Blue (EMB) agar. The plates were then incubated overnight at 37ºC. The color and morphology of the colonies were observed and recorded.

3.5.3 Oxidase Test

Single colony from each bacterial isolate was selected and transferred onto a filter paper with oxidase reagent and left for about 1 minutes to observe the color change for each of the bacterial. The isolates data was then recorded.

3.5.4 Catalase Test

Single pure colony from each bacterial isolate was transferred onto a glass. Two drops of 3% hydrogen peroxide were dripped onto the sample on glass slide. The formation of bubbles was observed and recorded.

3.5.5 Oxidation-Fermentation (O-F) Test

Single colony from each bacterial isolate was stabbed into two tubes of oxidation-fermentation medium supplemented with 1% glucose. One of the tubes was added with mineral oils to provide an anaerobic condition for the bacteria while other tube remained as aerobic condition. Both inoculated tubes were then incubated for 48 hours at 37ºC. The color changes was observed and recorded.

3.5.6 Simmon Citrate Test

Single colony from each bacterial isolates was selected and stabbed into a tube of Simmon citrate test in aerobic conditions. The tube was incubated for 48 hours at 37ºC. The color changes were observed and recorded.

3.5.7 Sulfide Indole Motility (S.I.M) Test

Single colony from each bacterial isolates was selected and stabbed into a tube of Sulfide Indole Motility test in aerobic conditions. The tube was incubated for 48 hours at 37ºC. The color changes were observed and recorded.

3.6 Total DNA Extraction

3.6.1 Fast Boiling Method

Two loopful of inoculum of bacterial culture was transferred into a sterile 1.5 mL centrifuge tube. It was resuspended with 0.5 mL of sterile distilled water and centrifuged with speed of 13,000 rpm for 2 minutes. The supernatants were discarded and resuspended with 0.5 mL of sterile distilled water was then centrifuged with speed of 13,000 rpm for two minutes. After discarded the supernatants, resuspended the pellet with 0.3 mL of sterile distilled water. The suspension was heated to 95ºC for 10 minutes at heat block followed by cooling on ice for 5 minutes. The sample was then centrifuged at speed of 13,000 rpm for two minutes and the supernatant was transferred into a new sterile microcentrifuge tube and stored at – 20ºC for future use.

3.7 Analysis of Total DNA

3.7.1 Determination of DNA Concentration and Purity through Agarose Gel Electrophoresis

The concentration and purity of the amplicons were measured by using Nanodrop 1000 (Thermo Scientific). Agarose gel electrophoresis was carried out to separate and check DNA sizes. To separate the DNA using agarose gel electrophoresis, DNA samples were mixed well with DNA staining dye, then loaded into respective wells in the gel and current was applied. An agarose gel of 0.8% (w/v) was used with the 100 bp DNA ladder as a molecular maker. After that, the gel was then viewed under UV transilluminator.

3.7.2 Polymerase Chain Reaction (PCR)

PCR was carried out to amplify the specific gene region for *bla*-TEM and *bla*-SHV genes. A sets of specific primers for *bla*-TEM (TEM_F and TEM_R) and *bla*-SHV (SHV_F and SHV_R) were used separately. Details of the primers were shown in Table 3.3. Table 3.4 showed the parameters of PCR reaction.

Table 3.3: Details of the Primers (TEM and SHV).

Primer	Length (bp)	Primer Sequences (5' to 3')	Expected Amplicon Size
bla-TEM_F	20	ATG AGT ATT CAA CAT TTC CG	~870 bp
bla-TEM_R	20	CTG ACA GTT ACC AAT GCT TA	
bla-SHV_F	20	TGG TTA TGC GTT ATA TTC GCC	~870 bp
bla-SHV_R	20	GGT TAG CGT TGC CAG TGC T	

Table 3.4: Parameters of PCR reaction.

Condition	**Temperature (ºC)**	**Time**	**Number of Cycles**
Initial Denaturation	94	3 minutes	1
	94	1 minutes	
Annealing	45	45 seconds	30
	72	1 minutes	
Final Extension	72	5 minutes	1

3.7.3 Agarose Gel Electrophoresis and Gel Purification of Amplicons

Amplicons were mix well with DNA staining dye and assessed in 2.0% (W/V) agarose gel. The 100 bp DNA ladder (SMOBIO) was used as a molecular marker of DNA. After electrophoresis, gel was viewed under UV transilluminator. PCR amplicons electrophoresed and the desired DNA fragment of approximately 870 bp were excised from the gel using sterile scalpel. The purification steps were carried out by following instructions on the QIAGEN manufacturer's manual. After

purification, the purified PCR products was assessed to determine the success of the purification process.

3.7.4 DNA Sequencing and Data Analysis.

Purified amplicons were outsourced to Apical Scientific Sdn. Bhd. (Malaysia) for direct DNA sequencing. Amplicons were sequenced using *bla*-TEM and *bla*-SHV specific forward primers respectively. The analysis of the DNA sequences Was performed using BlastX online data bases from National Centre for Biotechnology Information (NCBI). The obtained DNA sequences and the result of the alignment were recorded and tabulated.

CHAPTER 4

RESULTS

4.1 Cultivation, Isolation and Preliminary Screening of Bacterial from Soil Samples

Single colonies obtained from the serial dilution of soil samples on agar plates were isolated and streaked on Luria-Bertani agar plate supplemented with ampicillin. Fourteen morphologically different bacterial isolated from following locations soil samples from Kampar wet market, Lotus's Kampar, Taman Perak Indah and Westlake Garden. The morphology of bacterial isolates was tabulated in Table 4.1.

Figure 4.1: Bacterial isolates streak on Luria-Bertani agars supplemented with 50 µg/mL of ampicillin.

4.2 Preliminary Characterization of Bacterial Isolates

4.2.1 Gram Staining

All the bacterial isolates were stained with Gram's stain followed by observing under microscope with the help of oil immersion. For the bacterial cells that were stained with crystal violet, purple in coloration can be classified as Gram-positive bacteria while for the bacterial cells stained as pink color would be classified as Gram-negative bacteria. The structure and color of each bacterial cells were tabulated in Table 4.2.

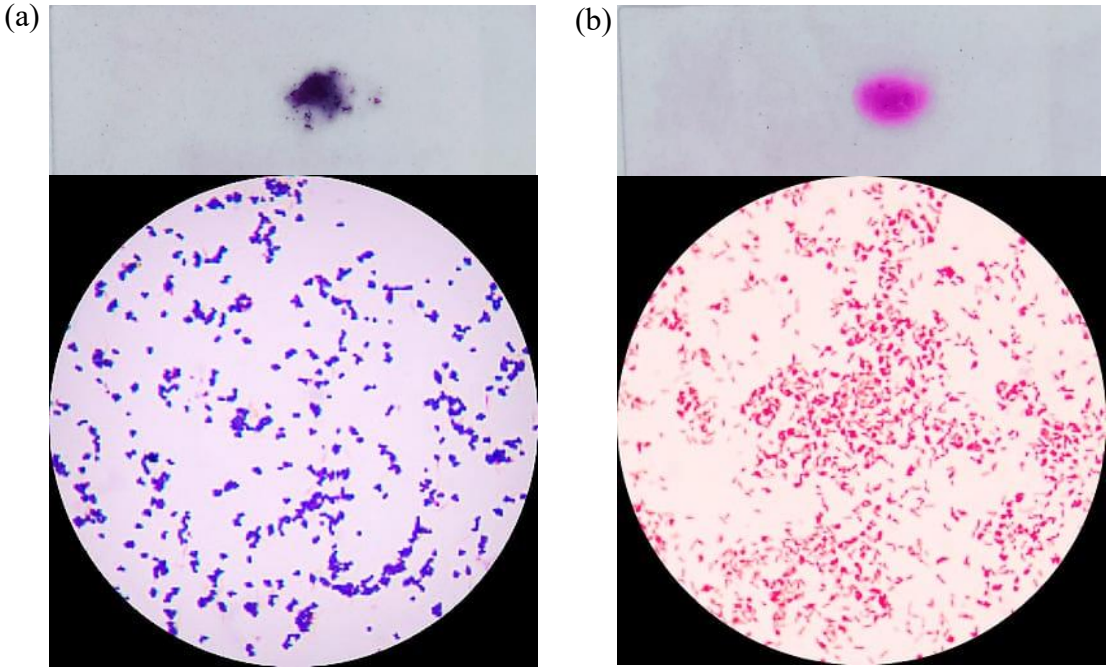

Figure 4.2: Bacterial cells viewed under microscope (1000x magnification) with oil immersion after Gram's staining on microscope glass slide. (a) Gram-positive bacteria and (b) Gram-negative bacteria.

Table 4.1: Morphological characteristics of bacterial isolates on Luria-Bertani (LB) agar supplemented with 50 µg/mL ampicillin.

