MOLECULAR CHARACTERISATION OF PLASMID-BORNE *bla*-SHV

HOMOLOGOUS GENES FROM ENTEROBACTERIACEAE

By

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ABSTRACT

MOLECULAR CHARACTERISATION OF PLASMID-BORNE *bla*-SHV

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The *bla*-SHV gene is commonly found in the family of Enterobacteriaceae such as *Klebsiella pneumoniae* and *Escherichia coli*. The bacterial strains are able to confer resistance to broad-spectrum penicillins such as ampicillin but not to the oxymino cephalosporins. The development of extended-spectrum cephalosporins or monobactams such as cefotaxime and ceftazidime against beta-lactamase producing bacteria has resulted in selection of bacteria which carry ESBL gene on their plasmids. This mutated gene is able to produce extended-spectrum of beta-lactamases which are active against oxyimino cephalosporins and monobactams. In this study, twenty-two bacteria isolates were selected for plasmid extraction using the alkaline lysis methodology. They were subjected to PCR amplification using *bla*-SHV specific primers. Only 17 extracted plasmids were shown to harbour plasmids containing *bla*-SHV genes, as demonstrated with the presence of gene fragments with expected size of approximately 870 bp. Out of the 17 plasmid-borne SHVs, 10 were chosen for subsequent gene purification. Subsequently, only six were selected for ligation into pGEM-T for transformation process. Two fragments labelled as TL9 and SB3 were successfully transformed

into *E. coli* strain JM109. The white bacterial colonies were randomly chosen for colony PCR amplification. The purified recombinant plasmids with the desired sized recombinant fragment were sequenced. Based on BlastX and BlastN results, TL9 was identified as non-ESBL *bla*-SHV-1 gene while SB3 was identified as penicillinase *OKP*-5 gene. This study suggested that gene encoding non-ESBLs such as *bla*-SHV-1 are located on plasmids in ESBL producing strains as demonstrated by TL9 bacterial isolate.

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DECLARATION

I hereby declare that the 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.

_____

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APPROVAL SHEET

This project report entitled "**MOLECULAR CHARACTERISATION OF PLASMID-BORNE** *bla***-SHV HOMOLOGOUS GENES FROM ENTEROBACTERIACEAE**" was prepared by NG WEI KHIANG and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **NG WEI KHIANG** (ID No: **08ANB03920**) has completed this final year project entitled "**MOLECULAR CHARACTERISATION OF PLASMID-BORNE *bla*-SHV HOMOLOGOUS GENES FROM ENTEROBACTERIACEAE**" supervised by Asst. Prof. Dr. Choo Quok Cheong (Supervisor) from the Department of Biological Science, Faculty of Science.

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

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LIST OF ABBREVIATIONS

bla-SHV	Beta-lactamase Sulfhydryl Variable
CaCl$_2$	Calcium Chloride
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum Beta-lactamase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani
PCR	Polymerase Chain Reaction
psi	pound-force per square inch
rpm	revolutions per minute
TBE	Tris/Borate/EDTA
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER 1

INTRODUCTION

Raw vegetables and fruits are important vehicles of foodborne illness. Through globalization of food distribution, contaminated food products have been reported to affect people in numerous countries. In recent years, the frequency of outbreaks associated with raw vegetable consumption has increased in developing countries. According to the Centre for Disease Control and Prevention (2011), 1 out of 6 Americans get sick, 128,000 are hospitalized and about 3,000 die from foodborne diseases each year. Foodborne diseases normally result from consumption of contaminated food and these include infections caused by pathogen or parasites that contaminate food at different level in food preparation process.

Foodborne disease such as diarrheal kills 1.8 million children every year worldwide (WHO, 2007). The most significant outbreaks were caused by consumption of raw vegetable. In year 1996, the outbreak of *Escherichia coli* O157:H7 in Japan were reported with approximately 10,000 cases with most cases involving school-age children. Further investigation suggested that radish sprouts were the main cause of the infection (National Institute of Health & Infectious Disease Control Division, Ministry of Health and Welfare of Japan, 1996).

The widespread use of antimicrobial agents in fighting bacterial diseases led to the emergence of bacteria strains that are resistant towards commonly used antibiotics (Levy, 1998). The family Enterobacteriaceae are well known to confer resistance to antimicrobial agents with serious infections due to the production of β-lactamase which could cleave β-lactam antibiotics (Paterson, 2006). In the early 1980s, the introduction of the third-generation cephalosporin into treatment caused further selection of bacteria strains that could produce extended spectrum beta-lactamases (ESBLs). These resistances include ceftriaxone, cefotaxime, ceftazidime and aztreonam (Bradford, 2001).

ESBL genes evolved from Class A beta-lactamases such as TEM-1 or SHV-1 which are generally plasmid-borne ESBL producers. Most SHV ESBLs are plasmid encoded by mobilization of non-ESBL *bla*-SHV-1 and *bla*-SHV-11 from the *K. pneumoniae* chromosome onto the plasmids (Ford & Avison, 2004). The occurrence of ESBL producing Enterobacteriaceae is of major concern in hospitals as they are responsible for urinary tract infection, septicaemia and hospital acquired pneumonia. So, various infection control precaution should be taken to minimize the rate of transmission and infections (Bhattacharya, 2006).

A total of 20 Enterobacteriaceae and 2 *Klebsiella sp.* isolates that were preliminary screened for *bla*-SHV gene were used in this study (Eo, 2010; Ng, 2011). The objectives of this study include:

- To screen the bacterial isolates with plasmid-borne *bla*-SHV gene,

- To amplify and sequence *bla*-SHV homologous gene,

- To obtain information of the *bla*-SHV homologous gene by performing BlastN and BlastX alignment search.

CHAPTER 2

LITERATURE REVIEW

2.1   Major Health Issues Involving Consumption of Vegetables

Based on the Department of Agriculture of United States (2009), the reason vegetables are eaten on daily basis is because they provide nutrients for growth and maintenance of health. Since most of the vegetables are naturally low in fat and calories, having a diet rich in vegetables may reduce risk for stroke and other cardiovascular diseases. For examples, a case study conducted in the Netherlands to investigate whether raw or processed vegetable consumption will affect the coronary heart disease (CHD) incidence showed that for a period of 10 years, the risk of CHD was 34% lower for participants with a high intake of total fruit and vegetables compared to the low intake participants (Oude Griep, Geleijnse, Kromhout, Ocke & Monique Verschuren, 2010). The study of stroke mortality in Hiroshima or Nagasaki showed that daily intake of green-yellow vegetables could reduce the risk of death from total stroke in men and women up to 26% as compared to those just having one intake or less per week (Sauvaget, Nagano, Allen & Kodama, 2003).