Soil Sample	Bacterial Isolate Designation	Appearance and Structure of Colony					
		Color	Shape	Elevation	Margin	Opacity	Texture
Kampar Pasar	3P	white	Circular	Convex	Entire	Translucent	Smooth
	7P	Ivory	Circular	Convex	Entire	Translucent	Smooth
Lotus's Kampar	1	Ivory	Circular	Convex	Entire	Translucent	Smooth
	2	Ivory	Circular	Convex	Entire	Translucent	Smooth
	3	Ivory	Circular	Convex	Entire	Translucent	Smooth
	P	Purple	Circular	Convex	Entire	Opaque	Rough
Taman Perak Kampar	5	Ivory	Circular	Convex	Entire	Translucent	Smooth
	6	Ivory	Circular	Convex	Entire	Translucent	Smooth
	7	Ivory	Circular	Convex	Entire	Translucent	Smooth
	8	Ivory	Circular	Convex	Entire	Translucent	Smooth
Westlake Garden Kampar	10	Ivory	Circular	Convex	Entire	Translucent	Smooth
	12	Ivory	Circular	Convex	Entire	Translucent	Smooth
	R	Red	Circular	Convex	Entire	Opaque	Smooth
	O	yellow	Circular	Convex	Entire	Opaque	Smooth

Table 4.2: Microscope observation (1000X Magnification) of bacterial cells after Gram stain.

Bacterial Isolate Designation	Structure of Cell	Color	Cell Type
3P	Bacillus	Pink	Gram-negative
7P	Bacillus	Pink	Gram-negative
1	Bacillus	Pink	Gram-negative
2	Bacillus	Pink	Gram-negative
3	Bacillus	Pink	Gram-negative
P	Bacillus	Pink	Gram-negative
5	Bacillus	Pink	Gram-negative
6	Bacillus	Pink	Gram-negative
7	Bacillus	Pink	Gram-negative
8	Bacillus	Pink	Gram-negative
10	Bacillus	Pink	Gram-negative
12	Bacillus	Pink	Gram-negative
R	Coccobacillus	Pink	Gram-negative
O	Coccus	Purple	Gram-positive

4.2.2 Selective and Differential Medium: Eosin Methylene Blue (EMB) Agar

Eosin Methylene Blue (EMB) agar is a selective and differential medium which inhibits the growth of gram-positive bacteria, a condition suitable for most Gram-negative bacteria to grow. This medium also differentiates lactose fermenter produce acids that lower the pH which give purple-black or metallic green sheen for bacteria colonies while non-lactose fermenter increases pH of agar by deamination of proteins giving pink or colorless to bacteria colonies. The morphological and appearance of bacterial isolates was tabulated in Table 4.3.

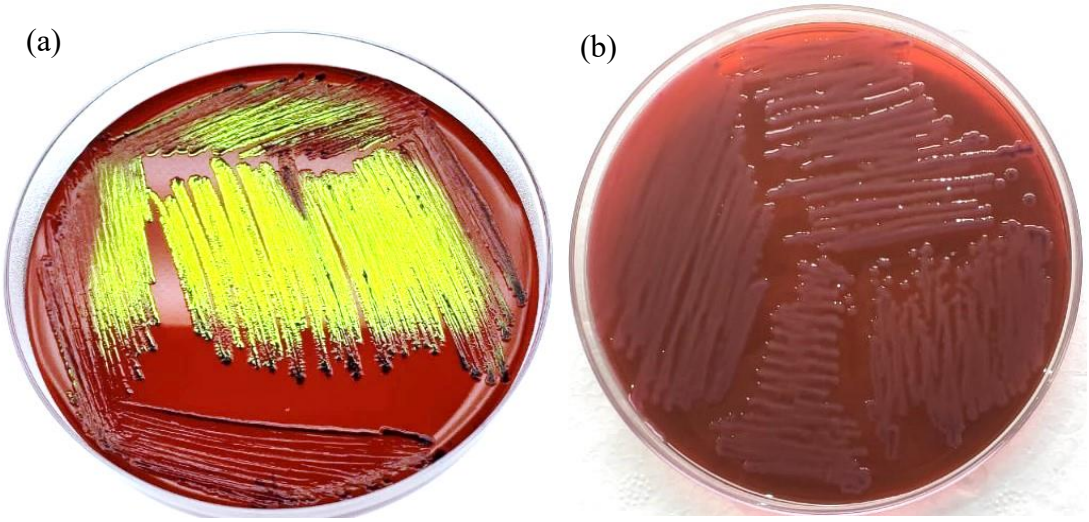

Figure 4.3: Bacterial isolates streaked on Eosin Methylene Blue (EMB) Agar. (a) Lactose fermenter and (b) non-Lactose fermenter.

Table 4.3: Morphological characteristics of bacterial isolates on EMB agar supplemented with 50 µg/mL ampicillin.

Bacterial Isolate Designation	**Color of colonies**	**Lactose (+)/non-Lactose fermenter (-)**	**Texture of colonies**
3P	Metallic Green Sheen	+	Smooth
7P	Dark Purple	+	Smooth
1	Pink to Purple	+	Smooth
2	Pink to Purple	+	Smooth
3	Metallic Green Sheen	+	Smooth
P	Purple center	+	Smooth
5	Metallic Green Sheen	+	Smooth
6	Colorless	-	Smooth
7	Pink to Purple	+	Smooth
8	Pink to Purple	+	Smooth
10	Purple Center	+	Smooth
12	Pink to Purple	+	Smooth
R	Colorless	-	Smooth
O	(No Growth)	NA	(No Growth)

4.2.3 Simmons Citrate Agar Test

Simmons Citrate Agar is a differential medium that can distinguish between bacteria that utilizes citrate acids as a carbon source and ammonium salt as a source of nitrogen. Positive citrate fermenter changes the color of medium from green to blue while non-citrate fermenter does not change the color of medium. The result for each bacterial isolates were shown in Table 4.4.

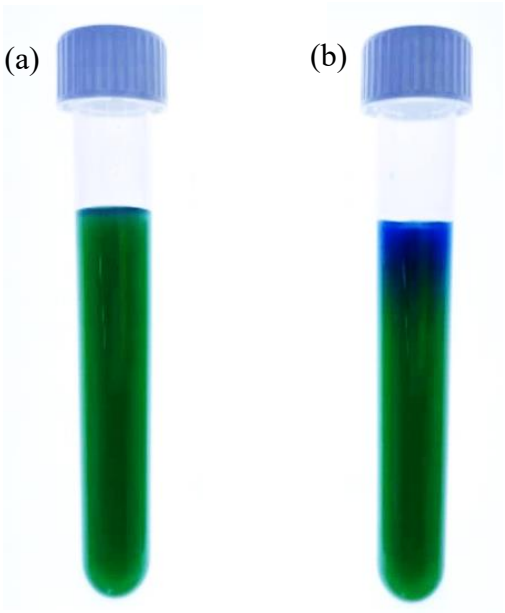

Figure 4.4: The figure showed the bacterial Isolates stabbed in Simmons Citrate Agar. (a) Tube is a negative control and (b) Tube was stabbed with bacterial that showing positive citrate test.

4.2.4 Oxidase Test

The color change in oxidase reagent will determine whether it's a positive or negative test for oxidase. Positive test occurred when reagent color changes from colorless to dark blue color, indicating the present of cytochrome C oxidase. For negative test, the reagent will remain colorless. The result for each bacterial isolates were shown in Table 4.4. The bacterial Isolates streaked on filter paper with oxidase reagent was shown in Figure 4.4.

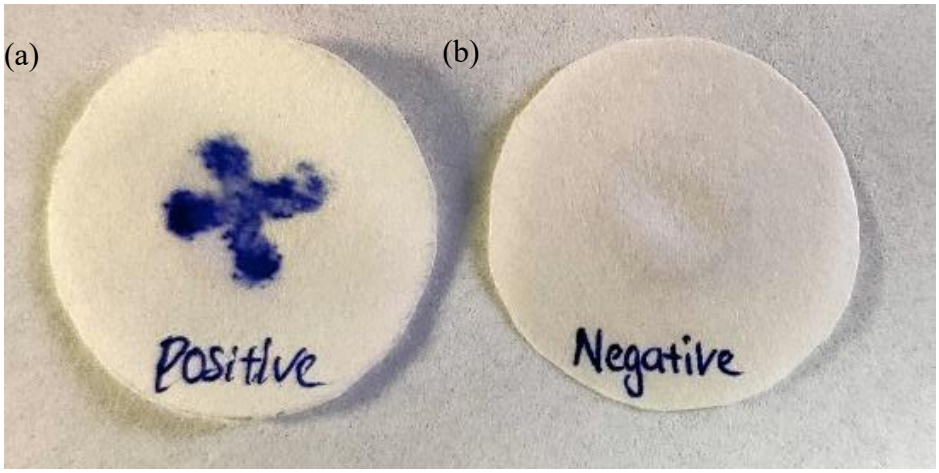

Figure 4.5: Bacterial Single Isolates streak on filter paper with oxidase reagent. (a) Positive oxidase test and (b) Negative control.

4.2.5 Catalase Test

Catalase test was used to access the ability of bacteria to produce catalase for neutralizing the non-radical Reactive Oxygen Species (ROS) such as hydrogen peroxide. For the positive reaction, bubble formation was observed when a few drops of 3% hydrogen peroxide was added onto the bacteria colony. In the negative reaction, no bubbles formation was observed when a few drops of 3% hydrogen peroxide onto the bacteria colony. The results for each bacterial isolates were shown in Table 4.4.