According to American Institute for Cancer Research and the World Cancer Research Fund (2007), it was estimated that 30 to 40 percent of all cancers can be prevented by taking an ideal meal or diet involving various types of vegetables and fruits. A review also stated that diet which includes selenium, folic acid,

vitamin B - 12, Vitamin D, chlorophyll, and antioxidants are essential elements to prevent against cancer (Donaldson, 2004). For example, a study showed that consumed green leafy vegetable was related with a lower risk of colorectal cancer for men (Park et al, 2007).

2.2 Emergence of Foodborne Illnesses

Foodborne illnesses are defined as any toxic or infectious diseases that are caused by agents that enter the body through ingestion of food. Although the outbreaks of some foodborne diseases are well known, they are considered emerging because these cases become more common throughout the whole world. For example, an outbreak of salmonellosis took place in United States of America that affected an estimated 224,000 persons in 1994 due to consumption of contaminated ice cream (World Health Organization, 2010).

Generally, the main agent that accounted for various foodborne diseases are those microorganisms or pathogen which colonized on the food for a period of time such as *Salmonella* bacteria, *Campylobacter* bacteria and *E. coli*. Various factors are contributed to the emerging of foodborne diseases across the world. The main reason is due to the globalization of the food supply which boosts up the potential for the transmission of foodborne bacteria to different population of people from various geographical areas. An example is the large outbreak of cyclosporiasis in North America in 1996, which was associated with the intake of imported

Guatemalan raspberries that infected with *Cyclospora cayetanensis* (Herwaldt & Ackers, 1997).

Nowadays, greater number of people especially those living in city tend to have their meals in restaurants, fast food outlets and street food vendors as it is more convenient and time saving. Most of the food stalls do not comply fully with the rules stated by government agency regarding the food safety and control. The unhygienic food preparation provides a suitable ground for the growth of foodborne pathogen and thus contamination might happen. For example, *Bacillus cereus* and *Staphylococcus aureus* were mostly detected based on a study which determining the prevalence of food contamination in fast food restaurants operating in Benin City, Nigeria. The study also showed that more than half of the participants did not attend any food hygiene and safety training to gain knowledge regarding the dangers of foodborne diseases (Isara, Isah, Lofor & Ojide, 2010).

A proportion of society believed that consuming raw food could improve the functioning of digestion system, promotes weight loss and reduced risk of heart disease. In reality, a number of infectious diseases were caused by microorganisms which were transmitted from unprocessed or uncooked vegetables to victims through daily food consumption. A study conducted in Netherlands showed that consumption of raw vegetables is a risk factor for *Campylobacter* infections (Verhoeff-Bakkenes et al, 2011). *Campylobacter* spp. were also found in raw salad vegetables 'ulam' obtained in Malaysia traditional

wet market and modern supermarkets in Selangor (Chai et al, 2007). Since raw vegetables might pose a certain health risk to consumers, it is advisable to consume processed foods in order to prevent infectious foodborne illness.

2.2.1   Infections and Pathogenicity of Enterobacteriaceae

Enterobacteriaceae consists of a large family of bacteria which are normally found in vegetables and could transmit to human through food consumption. They have simple nutritional requirements, ferment glucose, reduce nitrate and are catalase positive. This family includes a large number of gram-negative and also facultative anaerobic bacteria. For example, *Yersinia enterocolitica* being a member of this family, is able to cause infections that lead to death by invading the host tissues. In Japan, an outbreak of food poisoning caused by salad contaminated with *Y. enterocolitica* O:8 was reported in year 2004 (Sakai et al, 2005).

Lipopolysaccharide or endotoxin is the major component of the outer membrane of gram-negative bacteria. They indirectly secrete various inflammatory cytokines such as interleukin that could induce changes in human body which includes damage to the liver and kidney. These lipopolysaccharides typically consist of a hydrophobic domain known as lipid A, a distal polysaccharide (or O-antigen) and a non-repeating core oligosaccharide. The saccharide portion has different length and composition amongst the different gram-negative bacteria species (Raetz & Whitfield, 2002). These endotoxins poses very powerful biological effects when

entering the blood system of human and animals with symptoms ranging from fever, adult respiratory distress syndrome and endotoxin shock (Gorbet & Sefton, 2005).

2.3   Antimicrobial Agents

Generally, antimicrobial agents used in the treatment of infectious disease fall into two groups which are antibiotics and chemotherapeutic agents. Antibiotics are natural substances produced by certain groups of microorganism and they come in small amount that could inhibit other microorganisms. Chemotherapeutic agents are synthetic drugs produced from chemicals in the laboratory. Antimicrobial agents that kill the microbes directly are known as bactericidal whereas agents that prevent or inhibit the growth of microbes are known as bacteriostatic (Tortora, Funke & Case, 2010).

2.3.1   Antibiotic as Antimicrobial Agents

Antibiotics are occasionally used to describe any molecules produced in nature by bacteria and fungi that have the ability to kill or suppress the growth of microbes (Yim, Wang & Davies, 2006). Antibiotics are given to human and animals for treatments of infectious disease and as growth promotion especially in animals. The range of bacteria affected by a particular antibiotic is expressed in terms of spectrum of action. Antibiotics that affect a wide range of gram-positive or gram-negative bacteria are known as broad–spectrum antibiotics. Narrow–spectrum antibiotics are useful against either one of the bacteria group that was mentioned

earlier. For example, Vanocomycin acts against gram-positive bacteria of the genera *Staphylococcus, Bacillus* and *Clostridium* (Madigan, Martinko & Parker, 2003).

2.3.2 Mechanisms of Action of Antibiotics

Several mechanisms have been proposed to account for their protection against infection but are generally grouped into five categories which include inhibition of bacteria cell wall synthesis, protein synthesis, nucleic acid synthesis, essential metabolites synthesis and alteration of plasma membrane (Tortora et al, 2010).

Beta-lactam antibiotics such as penicillin and cephalosporins are examples of drug that inhibits the cell wall synthesis. It acts by preventing the synthesis of intact peptidoglycan causing the cell undergoes lysis process due to weakening of cell wall. Penicillin has little toxicity for host cells such as human cells because they do not have peptidoglycan cell walls to act on (Tortora et al, 2010). For examples, cephalosporin has been used widely for treatment of infections such as sinusitis, chronic bronchitis and pneumonia (DePestel et al, 2008). Major antibiotic families that inhibit protein synthesis include Tetracyclines, aminoglycosides, oxazolidonones, macrolides and stretogramins (Levy & Marshall, 2004). It is well reported that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome. Since tetracyclines are bacteriostatic agents, they bind and inhibiting 30S ribosomal subunit reversibly (Chopra, Hawkey & Hinton, 1992).