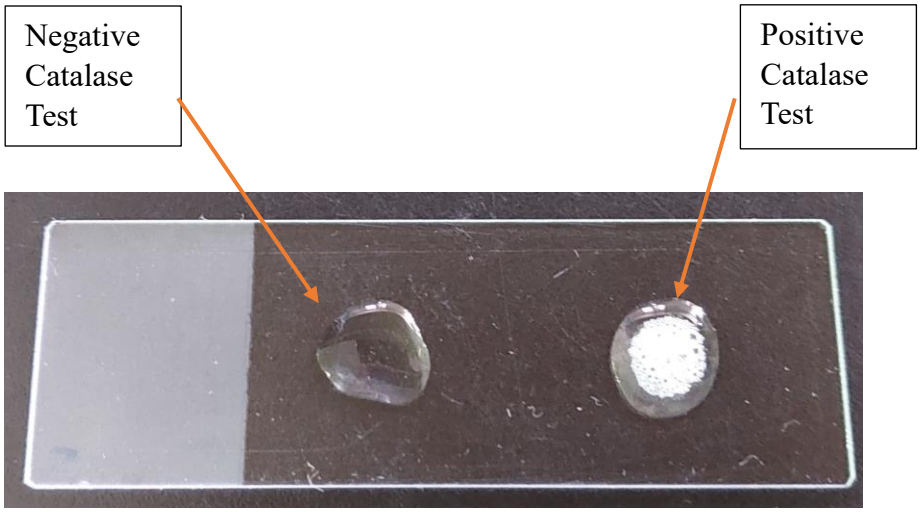

Figure 4.6: Bacterial isolates streaked on a glass slide, followed by addition of a few drops of 3% hydrogen peroxide. The formation of bubbles indicates positive catalase while negative catalase doesn't produce bubbles.

4.2.6 Oxidation-Fermentation (O-F) Test

The bacterial isolates stabbed in the oxidation-fermentation (O-F) medium under aerobic and anaerobic conditions with glucose as only carbon source was incubated for 24-48 hours at 37ºC. For O-F medium under aerobic conditions, color changes from green to yellow while tube under anaerobic conditions remain green color, indicating oxidative reaction. For O-F medium that changes color from green to yellow under aerobic and anaerobic conditions indicated fermentative reaction. If they are neither fermentation nor oxidation, both O-F medium under aerobic and anaerobic conditions remain blue or green in aerobic tube and green in anaerobic tube known as non-saccharolytic. The result for each bacterial isolates were shown in Table 4.5.

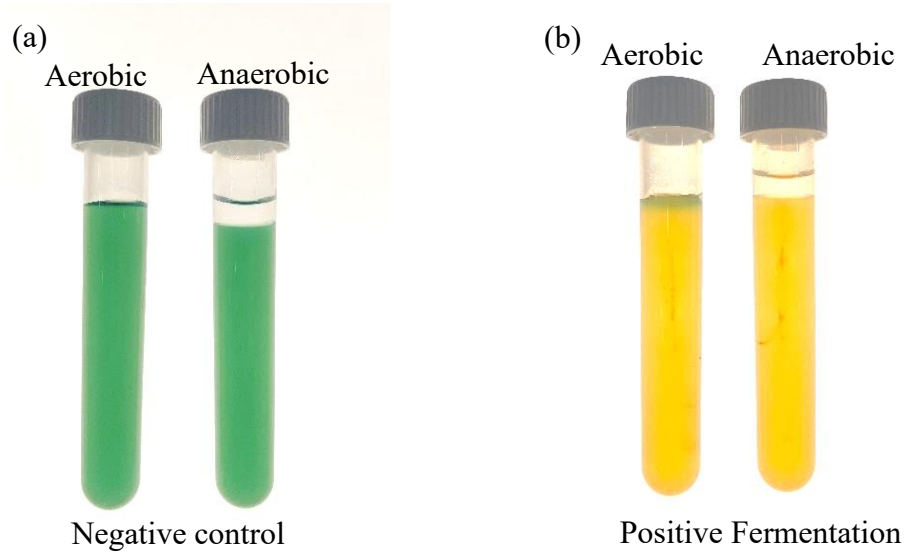

Figure 4.7: The aerobic and anaerobic conditions, both tubes showed yellow color which indicated fermentation metabolism. (a) Both tubes with O-F medium under aerobic and anaerobic conditions without bacterial inoculated acted as negative control. (b) Both tubes with O-F medium under aerobic and anaerobic conditions with bacterial inoculated acted as positive control whereby both tubes turned yellow, indicating fermentative reaction.

4.2.7 Sulfide Indole Motility (S.I.M) Test

Motility test is a test to access the ability of the bacterial isolates to move by itself with flagella in semi-solid motility medium. For positive motility, bacterial isolate spread throughout the medium by diffusion or hazy growth where the medium changes from transparent to become slightly opaque. For positive motility, bacterial isolate would confine to the stab line with sharp defined margin and surrounding of medium is clearly transparent. The results for each bacterial isolates were shown in Table 4.5.

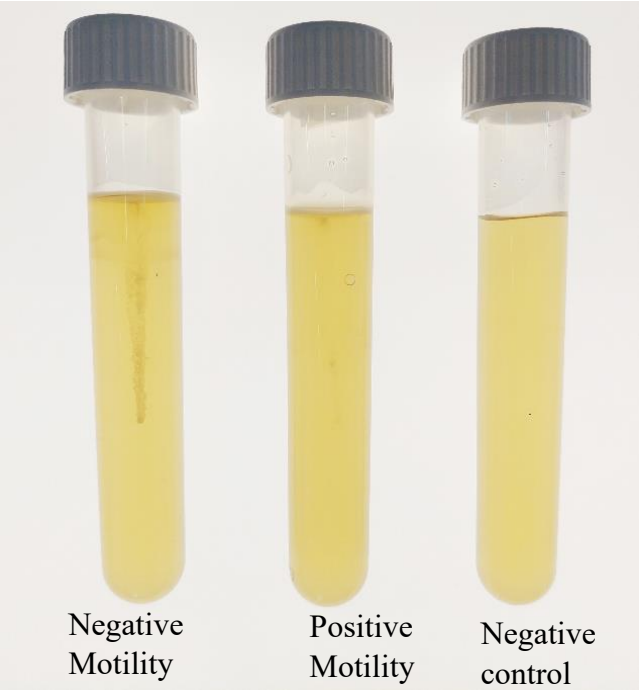

Figure 4.8: The motility test on SIM medium where negative control tube was not stabbed with bacterial isolates. Positive motility test on SIM medium was indicated as the entire stab-line spread throughout the tube. For the negative motility test, the bacterial remained in the stab-line without spreading.

Table 4.4: Reactions for Simmons citrate, oxidase and catalase tests on bacterial isolates.

Bacterial Isolate Designation	Simmons citrate test		Oxidase Test		Catalase Test	
	Observation	Reaction	Observation	Reaction	Observation	Reaction
3P	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
7P	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
1	No color change	Negative	No color change	Negative	Bubbles formed	Positive
2	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
3	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
P	No color change	Negative	Dark blue	Positive	Bubbles formed	Positive
5	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
6	Green to Blue	Positive	Dark blue	Positive	Bubbles formed	Positive
7	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
8	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
10	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
12	Green to Blue	Positive	No color change	Negative	Bubbles formed	Positive
R	No color change	Negative	No color change	Negative	Bubbles formed	Positive
O	No color change	Negative	Dark Blue	Positive	Bubbles formed	Positive

Table 4.5: Reactions for motility and oxidation-fermentation (O-F) tests on bacterial isolates.

Bacterial Isolate Designation	Motility Test		Oxidation-Fermentation (O-F) Test		
	Observation	Reaction	Observation (After 48 Hours)		Reaction
			Aerobic Condition	Anaerobic Condition	
3P	Spread throughout the tube	Positive	Yellow color formed	Yellow color formed	Fermentation
7P	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
1	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
2	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
3	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
P	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
5	Spread throughout the tube	Positive	Yellow color formed	Yellow color formed	Fermentation
6	Confined stab-line	Negative	Yellow color formed	Green color remained	Oxidation
7	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
8	Spread throughout the tube	Positive	Yellow color formed	Yellow color formed	Fermentation
10	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
12	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
R	Confined stab-line	Negative	Yellow color formed	Yellow color formed	Fermentation
O	Spread throughout the tube	Positive	Green color remained	Green color remained	Non-saccharolytic

4.3 Analysis of Extracted DNA and PCR Amplification

4.3.1 Total DNA Extraction

Total DNA of bacterial isolates were extracted. The concentration and purity of extracted DNA was shown in Table 4.6.

Table 4.6: Concentration and purity of extracted DNA.

Bacterial Isolate Designation	Concentration (ng/µL)	Purity (A_{260}/A_{280})
3P	387.1	1.82
7P	778.8	1.78
1	402.1	1.73
2	307.5	1.74
3	509.9	1.92
P	207.8	1.65
5	342.2	2.03
6	287.4	1.77
7	467.6	1.89
8	330.2	1.74
10	359.3	1.98
12	319.6	1.79
R	326.1	1.71
O	390.4	1.68

4.3.2 PCR Screening and Gel Purification of *bla*-TEM and *bla*-SHV Genes

Only one PCR amplicon showed approximately 870 bp DNA fragments when *bla*-TEM primers was used. On the other hand, four PCR amplicons showed approximately 870 bp DNA fragments when *bla*-SHV primers was used in PCR reaction. The gel image of *bla*-TEM and *bla*-SHV amplicons were shown in Figure 4.9 (a) and Figure 4.9 (b) respectively. The purified PCR product gel image was shown in Figure 4.10. The concentration and purity of the purified PCR product was tabulated in Table 4.7.