Sulfonamides were among the first synthetic bacteriostatic drugs which possess similar chemical structures to para-aminobenzoic acid (PABA). They inhibit synthesis of metabolites by competitively interfering with the incorporation of PABA during folic acid synthesis (Burkhart & Burkhart, 2009). Rifampicin is a broad spectrum antibiotic which inhibits the bacterial RNA polymerase and is effective against mycobacteria in the treatment of tuberculosis and leprosy because it able to penetrate freely into tissues and living cells (Campbell et al, 2001).

2.3.3 Example of Antibiotics

2.3.3.1 Vancomycin

Vancomycin was isolated from *Streptomyces orientalis*, a type of glycopeptides that disrupt the synthesis of peptidoglycan in gram-positive bacteria. It is normally used to treat infections caused by oxacillin-resistant staphylococci and other gram-positive bacteria resistant to β-lactam antibiotics (Murray, Rosenthal & Pfaller, 2009). Recently, there were reports of clinical failures with vancomycin when used against serious *S. aureus* infections with MIC values in the susceptible range. Recent study showed that mortality associated with MRSA bacteremia was higher when vancomycin was used for treatment of infection with strains that have high vancomycin MIC (Soriano et al, 2008). But, when vancomycin was used together with daptomycin in acute osteomyelitis model, the combinations were more effective in reducing MRSA bacterial number (Lefebvre et al, 2010).

2.3.4   Development of Antibiotic Resistance

The discovery of penicillin by Sir Alexander Fleming more than sixty years had lead the invention of different classes of antibiotics which useful for treating various infections caused by bacteria. Unfortunately, things have not been improving recently where more bacteria which were previously susceptible to common antimicrobials drugs, were reported to have developed resistance to a particular antibiotics (Levy, 2002).

A number of factors contributed to the development of bacterial resistance to antibiotics. The main reason is the abuse or misuse of antibiotics in hospital or community which resulted in selective pressure in the host, whereby it encourages the excessive growth of resistant strains that are able to survive with the presence of the antibiotics (Levy & Marshall, 2004). The chances for bacteria to develop resistance are higher if broad spectrum antibiotics were used to inhibit them. An example is the case study which involved prediction of antibiotic resistance in *S. pneumoniae* infections (Pantosti & Moro, 2005).

Antibiotics are commonly used in animals as part of the process to manufacture food, disease prevention, and promote growth which may also contribute to the development of antimicrobial resistance genes in the environment (Barza & Gorbach, 2002). A world-wide problem that had been accounted with the use of antibiotics in livestock is the emergence of salmonella that are resistant to antimicrobial drugs. For example, a 12-year old boy was infected with

ceftriaxone-resistant strains of salmonella acquired from cattle which is rare in United States (Fey et al, 2000).

2.3.5   Mechanisms of Antibiotic Resistance

Antibiotic resistance remains the serious challenge faced by global society especially to those developing countries. The emergence of multi-drug-resistance bacteria isolates even worsen the situation since these countries have limited knowledge and access in controlling these new rising issues that may affect the country population (Byarugaba, 2004). Bacteria may acquire resistance to antibiotics through a variety of mechanisms. For example, the antibiotic active efflux is capable of developing transport mechanism that pumps out the antibiotic molecules that had been penetrated into the cell thereby reducing the effectiveness of the antibody to demonstrate its antimicrobial activity (Hooper, 2005). Susceptible bacteria can acquire resistance to antibiotics via genetic mutation that could alter the protein binding site to which the antibiotic agent binds. Bacteria could also develop resistance by accepting new resistance genes from other bacteria through conjugation, transformation and transduction, also known as horizontal evolution (Tenover, 2006).

2.4   Beta-lactamase

Beta-lactamases are the general cause of bacterial resistance to β-lactam antimicrobial agents, such as penicillins, cephalosporins and carbapenems. This β-lactamase production has been detected and reported among the

Enterobacteriaceae which include *Haemophilus influenza, Pseudomonas aeruginosa* and also *E. coli*. Beta-lactamases catalyze the hydrolysis of the β-lactam ring by splitting the amide bond and thus disrupt the chemical structure of the antibiotics which is unable to inhibit bacterial cell wall synthesis (Buynak, 2006). The β-lactamase can be distributed as chromosomal enzymes or plasmid-mediated enzymes depend on the bacteria species. Chromosomal β-lactamases are almost ever-present in enterobacteria and their expression may be inducible or constitutive according to the strain. For example, in *E. coli*, the chromosomal AmpC is constitutively produced at a very low level due to weak promoter causing them susceptible to cephalosporins (Nelson & Elisha, 1999). Plasmid-mediated β-lactamases are commonly found in staphylococci, enterobacteria, *Haemophilus influenza*, and *Neisseria gonorrhoeae*. The classical types of plasmid-mediated β-lactamases generally found in enterobacteria are TEM-1, TEM-2, SHV-1 and OXA-1 (Livermore, 1995).

2.4.1 Classification of Beta-lactamase

Beta-lactamases are classified into several schemes which include amino acid sequence homology, molecular weight or substrate specificities. In the early years, Ambler was the first person who proposed this sequence-based classification scheme by classifying the identified β-lactamase into class A serine-β-lactamases and class B metallo-β-lactamases based on limited knowledge about available enzymes on those days (Ambler, 1980). Another two new classes of serine-β-lactamases identification were later introduced, which are class C

cephalosporinases (Jaurin & Grundstrom, 1981) and class D oxacillinases (Dale, Godwin, Mossakowska, Stephenson & Wall, 1985).

SHV and TEM β-lactamases are usually plasmid-mediated enzyme which are categorised under class A serine hydrolase and are prevalent among gram-negative bacteria (Cantu, Huang & Palzkill, 1996). These enzymes hydrolyse penicillin and cephalosporins but unable to hydrolyse extended-spectrum antibiotics such as ceftazidime and azteronam. Some class A β-lactamases have evolved to accept expanded spectrum antibiotics as substrate due to mutation and become extended spectrum beta lactamase (ESBL) (Du Bois, Marriott & Amyes, 1995).

Class B β-lactamases are metallo-enzymes that require zinc ions for their activity and able degrade all classes of β-lactams except monobactams. The most significant characteristic is their carbapenemase activity which has the ability to hydrolyze carbapenem, a broad-spectrum antibiotic used to control bacteria that produce serine-β-lactamases (Bebrone, 2007).

Class C serine-β-lactamases are also known as AmpC β-lactamases. They tend to be chromosomal although several plasmid-borne classes were observed (Barlow & Hall, 2002). This class C are widely distributed among Enterobacteriaceae and mediate resistance to cephalosporins, oxyiminocephalosporins, and aztreonam (Nordmann, 1998). The class D β-lactamases gene are located both on plasmids

and in chromosome of a wide range of gram-negative bacteria and they are able to hydrolyze oxacillin and penicillins (Sanschagrin, Couture & Levesque, 1995).

2.4.2 The Extended Spectrum Beta Lactamases (ESBLs)

The development and introduction of extended-spectrum cephalosporins in the early 1980 was considered breakthrough to fight against beta-lactamase-mediated bacteria that were resistance to common antibiotics present at the early days (Medeiros, 1997). Few years later in Germany, gram-negative bacteria such as *Klebsiella ozaenae* was reported producing mutated version of plasmid-mediated β-lactamase which was resistant to third-generation cephalosporins (Kliebe, Nies, Meyer, Tolxdorff-Neutzling & Wiedemann, 1985). The isolated mutated β-lactamase gene showed a single nucleotide mutation compared to the gene encoding TEM-1, TEM-2, and SHV-1 class A β-lactamases (Sirot, 1995). This new β-lactamases were then categorized as extended spectrum beta lactamases (ESBLs) which are able to hydrolyze penicillins, first-, second-, and third-generation cephalosporins such as ceftriaxone, cefotaxime, ceftazidime and aztreonam but they can be inhibited by clavulanic acid (Paterson & Bonomo, 2005).

ESBL genes are generally carried by plasmid and some of them are situated within transposable elements which significantly facilitate the spreading between different bacterial strains or DNA replicons (Heritage, Hawkey, Todd & Lewis, 1992). The dissemination of plasmids among members of the family

Enterobacteriacea, for example *Escherichia coli* and *Klebsiella pneumoniae* play a critical role in the major outbreak of ESBL especially in Europe (Coque, Baquero & Canton, 2008). The spread of ESBL within a single environment has been identified and it was due to appearance of the same gene in unrelated plasmids (Bradford, Cherubin, Idemyor, Rasmussen & Bush, 1994). The growing prevalence of multidrug-resistant organisms was attributed by the presence of ESBL encoding gene in the same plasmids which also carrying genes for resistance to other antibiotics such as aminoglycosides, trimethoprim, sulphonamids, tetracyclines and chloramphenicol (Pitout, Nodmann, Laupland & Poirel, 2005). This multidrug-resistant ESBL-producing strain can survive over long period of time and able to transfer between different wards and hospitals causing wide spread of nosocomial outbreaks (Rice, Eckstein, DeVente & Shlaes, 1996).

2.4.2.1 SHV-type ESBLs

The SHV-type ESBLs are commonly found in clinical isolates compared to any other type of ESBLs. SHV-1 β-lactamase is generally found in *K. pneumoniae*. About 20% of the plasmid-mediated ampicillin resistance was found in this species (Tzouvelekis & Bonomo, 1999). In some strains of *K. pneumoniae*, *bla*SHV-1 gene was integrated into the bacterial chromosome (Livermore, 1995). The first reported SHV-type ESBL was in 1983 and it was designated as SHV-2 β-lactamase because the sequencing results specified a glycine to serine

substitution at position 238 which account for the extended-spectrum characteristics (Knothe, Shah, Krcmery, Antal & Mitsuhashi, 1983).

Within years, these SHV-type ESBLs were increasingly been detected in the family of Enterobacteriaceae which gave rise to various outbreaks. Some of the examples of outbreak include infection by *C. diversus*, and *P. aeruginosa* (Harrif-Heraud, Arpin, Benliman & Quentin, 1997; Naas, Philippon, Poirel, Ronco & Nordmann, 1999). Almost all of the SHV variants possessing an ESBL phenotype have mutation at position 238 but some of the variants related to SHV-5 have an additional glutamate to lysine substitution at position 240. Generally, the substitution at position 238 showed high level of resistance to cefotaxime and a slight resistance to ceftazidime, while the presence of substitutions at both positions 238 and 240 confers high level of resistance to ceftazidime and an additional increase in cefotaxime (Huletsky, Knox & Levesque, 1993).

2.4.2.2 Plasmid-borne SHV type beta-lactamase

The first reported plasmid-encoded β-lactamases which capable of hydrolyzing the extended-spectrum cephalosporins such as cefotaxime and ceftazidime was published in 1983, known as SHV-2. It was isolated from *Klebsiella ozaenae* and differed from the parent enzyme SHV-1 by replacement of glycine by serine at position 238 (Knothe et al, 1983). In year 1989, another variant of SHV type beta-lactamase was found in clinical isolates of *Klebsiella pneumoniae* known as SHV-3, which differs from SHV-1 in two positions. The gene that responsible to

encode SHV-3 beta-lactamase was found on plasmid and responsible for transferable cefotaxime resistance between *E. coli* and *Klebsiella pneumoniae* (Nicolas, Jarlier, Honore, Phillippon & Cole, 1989).

2.4.2.3   Current Research in Plasmid-borne *bla*-SHV

Recent study showed that all extant plasmid-borne *bla*-SHV were descended from one of two *Klebsiella pneumoniae* genomes to plasmid mobilization events facilitated by IS26 (Ford & Avison, 2004). The plasmid-borne genes encoding SHV-1, SHV-2 and SHV-5 would have an IS26 insertion 2 kbp upstream of the *bla*-SHV coding region (Gutmann et al, 1989). IS26 which reside in *bla*-SHV promoter are generally found on the plasmids that encode SHV-11, SHV-2a and SHV-12 (Nuesch-Inderbinen, Kayser & Hachler, 1997). Since year 1983, a total of 112 SHV enzymes have been identified due to nucleotide substitutions and non-synonymous mutation of T92A but not all of them code for ESBL enzymes (Mendonca, Nicolas & Canica, 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial Strains

This study was performed using 20 Enterobacteriaceae and 2 *Klebsiella pneumoniae* bacterial isolates that were previously screened and classified by Ng (2011) and Eo (2010) from 4 types of vegetables. The bacterial strains were isolated from lettuce, bean sprout, carrot and spring onion.

3.1.2 Chemical Reagents and Equipments

The chemical reagents and equipments used for this study were provided by the Department of Biological Science of Universiti Tunku Abdul Rahman. The reagents and equipments were allocated at Final Year Project laboratory, Microbiology laboratory, Molecular Biology laboratory and also Post-graduate laboratory. The details of the chemicals and equipments were listed in Table 3.1 and Table 3.2.

Table 3.1 List of chemical reagents and their manufacturers.