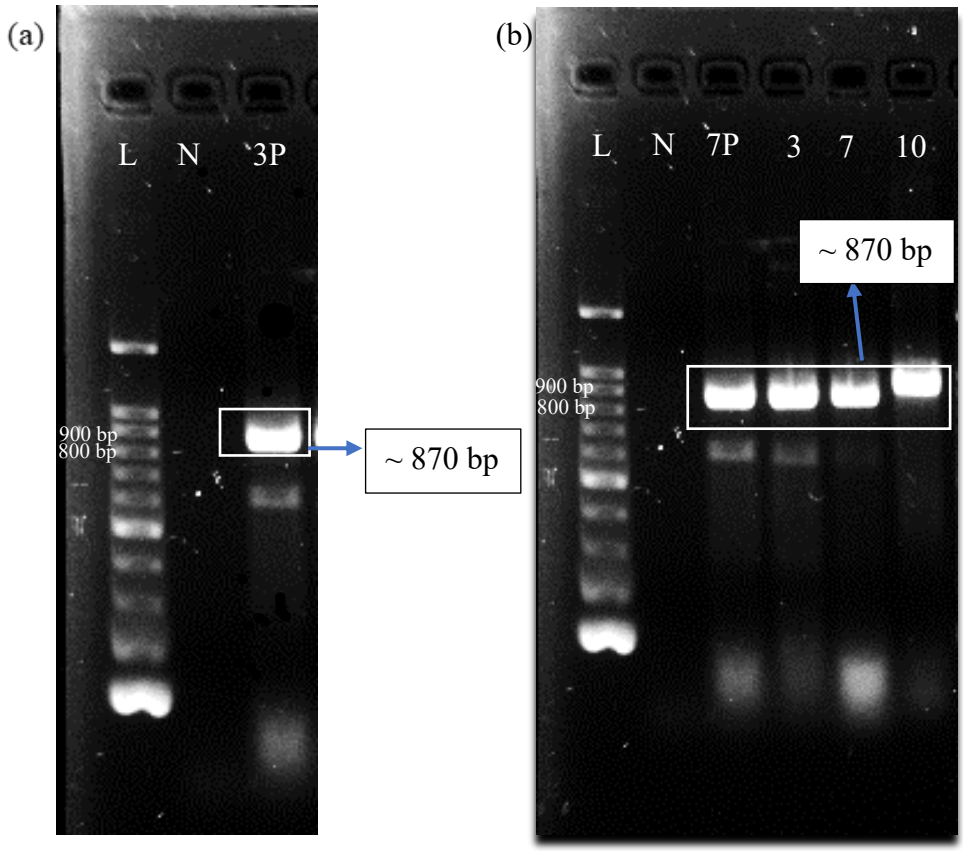

Figure 4.9: Gel image (a) PCR amplicon after amplication with *bla*-TEM primers. Gel image (b) showed PCR amplicons after amplification with *bla*-SHV primers. Lane L represents the 100 bp DNA ladder with concentration of 2 µg. Lane N was negative control.

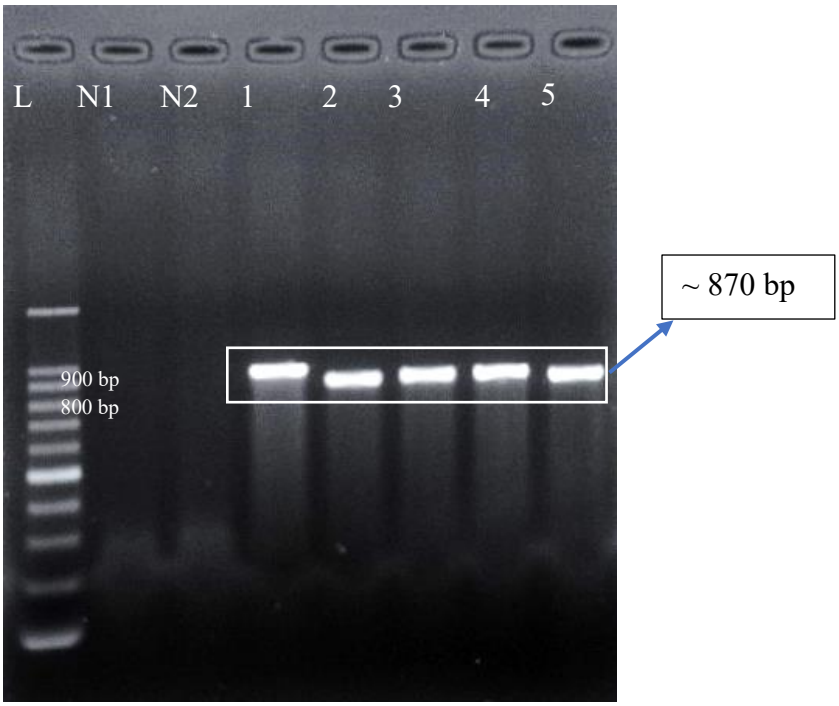

Figure 4.10: Gel image of purified PCR product for *bla*-TEM and *bla*-SHV

Lane L represents the 100 bp DNA ladder with concentration of 2 µg. N1 and N2 represent the negative controls. Lane 1 represents the purified PCR product for *bla*-TEM amplicon from 3P bacterial isolate. Lane 2, 3, 4, 5 represents the purified PCR product for *bla*-SHV amplicon from bacterial isolate 7P, 3, 7, and 10, respectively.

Table 4.7: Concentration and purity of purified PCR products.

Bacterial Isolate Designation	**Concentration (ng/µL)**	**Purity (A_{260}/A_{280})**
3P	35.4	1.99
7P	49.3	1.80
3	57.3	1.88
7	45.1	1.86
10	53.0	1.92

4.4 Molecular Characterization of *bla*-TEM and *bla*-SHV Homologous Genes

4.4.1 DNA Sequencing and BlastX Analysis

The purified PCR products of bacterial isolate 3P, 7P, 3, 7 and 10 was outsources to 1st Base Sdn Bhd for direct sequencing. The PCR product from 3P bacterial isolate was sequenced using *bla*-TEM specific forward primer. For PCR product from 7P, 3, 7 and 10 bacterial isolates were sequenced using *bla*-SHV specific forward primer. The resultant DNA sequences was analyzed using BlastX program and showed in Table 4.8. The obtained DNA sequences using *bla*-SHV specific forward primer was analyzed using BlastX program shown in Tables 4.9, 4.10, 4.11 and 4.12.

4.4.2 Phylogenetic Analysis

The nucleotide sequences were translated into protein sequences using BlastX. The protein sequences were trimmed and then aligned using Cluster X program to generate the phylogenetic tree. Figure 4.15 shown the alignment of trimmed proteins sequences for bacterial isolates 7P, 3, 7 and 10. The Figure 4.16 shown the phylogenetic tree for protein sequences for bacterial isolates 7P, 3, 7 and 10.

4.4.3 Protein Sequences Alignment Analysis

The nucleotide sequences were translated into protein sequences using BlastX. The protein sequences were trimmed and then aligned using Cluster X program to generate figure for Pairwise alignment and Multiple Sequences Alignment.

Table 4.8: DNA sequences using *bla*-TEM specific forward primer and the corresponding top 3 highest similarity BlastX alignment results.

Bacterial Isolate	Sequences	BlastX Alignment Results				
		Highest BlastX Identity	Accession Number	Identities	Score (bits)	E-value
3P	>3P NNNNNNTTCNTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTC ACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAG TTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAG CGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCC AATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTA TCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCAT ACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCAC AGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATG CAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTT ACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTT TTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTT GGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGT GACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAA ACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACA ATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACT TCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAA ATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGC ACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTA CACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGAC AGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGTAAAC CTGTCAGAA	TPA: TEM family class A beta-lactamase [*Enterobacter hormaechei*]	HCD1591924.1	98.92%	534	0.0
		TEM family class A beta-lactamase [*Escherichia fergusonii*]	UAW45603.1	99.64%	531	0.0
		TEM family class A beta-lactamase [*Escherichia coli*]	MCZ5570120.1	99.64%	531	0.0

Table 4.9: DNA sequences using *bla*-SHV specific forward primer and the corresponding top 3 highest similarity BlastX alignment results.