Chemical reagents	Manufacturer
Absolute Ethanol	R & M
Agarose powder	CAMBREX
Ethylenediaminetetraacetic acid (EDTA)	SYSTERM®
Glacial acetic acid	SYSTERM®
Glucose	Fisher Scientific
Luria-Bertani (LB) Agar	MERCK
Luria-Bertani (LB) Broth	MERCK
Potassium Acetate	MERCK
Sodium Hydroxide	R&M
Sodium Dodecyl Sulphate	Fisher scientific
Tris(hydroxymethyl)aminomethane (Tris)	Thermo Scientific

Table 3.2 List of equipments and their manufacturers.

Equipments	Manufacturer
Autoclave machine	HIRAYAMA
Centrifuge machine	Sigma
Electronic balance	Adventurer^{TM} Pro
Freezer	PENSONIC
Gel Electrophoresis Set	BAYGENE
Hot plate	LMS
Incubator	Memmert
Incubator shaker	LabTech
Laminar flow hood	Isocide^{TM}
Microcentrifuge	TOMY
Micropippette set	Thermo Scientific
Microvaccum	TOMY
Microwave oven	Sanyo
Nanodrop 1000	Thermo Electron Corporation
PCR machine (thermal cycler)	Eppendorf
Refrigerator	Toshiba
Spectrophotometer	BIO-RAD SmartSpec^{TM}
UV transilluminator	UVP
Water bath	Memmert

3.2   General Methods

3.2.1   Preparation of Media and Reagents

All the growth media and chemical solutions were prepared according to preparation guidelines on the bottles by using double distilled water and kept in autoclaved Schott bottles. The prepared media and agar were kept at room temperature unless storage at 4°C is required. Molten agar was poured into sterile petri dishes after autoclaved and allowed to cool down before stored at room temperature or in refrigerator. Media supplemented with antibiotic were mixed well before pouring into petri dishes. Methods for media and buffer preparation are stated in Table 3.3.

3.2.2   Sterilisation

Sterilisation of all the media solutions and tools used throughout this study were achieved through autoclaving or filtration. Liquid media were autoclaved at 121°C at 15 psi for 20 minutes. Autoclaved apparatus were dried at 70°C in an oven prior to use. Heat sensitive chemicals such as ampicillin and glucose were filter-sterilized using a 0.20 µm membrane filter.

Table 3.3 Preparation of Media and Reagents.

Media and reagents	Method of preparation
Luria-Bertani (LB) Agar	An amount of 37.0g of LB agar powder was weighed, dissolved and topped up with deionised distilled water to 1L.
Luria-Bertani (LB) Broth	An amount of 20.0g of LB broth powder was weighed, dissolved and topped up with deionised distilled water to 1L.
Media with ampicillin(50µg/mL)	An amount of 1mL of ampicillin stock (50mg/mL) was taken and added into 1L of media that had been cooled down to approximately 50°C. Mixed the solution evenly.
Solution I 25mM Tris-Cl (pH 8.0) 10mM EDTA (pH 8.0) 50mM glucose	An amount of 1.25mL of 1M Tris-Cl and 1.00mL of 0.5M EDTA were added into 45.25mL of deionised distilled water before 2.5mL of filtered sterilised glucose was added into the solution to give a final volume of 50mL solution.
Solution II 0.2M NaOH 1.0% (w/v)SDS	An amount of 1.0mL of 10M NaOH and 5.0mL of 10% SDS were added into 44.0mL of deionised distilled water to make a final volume of 50.0mL solution.
Solution III 5.0M Potassium acetate Glacial acetic acid Distilled water	An amount of 60.0mL of 5M potassium acetate and 11.5mL of glacial acetic acid were added into 28.5mL of distilled water to make a final volume of 100.0mL solution.

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3.3   Alkaline Lysis

The alkaline lysis methodology was used to extract the bacterial plasmid DNA (Joly, 1996). A single colony of the desired bacterial strain was inoculated into 10 ml of LB broth supplemented with 50 µg/mL of ampicillin. The inoculum was grown in an incubator shaker overnight with 200 rpm agitation at 37°C. A volume of 5 ml of the overnight inoculum was transferred into a tube and then centrifuged at 13,000 rpm for 15 minutes at room temperature. The supernatant was discarded.

The pellet was re-suspended in 200 µl of Solution I and the mixture was transferred into a new microcentrifuge tube. A volume of 10 µl of lysozyme (10mg/ml) was added into the tube and gently mixed. A volume of 200 µl of cold Solution II was added into the tube and inverted gently before incubation on ice for 5 minutes. An volume of 300 µl of Solution III was added into the tube and mixed gently before incubation on ice for another 5 minutes. The mixture was centrifuged at 13,000 rpm for 15 minutes and the supernatant was transferred into a fresh microcentrifuge tube.

Equal volume of 95% ethanol was mixed to the supernatant and incubated at room temperature for 15 minutes before subjected to centrifugation at 13,000 rpm for 5 minutes. The alcohol and supernatant were discarded and 500 µl of 70% ethanol was added to wash the remaining pellet. The ethanol was aspirated and the pellet was vacuum-dried using Microvaccum for around 5 minutes. The dried pellet was re-suspended with 50 µl of sterile deionised distilled water and stored

in -20°C until use. The extracted plasmid DNA concentration and purity were determined using Nanodrop 1000 (Thermo Electron corporation).

3.4   Polymerase Chain Reaction (PCR) and Purification

3.4.1   Oligonucleotides for PCR Amplification

The PCR amplification was performed using *bla*-SHV specific primers. Table 3.4 showed the length and sequence of the primers used

Table 3.4 Details of the *bla*-SHV primers used in this study.

Primer names	Length (bp)	Sequence (5' – 3')	Approximate fragment size
bla-SHV_F	21	TGG TTA TGC GTT ATA TTC GCC	~870bp
bla-SHV_R	19	GGT TAG CGT TGC CAG TGC T	

*Note: F: forward, R: reverse

Primers reference: Ng (2011)

3.4.2   Preparation of PCR Mixtures for Beta-lactamase (*bla*-SHV) Gene Amplification

The detection of *bla*-SHV gene in different isolates of Enterobacteriaceae was performed by PCR amplification. The PCR pre-mix solution was prepared by pipetting 1X PCR buffer, 2 mM $MgCl_2$, 0.4 nM forward primer, 0.4 nM reverse primer, 0.2 mM dNTP mixture and deionised distilled water into a sterile

microcentrifuge tube. Upon completing addition of the pre-mix, the PCR concoction was distributed to each PCR tube and mixed with 250 ng of plasmid DNA which served as template for the PCR reaction. An amount of 0.04 U *Taq* polymerase was added last before all the samples were placed into a pre-programmed thermal cycler machine. A negative control was prepared for every batch to ensure no DNA contamination by substituting the plasmid DNA template with sterile distilled water.

The following parameter was used for PCR amplification of *bla*-SHV gene:

Temperature (°C)	Time (Sec)		
96:	300	Initial denaturation	
96:	30		
50:	30	Annealing	30 cycles
72:	60		
72:	300	Final extension	

3.4.3 Gel Electrophoresis of PCR Products

The amplified PCR products were separated by agarose gel electrophoresis. An aliquot of 2 µl of PCR product was mixed with 1 µl of loading dye. The mixture was electrophoresed in 1.5% (w/v) TBE agarose gel. The GeneRuler™ 100 bp DNA Ladder (Fermentas) was used as the molecular weight marker in every agarose gel. The current used for gel electrophoresis was 80 V and the duration for the front dye to reach 3/4 of the length of the gel was around 40 minutes. The

gel was stained with ethidium bromide and viewed under UV transilluminator (UVP) after completed the gel electrophoresis process.

3.4.4   Agarose Gel Purification

The amplified PCR products were purified using *GeneJet*^{TM} *Gel Extraction Kit* (Fermentas Life Science) based on the manufacturer's manual. This kit allowed rapid and purification of DNA fragments from standard or low melting point agarose gels which were electrophoresed either in 1X TAE or TBE buffers. A sterile scalpel was used to excise gel slice containing the desired DNA fragment into a pre-weighed microcentrifuge tube and the weight of the gel slice was recorded.

Binding buffer was added to the tube according to 1:1 volume of the gel slice. The gel mixture was incubated at 50 – 60°C for 10 minutes until the gel slice was completely dissolved. A volume of 800 µl of solubilised gel solution was transferred to the *GeneJet*^{TM} *Purification Column* and subjected to centrifugation for around 2 minutes. The flow-through was discarded and the column was placed back into the same collection tube. The excess solubilised gel solution could be added to the column in stages and proceed to centrifugation.

For sequencing purposes, 100 µl of *Binding Buffer* was added to the purification column and centrifuged it for 2 minutes. The flow-through was discarded and the column was placed back to the same tube. An amount of 700 µl of *Wash Buffer*

which had been diluted with ethanol was added to the column and centrifuged it for 2 minutes. The flow-through was discarded and the column was placed back to the same tube. The empty column was centrifuged for an additional 2 minutes to completely remove residual *Wash Buffer*. The column was transferred into a sterile microcentrifuge tube. A volume of 50 µl of *Elution Buffer* was added to the column and centrifuged it for 2 minutes. The purification column was discarded and the purified DNA was stored at -20°C. The purified DNA concentration and purity were determined using Nanodrop 1000 (Thermo Electron Corporation).

3.5   Ligation of PCR Fragments to Vector

The vector used in this study was pGEM®-T Easy vector system manufactured by Promega. The purified PCR fragments were ligated into the pGEM®-T based on the manufacturer's manual. The ligation compounds were prepared by pipetting 3 µl of purified PCR products, 1 µl of 50 ng pGEM®-T Easy vector, 1 µl of 3U T4 ligase and 5 µl of 2x Rapid Ligation Buffer into a PCR tube. This ligation mixture was incubated overnight at 4°C and could be stored at -20°C before proceeding to transformation.

3.6   Transformation

3.6.1   Preparation of Competent Cells

The transformation was carried out using *E. coli* strain JM109 competent cells. A single colony of *E. coli* was inoculated into 5 mL of LB medium and incubated overnight at 37°C with agitation at 200 rpm. An amount of 300 µl of the

overnight inoculum was transferred into 15 mL of fresh LB medium and further agitated at 200 rpm at 37°C until it reached an OD_{600} of between 0.5 – 0.6. The cells that fulfilled the OD_{600} were pelleted by centrifugation at 4000 g for 5 minutes at 4°C.

After the supernatant was discarded, the cell pellet was resuspended in 2 mL of cold 0.1 M $CaCl_2$ and incubated on ice water for 2 hours. The competent cells were kept on ice before proceeding to the transformation process. For long term storage, the competent cells were mixed with an equal volume of 40% (v/v) sterile glycerol and dispensed into 1 mL aliquots in pre-chilled microcentrifuge tubes and stored at -70°C.

3.6.2 Transformation of Competent Cells

The sterile microcentrifuge tube was pre-chilled before 200 µl of competent cells were added into it. The plasmid pUC19 was used as the positive control and for the negative control, no pUC19 was added to mix with the competent cells. For positive control tube, 1 µl of pUC19 was added into the competent cells. A volume of 2 µl of ligation mixture (Section 3.5) was mixed with the competent cells and all the tubes were then incubated on ice water for an hour.

The tubes were then subjected to heat-shock at 42°C for exactly 90 seconds and immediately incubated on ice for an additional 5 minutes. A volume of 900 µl of LB medium was added to the tube and incubated at 37°C for 45 minutes with

agitation at 80 rpm. The cells were pelleted at 6000 g for 10 minutes and resuspended in 100 µl LB medium. The agar plates containing 50 µg/mL of ampicillin were spread with 20 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-gal before the transformed cells were plated onto agar plates using sterilised hockey stick. The plates were incubated at 37°C for 16 – 18 hours and selection of the transformed bacteria was based on the blue-white colonies formed on the plate.

3.7   Colony PCR

The colony PCR was performed to screen transformed bacterial colonies that were suspected to harbour recombinants plasmid with desired DNA inserts. The PCR pre-mix solution was prepared by pipetting 1X PCR buffer, 2 mM $MgCl_2$, 0.4 µM T7 forward primer, 0.4 µM SP6 reverse primer, 200 µM dNTP mixtures and deionised distilled water into a sterile microcentrifuge tube. The desired bacterial colony was picked gently using a sterile toothpick and dispensed into the PCR concoction. An amount of 0.04 U *Taq* polymerase was added last to make a final volume of 25 µl before the sample was placed into a pre-programmed thermal cycler machine. The following parameter was used for PCR screening of recombinant plasmids:

Temperature (°C)	Time (Sec)		
96:	180	Initial denaturation	
96:	30		
50:	15	Annealing	30 cycles
60:	150		
60:	600	Final extension	

The PCR product was assessed using 1.5% (w/v) agarose gel using GeneRuler™ 100 bp DNA Ladder (Fermentas) after the amplification process. The gel was stained with ethidium bromide and viewed under UV transilluminator (UVP) after completed the gel electrophoresis.