Bacterial Isolate	Sequences	BlastX Alignment Results				
		Highest BlastX Identity	Accession Number	Identities	Score (bits)	E-value
7P	>7p NNNNNNNNNCTGTTAGCACCCTGCCGCTGGCGGTACACGCCA GCCCGCAGCCGCTTGAGCAAATTAAACAAAGCGAAAGCCAGC TGTCGGGCCGCGTAGGCATGATAGAAATGGATCTGGCCAGCGG CCGCACGCTGACCGCCTGGCGCGCCGATGAACGCTTTCCCATG ATGAGCACCTTTAAAGTAGTGCTCTGCGGCGCAGTGCTGGCGC GGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACT ATCGCCAGCAGGATCTGGTGGACTACTCGCCGGTCAGCGAAA AACATCTTGCCGACGGCATGACGGTCGGCGAACTCTGCGCCG CCGCCATTACCATGAGCGATAACAGCGCCGCCAATCTGCTGCT GGCCACCGTCGGCGGCCCCGCAGGATTGACTGCCTTTTTGCGC CAGATCGGCGACAACGTCACCCGCCTTGACCGCTGGGAAACG GAACTGAATGAGGCGCTTCCCGGCGACGCCCGCGACACCACT ACCCCGGCCAGCATGGCCGCGACCCTGCGCAAGCTGCTGACC AGCCAGCGTCTGAGCGCCCGTTCGCAACGGCAGCTGCTGCAG TGGATGGTGGACGATCGGGTCGCCGGACCGTTGATCCGCTCCG TGCTGCCGGCGGGCTGGTTTATCGCCGATAAGACCGGAGCTGG CGAACGGGGTGCGCGCGGGATTGTCGCCCTGCTTGGCCCGAA TAACAAAGCAGAGCGCATTGTGGTGATTTATCTGCGGGATACG CCGGCGAGCATGGCCGAGCGAAATCATCAAATCGTCGGTATTG TCGCGGCGCTGATCGAGCCCTGGCAATGCTAGCCAANTCTTGG NTCTGTTTGTCTNNTGGGAGTANTGGCATTGTATTATGTTATGT TGNAGGAGAGAGAGGAATATAATACGCTTTC	SHV family class A beta-lactamase [*Klebsiella pneumoniae*] SHV family class A beta-lactamase [*Klebsiella pneumoniae*] TPA: SHV family class A beta-lactamase [*Klebsiella pneumoniae*]	WP_130958456.1 EKW1754973.1 HBQ2376680.1	97.82% 97.81% 97.81%	545 544 544	0.0 0.0 0.0

Table 4.10: DNA sequences using *bla*-SHV specific forward primer and the corresponding top 3 highest similarity BlastX alignment results.

Bacterial Isolate	Sequences	BlastX Alignment Results				
		Highest BlastX Identity	Accession Number	Identities	Score (bits)	E-value
3	>3 NNNNNCTGATGCCGNNTGCCACTGGCGGTATTCGCCAGCCCTCAGCCGCT TGAGCAGATTAAAATCAGCGAAGGTCAGCTGGCGGGCCGGGTGGGCTAT GTTGAAATGGATCTGGCCAGCGGCCGCACGCTGGCCGCCTGGCGCGCCA GTGAGCGCTTTCCGCTGATGAGCACCTTTAAAGTGCTGCTCTGCGGCGCG GTGCTGGCCCGGGTGGATGCCGGCGACGAACAGCTGGATCGGCGGATCC ACTACCGCCAGCAGGATCTGGTGGACTACTCCGCGGTCAGCGAAAAACAC CTTGCCGACGGGATGACCGTTGGCGAACTCTGCGCCGCCGCCATCACCAT GAGCGACAACAGCGCCGGCAATCTGCTGTTGAAGAGCGTCGGCGGCCCC GCGGGATTGACCGCTTTTCTGCGCCAGATCGGTGACAACGTCACCCGCCT TGACCGCTGGGAAACGGAACTCAATGAGGCGCTTCCCGGCGACGTGCGC GACACCACCACCCCGGCCAGCATGGCCACCACCCTGCGCAAGCTGCTAAC CACCCCCTCTCTGAGCGCCCGTTCGCAGCAGCAGCTGCTGCAGTGGATGG TTGACGACCGGGTGGCCGGCCCGTTGATCCGCGCCGTGCTGCCGGCGGG CTGGTTTATCGCCGATAAAACCGGGGCCGGTGAGCGGGGCTCACGCGGC ATTGTCGCCCTGCTCGGCCCGGACGGCAAAGCGGAGCGTATCGTGGTGAT CTATCTACGGGATACCGCGGCGACCATGGCCGAACGTAACCAGCAGATCGC CGGGATAGGCGCGGCGCTGATCGAGCACTGGCAACGCTAACCACTATTGC AGTATATGTTNNNTCTAACTGGGCTCCTTTAAATTTGGTGTTAAANTTTGTG	Broad-spectrum class A beta-lactamase OKP-B-2 [*Klebsiella*]	WP_063860914.1	99.63%	519	0.0
		SHV-LEN [uncultured bacterium]	AMJ38059.1	99.26%	517	0.0
		broad-spectrum class A beta-lactamase OKP-B-6 [*Klebsiella*]	WP_004205995.1	99.26%	517	0.0

Table 4.11: DNA sequences using *bla*-SHV specific forward primer and the corresponding top 3 highest similarity BlastX alignment results.

Bacterial Isolate	Sequences	BlastX Alignment Results				
		Highest BlastX Identity	Accession Number	Identities	Score (bits)	E-value
7	>7 NNCNNNCNGATGCCNCCTGCCACTGGCGGTATTCGCCAGCCCT CAGCCGCTTGAGCAGATTAAAATCAGCGAAGGTCAGCTGGCGG GCCGGGTGGGCTATGTTGAAATGGATCTGGCCAGCGGCCGCAT GCTGGCCGCCTGGCGCGCCAGTGAGCGCTTTCCGCTGATGAGC ACCTTTAAAGTGCTGCTCTGCGGCGCTGTGCTGGCCCGGGTGG ATGCCGGCGACGAACAGCTGGATCGGCGGATCCACTACCGCCA GCAGGATCTGGTGGACTACTCCCCCGTCAGCGAAAAACACCTT GCCGACGGGATGACCGTTGGCGAACTCTGCGCCGCCGCCATCA CCATGAGCGACAACAGCGCCGGCAATCTGCTGTTGAAGAGCGT CGGCGGCCCCGCGGGATTGACCGCTTTTCTGCGCCAGATCGGT GACAACGTCACCCGCCTTGACCGCTGGGAAACGGAACTCAATG AGGCGCTTCCCGGCGACGTGCGCGACACCACCACCCCGGCCA GCATGGCCACCACCCTGCGCAAGTTGCTAACCACCCCCTCTCT GAGCGCCCGTTCCCAGCAGCAGCTGCTGCAGTGGATGGTGGAC GACCGGGTGGCCGGCCCGTTGATCCGCGCCGTGCTGCCGGCGG GCTGGTTTATCGCCGATAAAACCGGGGCCGGTGAGCGGGGCTC ACGCGGCATTGTCGCCCTGCTCGGCCCGGACGGCAAAGCGGA GCGTATCGTGGTGATCTATCTGCGGGATACCGCGGCGACCATGG CCGAACGTAACCAGCAGATCGCCGGGATAGGCGCGGCGCTGAT CGAGCACTGGCAANGCTAACCAAT	broad-spectrum class A beta-lactamase OKP-B-1 [*Klebsiella*]	WP_032456002.1	100.00%	521	0.0
		OKP family broad-spectrum class A beta-lactamase [*Klebsiella quasipneumoniae*]	WP_049092654.1	99.63%	520	0.0
		OKP family broad-spectrum class A beta-lactamase [*Klebsiella quasipneumoniae*]	WP_163619144.1	99.63%	520	0.0

Table 4.12: DNA sequences using *bla*-SHV specific forward primer and the corresponding top 3 highest similarity BlastX alignment results.

Bacterial Isolate	Sequences	BlastX Alignment Results				
		Highest BlastX Identity	Accession Number	Identities	Score (bits)	E-value
10	>10 NNNNNCCTGTTAGCNCCCTGCCACTGGCGGTAGACGCCGGTCC ACAGCCGCTTGAGCAGATTAAACAAAGCGAAAGCCAGCTGTC GGGCCGCGTGGGGATGGTGGAAATGGATCTGGCCAGCGGCCG CACGCTGGCGGCCTGGCGCGCCGATGAACGCTTTCCCATGGTG AGCACCTTTAAAGTGCTGCTGTGCGGCGCGGTGCTGGCGCGG GTGGATGCCGGGCTCGAACAACTGGATCGGCGGATCCACTACC GCCAGCAGGATCTGGTGGACTACTCCCCGGTCAGCGAAAAAC ACCTTACCGACGGGATGACGATCGGCGAACTCTGCGCCGCCGC CATCACCCTGAGCGATAACAGCGCTGGCAATCTGCTGCTGGCC ACCGTCGGCGGCCCCGCGGGATTAACTGCCTTTCTGCGCCAGA TCGGTGACAACGTCACCCGTCTTGACCGCTGGGAAACGGCAC TGAATGAGGCGCTTCCCGGCGACGCGCGCGACACCACCACCC CGGCCAGCATGGCCGCCACGCTGCGCAAACTACTGACCGCGC AGCATCTGAGCGCCCGTTCGCAACAGCAACTCCTGCAGTGGAT GGTGGACGATCGGGTTGCCGGCCCGCTGATCCGCGCCGTGCTG CCGCCGGGCTGGTTTATCGCCGACAAAACCGGGGCTGGCGAA CGGGGTGCGCGCGGCATTGTCGCCCTGCTCGGCCCGGACGGC AAACCGGAGCGCATTGTGGTGCTCTATCTGCGGGATACCCCGG CGAGTATGGCCGAGCGTAATCAACATATCACCGGGATCGGCGC AGCGCTGATCGAGCACTGTCGACGCGTAACCACGACCTGCTG GGATTTGCCATGTGAAGTGTAGATTCTCTAGTTTATACACG	class A beta-lactamase LEN-10 [Klebsiella variicola]	WP_022065596.1	97.82%	541	0.0
		MULTISPECIES: class A beta-lactamase LEN-18 [Klebsiella]	WP_063860820.1	97.45%	540	0.0
		MULTISPECIES: class A beta-lactamase LEN-27 [Klebsiella]	WP_008804981.1	97.45%	540	0.0

```
CLUSTAL O(1.2.4) multiple sequence alignment


1TEM_1|Chain      HPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRID  60
3P                HPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRPEERFPMMSTFKVLLCGAVLSRVD  60
                  ************************************************************:*

1TEM_1|Chain      AGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGP  120
3P                AGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVRELCSAAITMSDNTAANLLLTTIGGP  120
                  ************************************************************

1TEM_1|Chain      KELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPVAMATTLRKLLTGELLTLASRQ  180
3P                KELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTMPAAMATTLRKLLTGELLTLASRQ  180
                  ************************************.***********************

1TEM_1|Chain      QLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTG  240
3P                QLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGSRGIIAALGPDGKPSRIVVIYTTG  240
                  ************************************************************

1TEM_1|Chain      SQATMDERNRQIAEIGASLIKH        262
3P                SQATMDERNRQIAEIGASLIKH        262
                  **********************
```