3.8   Recombinant Plasmid Extraction

The bacterial recombinant plasmid was extracted using commercial *Plasmid DNA Purification Kit* (INTRON) based on the manufacturer's manual. A single colony from a freshly streaked bacterial plate was picked and dipped it into LB broth supplemented with 50 µg/mL of ampicillin for overnight incubation. An amount of 5 mL of bacterial culture was harvested by centrifugation at 13,000 rpm for 30 seconds at room temperature and the supernatant was then discarded. The pellet was resuspended with 250 µl of *Resuspension Buffer* until no clumps of the cell pellet remained.

A volume of 250 µl of *Lysis buffer* was added to the suspension and gently mixed by inverting the tube several times with cap closed. The tube was added with 350 µl of chilled *Neutralization Buffer* and mixed gently by inverting the tube several times before proceed to centrifugation at 13,000 rpm for 10 minutes at 4°C. The supernatant was transferred into the column attached to collection tube and centrifuged at 13,000 rpm for 60 seconds. The filtrate in collection tube was discarded and column was placed back in the same collection tube. An amount of 500 µl of *Washing buffer A* was added and centrifuged at 13,000 rpm for 60 seconds. The filtrate was discarded and column was placed back in the same collection tube.

An amount of 700 µl of *Washing Buffer B* was added and centrifuged at 13,000 rpm for 60 seconds. The filtrate was discarded and column was placed back in the same collection tube. Centrifugation was performed again at 13,000 rpm for 60 seconds to dry the filter membrane. The column was transferred into a clean and sterile centrifuge tube followed by addition of 50 µl of pre-warmed *Elution Buffer* before it was left for stand for 1 minute. The column, along with the tube, was being centrifuged at 13,000 rpm for 60 seconds to collect the eluted plasmid and stored at -20°C. The extracted plasmid DNA concentration and purity were determined using Nanodrop 1000 (Thermo Electron corporation). The plasmid DNA was out-sourced for sequencing after confirmation of the desired size of the recombinant plasmid was achieved.

3.9 BlastX and BlastN Alignment Analysis

The extracted recombinant plasmids were sequenced using T7 and SP6 promoter primers. Sequencing was out-sourced to 1st Base Laboratories Sdn. Bhd. The resulting DNA sequences were then aligned with BlastX and BlastN programme which is available in the National Centre for Biotechnology Information (NCBI) website.

RESULTS

4.1   Plasmid DNA Extraction Using Alkaline Lysis Methodology
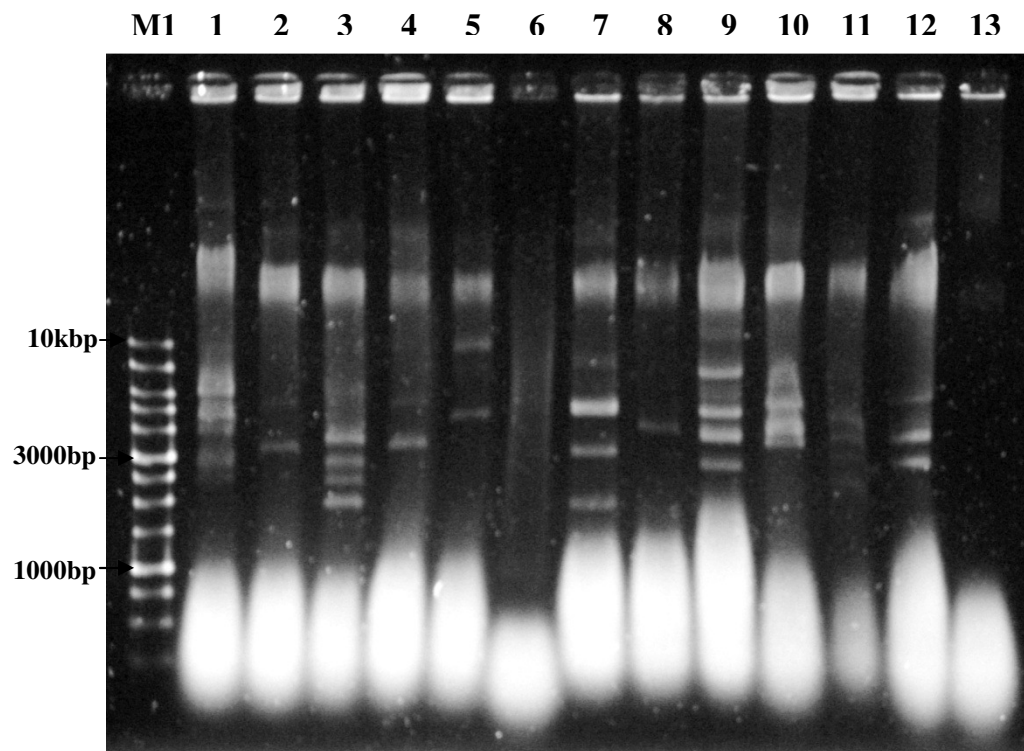
Figure 4.1 The gel image of the extracted plasmid DNA from 13 isolates.

Lane M1 is the 1kb DNA Ladder (Fermentas). Lane 1 to 13 represents the
bacterial isolates TL1, TL6, TL9, TL10, TL11, SB1, SB3, SB4, SB5, SB6, SB7,
SB8, and SB9, respectively.
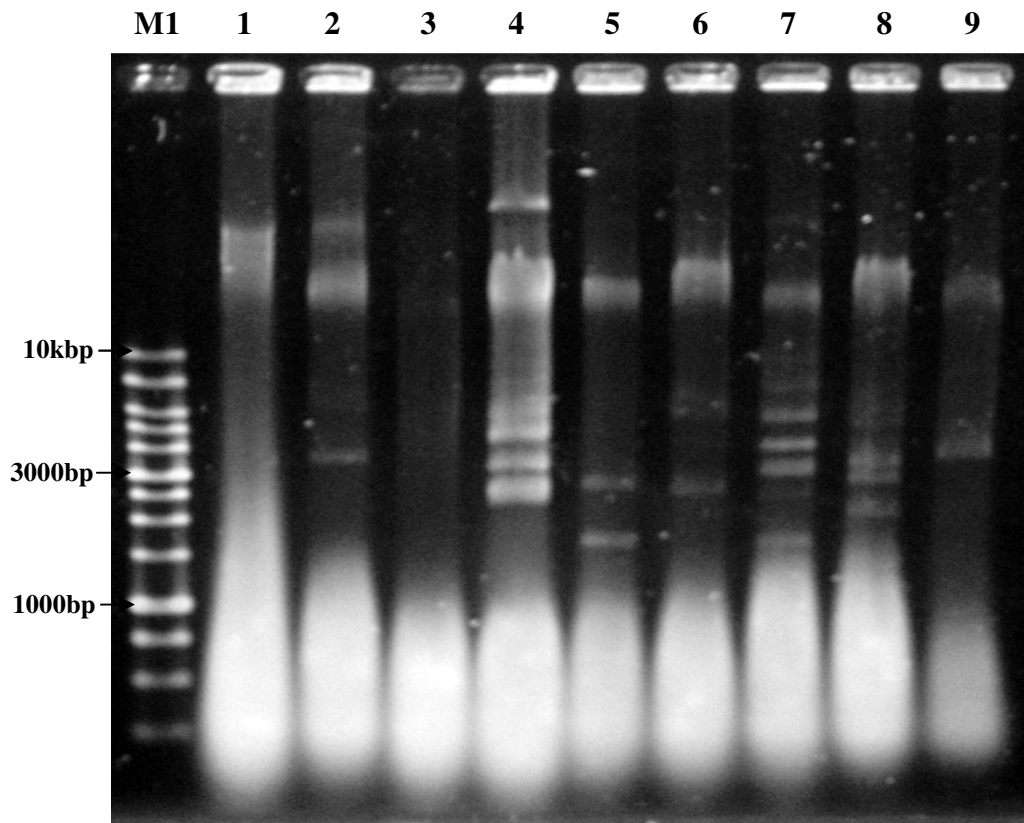
Figure 4.2 The gel image of the extracted plasmid DNA from 9 isolates.