Figure 4.11: Pairwise alignment of translated nucleotide sequences of TEM-1 (1TEM_1) with bacteria isolate 3P translated sequence.

```
CLUSTAL O(1.2.4) multiple sequence alignment


3_SHV_F           PLAVFASPQPLEQIKISEGQLAGRVGYVEMDLASGRTLAAWRASERFPLMSTFKVLLCGA    60
7_SHV_F           PLAVFASPQPLEQIKISEGQLAGRVGYVEMDLASGRMLAAWRASERFPLMSTFKVLLCGA    60
pdb|1VM1|A        PLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKVVLCGA    60
                  ****.**********:**.**:**** :******** *:****.****:******:****

3_SHV_F           VLARVDAGDEQLDRRIHYRQQDLVDYSAVSEKHLADGMTVGELCAAAITMSDNSAGNLLL    120
7_SHV_F           VLARVDAGDEQLDRRIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAGNLLL    120
pdb|1VM1|A        VLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAANLLL    120
                  ************:*:************ *************************.****

3_SHV_F           KSVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDVRDTTTPASMATTLRKLLTTPSL    180
7_SHV_F           KSVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDVRDTTTPASMATTLRKLLTTPSL    180
pdb|1VM1|A        ATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRL    180
                   :**********************************.**********:*******:  *

3_SHV_F           SARSQQQLLQWMVDDRVAGPLIRAVLPAGWFIADKTGAGERGSRGIVALLGPDGKAERIV    240
7_SHV_F           SARSQQQLLQWMVDDRVAGPLIRAVLPAGWFIADKTGAGERGSRGIVALLGPDGKAERIV    240
pdb|1VM1|A        SARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARGIVALLGPNNKAERIV    240
                  *****:****************:******************:*********:.******

3_SHV_F           VIYLRDTAATMAERNQQIAGIGAALIE     267
7_SHV_F           VIYLRDTAATMAERNQQIAGIGAALIE     267
pdb|1VM1|A        VIYLRDTPASMAERNQQIAGIGAALIE     267
                  ******* *:*****************
```

Figure 4.12: Multiple sequences alignment of translated nucleotide sequences of SHV-1 (1VM1) with bacteria isolate 3 and 7 translated sequences.

```
CLUSTAL O(1.2.4) multiple sequence alignment

10_SHV_F        PLAVDAGPQPLEQIKQSESQLSGRVGMVEMDLASGRTLAAWRADERFPMVSTFKVLLCGA    60
7p_SHV_F        PLAVHASPQPLEQIKQSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKVVLCGA    60
pdb|1VM1|A      PLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADERFPMMSTFKVVLCGA    60
                ****.*.******** ***********:**********:**********:*****:****

10_SHV_F        VLARVDAGLEQLDRRIHYRQQDLVDYSPVSEKHLTDGMTIGELCAAAITLSDNSAGNLLL    120
7p_SHV_F        VLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAANLLL    120
pdb|1VM1|A      VLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCAAAITMSDNSAANLLL    120
                ******** ***:*:******************:****:*********:*****.****

10_SHV_F        ATVGGPAGLTAFLRQIGDNVTRLDRWETALNEALPGDARDTTTPASMAATLRKLLTAQHL    180
7p_SHV_F        ATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRL    180
pdb|1VM1|A      ATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPASMAATLRKLLTSQRL    180
                ************************** **************************:*:*

10_SHV_F        SARSQQQLLQWMVDDRVAGPLIRAVLPPGWFIADKTGAGERGARGIVALLGPDGKPERIV    240
7p_SHV_F        SARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARGIVALLGPNNKAERIV    240
pdb|1VM1|A      SARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARGIVALLGPNNKAERIV    240
                *****:*****************:*** ************************:.* ****

10_SHV_F        VLYLRDTPASMAERNQHITGIGAALIE     267
7p_SHV_F        VIYLRDTPASMAERNHQIVGIVAALIE     267
pdb|1VM1|A      VIYLRDTPASMAERNQQIAGIGAALIE     267
                *:*************::*.** *****
```

Figure 4.13: Multiple sequences alignment of translated nucleotide sequences of SHV-1 (1VM1) with bacteria isolate 7P and 10 translated sequence.

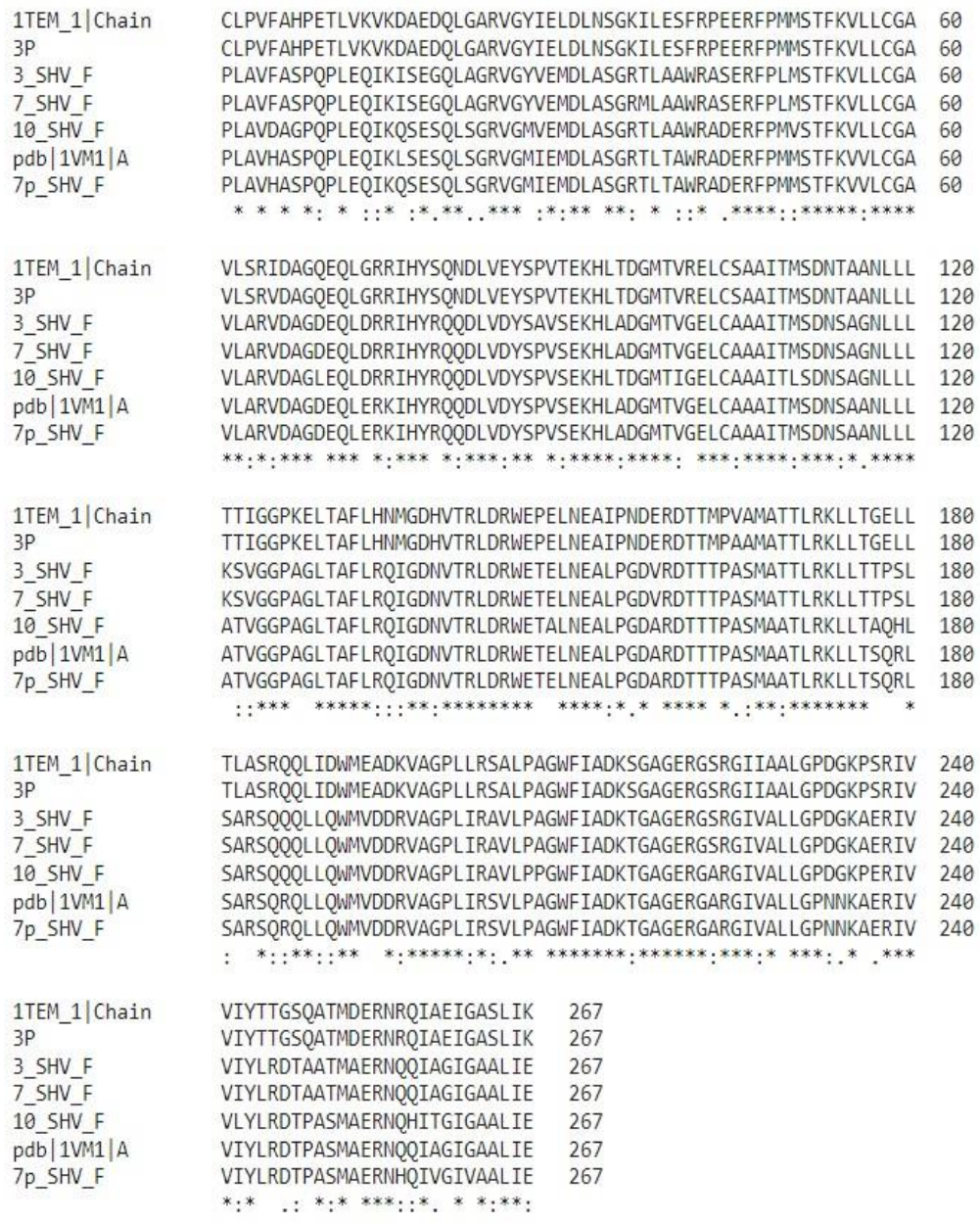

Figure 4.14: Multiple sequences alignment of translated nucleotide sequences. 1VM1 was SHV-1 protein sequence while 1TEM_1 was TEM-1 protein sequences. The alignment also includes translated SHV proteins sequences for bacterial isolates 7P, 3, 7 and 10 together with translated TEM proteins sequences for bacterial isolate 3P.

Figure 4.15: The alignment of translated SHV proteins sequences for bacterial isolates 7P, 3, 7 and 10.

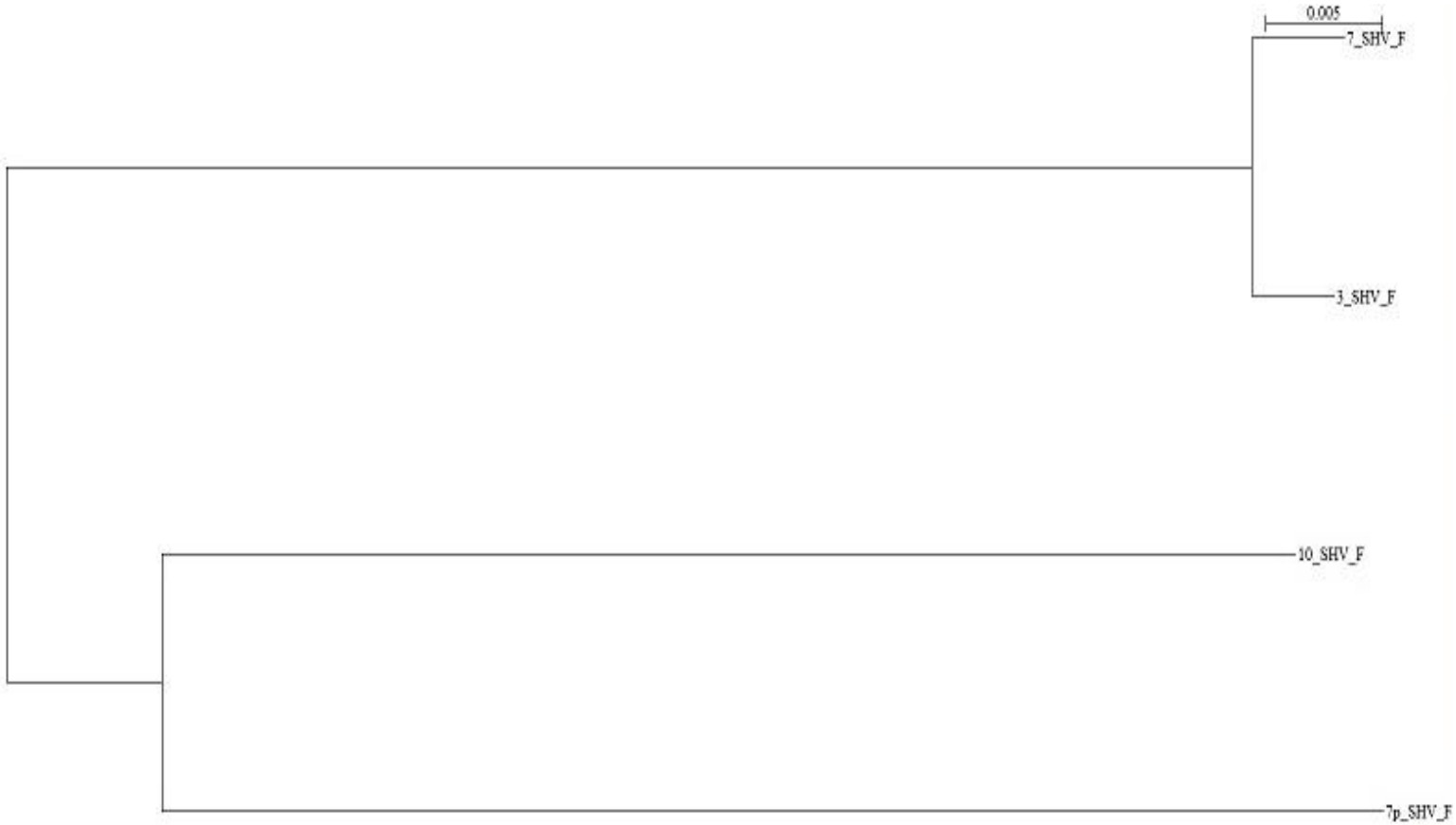

Figure 4.16: Phylogenetic tree for bla-SHV protein sequences from bacterial isolates 7P, 3, 7 and 10

CHAPTER 5

DISCUSSION

5.1 Cultivation, Isolation and Preliminary Screening of Bacterial Isolates from Soil Samples

In this study, the isolation and selection of possible bacterial isolates from the soil samples were carried out using Luria-Bertani agar supplemented with 50 µg/ mL ampicillin. The purpose of using Luria-Bertani agar supplemented with ampicillin was to screen for potential bacteria that was capable of producing beta-lactamases. Besides that, the composition of Luria-Bertani agar was suitable and commonly used in bacterial cultivation as most of the bacteria can grow well in such medium. Fourteen bacterial isolates with different morphological of characteristics were successfully isolated from soil samples and as shown in Table 4.1.

5.2 Preliminary Characterization of Bacterial Isolates

All the bacterial isolates were further characterized and differentiated by using of EMB agar and selected biochemical tests. There included Gram staining, citrate test by Simmons citrate agar, oxidase, catalase, oxidation-fermentation (O-F) and motility tests.

5.2.1 Gram Staining

Gram staining was used to classify the bacteria into two groups: Gram-positive and Gram-negative bacteria. The Gram-positive bacteria will retain the purple color from crystal violet dye due to thicker cell wall made from peptidoglycan. The decolorized step dehydrates peptidoglycan layer that causes shrinking which trapping the crystal violet-iodine complex. For Gram-negative bacteria, the pink color of counterstain from safranin dye was retained as it is not able to retain the crystal violet-iodine complex due to thinner peptidoglycan cell wall.

All thirteen bacterial isolates were classified as gram-negative bacteria after Gram staining, with one exceptional that identified as Gram-positive bacterial designated “O”.

5.2.2 Selective and Differentiation Medium: Eosin Methylene Blue (EMB) Agar

Eosin Methylene Blue (EMB) agar is a selective and differential medium which contain methylene blue dye that can inhibit the growth of Gram-positive bacteria but allowing Gram-negative bacteria to grow. Eosin dye will acts as pH indicator that responds to changes in pH from colorless to black under acidic condition. The lactose in the EMB agar can used to differentiate the lactose and non-lactose fermenters by reacting with both methylene blue and eosin dye to produce a colored colony. For the Gram-negative bacteria lactose fermenter, acid will be produced by giving color between pink to purple or metallic green sheen. Non-lactose fermenter does not produced acid which form colorless colonies (Lal and Cheeptham, 2007).

All eleven bacterial isolates were classified as Gram-negative lactose fermenter. Two bacterial isolates were classified as Gram-negative non-lactose fermenter and one did not growth on the EMB agar.

5.2.3 Simmons Citrate Agar Test

Simmons Citrate Agar is a medium used to distinguish members of *Enterobacteriaceae* family based on the metabolic by product. The medium is supplemented with citrate salt that could be utilized by bacteria as a carbon and energy source. This would subsequently increase the pH of the medium that causes pH indicator as bromothymol blue having color changes from green to blue. Sodium citrate was used as a carbon and carbon dioxide was produced. Reaction with sodium ion led to the formation sodium carbonated which is alkaline leading to increase the pH of the medium to produce blue color (MacWilliams, 2009). Blue color indicates the positive citrate test while no color changes indicate negative citrate test.

All ten bacterial isolates were classified as positive citrate test while four bacterial isolates classified as negative citrate test.

5.2.4 Oxidase Test

Oxidase test is a biochemical test used to determine the presence of cytochrome c oxidase. The reagent contains 1% tetra-methyl-p-phenylenediamine dihydrochloride which is an electron-rich compound. This enzyme would react with tetramethyl-p-phenylenediamine (TMPD) by oxidation whereby the

electron will be donated to cytochrome c oxidase to produce oxidized TMPD radical which is indicated as dark blue color (Shields and Cathcart, 2010).

Among fourteen bacterial isolates, three bacterial isolates were classified as positive oxidase test while the other 11 bacterial isolates showed negative oxidase test.

5.2.5 Catalase Test

Catalase test was used to the determine presence of catalase enzyme that can neutralize the toxic substances produced during the respiration such as hydrogen peroxide in both aerobic and facultative anaerobic bacteria. High amount of this byproduct accumulation can damage the cell and subsequently lead to cell death. In order to survive, catalase enzyme was produced by bacteria as a defense mechanism to neutralize hydrogen peroxide by degradation it to form water and oxygen (Reiner, 2010). The oxygen was the main component that give rise to the bubble formation indicating the presence of catalase enzyme.

All fourteen bacterial isolates shown positive catalase test where they are able to produce catalase enzyme as detoxification mechanism.

5.2.6 Oxidation-Fermentation (O-F) Test

Oxidation-fermentation test is a basal medium is supplemented with different monosaccharide to test for the ability of bacteria to metabolize the carbohydrate under oxidation or fermentation condition. In this study, glucose was used as a

only carbohydrate in the oxidation-fermentation test. Oxidation was allowed to occur in the aerobic condition while fermentation occurred in anaerobic conditions. The production of metabolites that reduce the pH in both aerobic and anaerobic conditions can be detected by the pH indicator which is bromothymol blue. The acidic metabolites produced can turn the color of bromothymol blue from green to yellow. Bacteria undergoes oxidation in aerobic condition only know as oxidative bacteria. Bacteria undergoes oxidation and fermentation in aerobic and anaerobic conditions are known as fermentative bacteria. On the other hand, bacteria undergoes neither oxidation nor fermentation are known as non-saccharolytic bacteria, they do not metabolize glucose or other carbohydrates in both aerobic and anaerobic conditions (Hanson, 2008).

Among fourteen bacterial isolates, only twelve bacterial isolates shown positive oxidation and fermentation of glucose in the O-F test. While only one bacterial isolate showed positive oxidation while the other one showed non-saccharolytic for glucose supplemented O-F test.

5.2.7 Sulfide Indole Motility (S.I.M) Test

Motility test medium is used to determine whether the bacteria have the ability to move with the presence of flagella like propeller in the medium. For motile bacteria that is motile, the bacteria can spread out from the stab-line throughout the medium after 48 hours of incubation. For bacteria that is non-motile, the bacteria will be confined in the stab-line and do not spread across the medium (Shields and Cathcart, 2011). Among fourteen bacterial isolates, only four

bacterial isolates showed positive motility test. The other ten bacterial isolates shown negative motility test.

5.3 Total DNA Extraction

The DNA from bacterial isolates was extracted by using the fast-boiling method. This method was chosen as it was easy to perform, cost effective, high quality and remarkable quantity of DNA can be obtained. Total DNA extracted from all the bacterial isolates were measured with Nanodrop 1000, giving a concentration of DNA ranging from 207.8 ng/µL to 778.8 ng/µL with the purity of DNA between 1.65 to 2.03. The 260 nm ratio 280 nm indicated the purity of DNA extracted. The purity with a rate of less than 1.8 was due to protein contamination while for the purity more than 1.8 was due to contamination of RNA.

5.4 Polymerase Chain Reaction and Gel Electrophoresis

The polymerase chain reaction is a molecular technique that been widely used to detect the specific region by amplification of the region of a DNA molecule with suitable primers. The technique involves the use of *Taq* polymerase, dNTPs, specific primers under series of repeating cycle that involved denaturation, takes annealing and extension of DNA polymer.

In this study, both *bla*-TEM and *bla*-SHV primers were used for the amplification of specific regions for beta-lactamase. Both set of primers gave

the approximate amplicons sizes of 870 bp for all samples. The gel image for *bla*-TEM amplification was shown in Figure 4.9 (a) and gel image for *bla*-SHV amplification was shown in Figure 4.9 (b).

By analyzing the gel image for *bla*-TEM amplification, only one bacterial isolate showed the band size of amplicon with approximately 870 bp. For gel image for *bla*-SHV amplification, four bacterial isolates showed band size of amplicon at approximately 870 bp. The presence of the faint band at the gel image under the amplicons was a amplicon that due to non-specific amplification while for the faint clump at the bottom of the gel could be due to primer dimmer. PCR products that show positive *bla*-TEM amplification corresponded to 3P while PCR products for *bla*-SHV amplification were 7P, 3, 7, and 10.

5.5 DNA Purification

DNA purification is an important step to purify the target amplicon to eliminate the unnecessary contaminant like primer-dimer, non-specific amplification of unwanted amplicons. This is to ensure that the specific homologous gene was obtained.

After DNA purification, the amplicons were then analyzed by agarose gel electrophoresis. The clear distinct band of approximately 870 bp was observed as shown in Figure 4.10. The concentration and purity measured by Nanodrop 1000 was ranged between 35.4 ng/μL to 57.3 ng/μL and with the purity ranged of 1.80 to 1.99.

5.6 DNA Sequencing and BlastX Analysis

Based on the BlastX analysis results, the nucleotide sequences were translated into protein sequences. The translated sequences of each bacterial isolates were compared to the NCBI data base. The translated sequences of bacterial isolates designated as 3P was confirmed related to the TEM family class A beta-lactamase with 99.64% identity to *Escherichia* and 98.92% identity to *Enterobacter* family. For the bacterial isolate 3P, the translated sequence showed highly similar to SHV family class A beta-lactamase. It has 97.81% to 97.82% identity to *Klebsiella pneumoniae*. Bacterial isolate designated as 3 was highly similar to *Klebsiella pneumoniae* with broad-spectrum class A beta-lactamase OKP-B-2 and OKP-B-6 with identity of 99.63% and 99.26% respectively. It also has an identity of 99.26% to SHV-LEN type beta-lactamase from unculture bacterium. On the other hand, bacterial isolate 7 was highly similar to broad-spectrum class A OKP-B-1 beta-lactamase and OKP family broad-spectrum class A beta-lactamase with identity of 100% and 99.63% respectively. It is belonging to *Klebsiella quasipneumoniae*. Lastly, bacterial isolate 10 was similar to class A beta-lactamase LEN-10, LEN-18 and LEN-27 with identity of 97.82%, 97.45% and 97.45% respectively. It is belonged to *Klebsiella spp.*

5.7 Multiple Sequences Alignment and Pairwise Alignment Analysis

From the pairwise alignment analysis of Figure 4.11, the bacterial isolate 3P have conservative amino acids substitution at I59V and semi-conservative amino acids substitution V159A when compared with TEM-1 protein sequence. Result from multiple sequences alignment (MSA) of Figure 4.12, the bacterial isolate 3 and 7 both have non-conservative amino acids substitution at M28Y, A121K, Q178P and R179S but bacterial isolate 3 has additional non-conservative amino acids substitution at P88A while bacterial isolate 7 has additional non-conservative amino acids substitution at T38M and P248A. From Figure 4.13, bacterial isolate 7P has non-conservative amino acids substitution at D69L, E149A, A208P, A236P while bacterial isolate 10 has non-conservative amino acids substitution at G262V when compared with SHV-1. The bacterial isolate 7P and 10 has common non-conservative amino acids substitution at L16Q. On the other hand, both TEM-type and SHV-type have conserved serine β-lactamase motifs responsible for catalytic activity or substrate binding. The conserved class A serine beta-lactamase motifs are responsible for catalytic activity are S-X-X-K, S-D-N, and K-(S/T)-G. By analyzing MSA showed in Figure 4.14, TEM-1, SHV-1, 3P, 7P, 3, 7, and 10 have the same motifs responsible for catalytic activity which is S-T-F-K, S-D-N, and only different between TEM-type related beta-lactamase was K-S-G while SHV-type related beta-lactamase was K-T-G.

5.8 Phylogenetic Analysis

Using the phylogenetic analysis, the bacterial isolate 3 and 7 was sister group. as non-conservative amino acids substitution was 2 location which are T37M and A89P. The bacterial isolate 7P and 10 was sister group as non-conservative amino acids substitution was located at L69D, E149A, A208P, A236P and V262G. The bacterial isolate 7P and 10 was closer to the SHV-1 as the evolutionary distance was lesser when compared with the relationship between SHV-1 and bacterial isolate 3 and 7. Lastly, the phylogenetic tree showed in Figure 4.16, beta-lactamase of bacterial isolate 7 and 3 shared a common ancestor while 7P and 10 shared a common ancestor.

5.9 Future Studies

For future studies, more biochemical tests should be included in characterization of the bacterial isolates obtained from soil samples for more detailed investigation. API 20 bacterial identification kit can also be used in characterization of the isolated bacterial isolated. Besides that, other beta-lactamase specific primers can be used to amplify other possible beta-lactamases genes and not only restricted to *bla*-TEM and *bla*-SHV. Next, the antibiotics susceptibility tests could also be include and screen for the extended-spectrum beta-lactamase for further identification. The 16S rRNA gene sequencing could be carry out in future studies to further validate its identity of the bacterial strains.

CHAPTER 6

CONCLUSION

In this project, a total of fourteen ampicillin resistant bacterial isolates were isolated. These bacterial isolates were then subjected to selected biochemical tests. Eleven fulfilled the requirements of the *Enterobacteriaceae* family which are Gram-negative, catalase-positive, oxidase-negative and fermentation reaction. Only one bacterial isolate, 3P was correspond of bla-TEM homologous gene while the other four bacterial isolates designated as 7P, 3, 7, and 10 consisted of bla-SHV homologous genes with an expected band size of 870 bp.

The DNA sequencing results were carried out using BlastX, multiple sequence alignment and phylogenetic tree analysis. BlastX results showed that translated DNA sequences of bacterial isolate 3P illustrated high similarities to TEM class A beta-lactamase belonging to the *Escherichia* genus. For bacterial isolate 7P, it has a high similarity to SHV class A beta-lactamase from *Klebsiella pneumoniae*. Bacterial isolate 10 showed high similarity to LEN class A beta-lactamase gene fragment of *Klebsiella pneumoniae*. Besides that, the translated gene fragment 3 and 7 illustrated high similarity to broad spectrum OKP-B-2 and OKP-B-1 beta-lactamase of *Klebsiella* genus respectively.

The analysis of multiple sequence alignment of translated DNA of 3, 7 and SHV-1 bacteria showed non-conservative amino acids substitution at seven locations. They are M27Y, T37M, P88A, A121K, Q178T, R179S, P248A. For the alignment of 7P,

10 and SHV-1 showed non-conservative amino acids substitution were showed at five locations. They corresponded to D69L, E49A, A208P, A235P, and G262V. Phylogenetic analysis demonstrated that translated sequences of 7P and 10 is a sister group that shared a common ancestor with shorter evolutionary distance as compared to sister group of 3 and 7. The analysis also suggested that bacterial isolates 7P was more closely related to SHV-1 when compared to bacterial isolates 3 and 7 while bacterial isolates 7P was slightly heterogeneous.

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