Lane M1 is the 1kb DNA Ladder (Fermentas). Lane 1 to 9 represents the bacterial

isolates SL1, SL4, SL5, SL6, SL7, SL8, SL9, C3, and S3, respectively.

4.2   Purity and Concentration of the Plasmid DNA

The purity and concentration of all the extracted plasmid DNA was determined using spectrophotometer and absorbance readings were recorded down as below.

Table 4.1 Absorbance reading taken using spectrophotometer.

Bacterial Isolates	A_{260}	A_{280}	A_{260}/A_{280}	Concentration (ng/µl)
TL1	0.738	0.463	1.702	1668
TL6	2.170	1.742	1.297	4675
TL9	1.611	0.947	1.791	3758
TL10	2.264	1.523	1.574	5080
TL11	0.802	0.595	1.429	1725
SB1	2.137	1.630	1.314	5302
SB3	2.086	1.093	1.912	5205
SB4	1.534	0.783	1.977	3800
SB5	0.496	0.294	1.709	1218
SB6	1.902	1.534	1.298	4005
SB7	1.134	0.845	1.470	2260
SB8	0.518	0.284	1.900	1235
SB9	2.424	1.392	1.758	5985
SL1	0.422	0.250	1.771	988
SL4	0.532	0.367	1.532	1188
SL5	0.620	0.487	1.289	1485
SL6	0.778	0.620	1.265	1888
SL7	0.592	0.436	1.456	1245
SL8	0.318	0.198	1.678	743
SL9	0.555	0.344	1.676	1308
C3	0.211	0.125	1.761	498
S3	0.482	0.369	1.471	883

4.3   Detection of the Existence of *bla*-SHV Gene on Plasmid DNA through PCR Screening

(a)

(b)
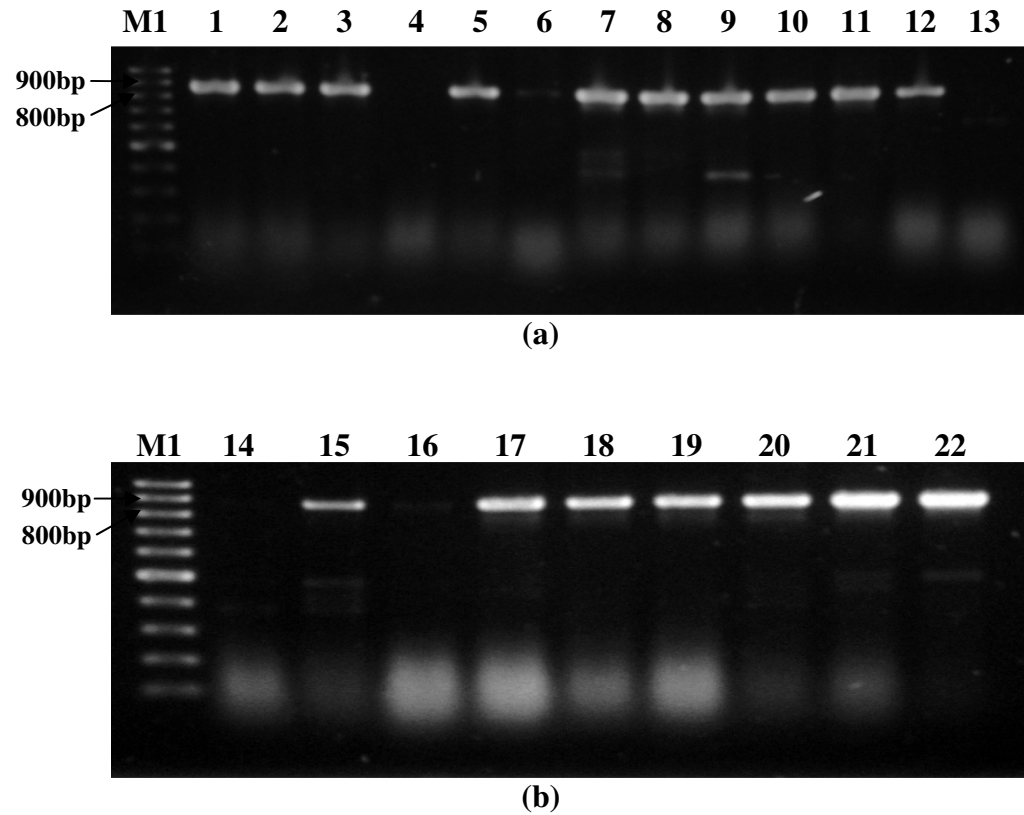
Figure 4.3 The gel photos of (a) and (b) were the PCR products amplified using *bla*-SHV primers.

Lane M1 is the 100 bp DNA Ladder (Fermentas). Lane 1 to 22 represent the PCR product for bacterial isolates TL1, TL6, TL9, TL10, TL11, SB1, SB3, SB4, SB5, SB6, SB7, SB8, SB9, SL1, SL4, SL5, SL6, SL7, SL8, SL9, C3, and S3. The expected band size is approximately 870 bp.

Table 4.2 Summary of the PCR screening for *bla*-SHV gene on the plasmids.

| Bacterial Isolates	PCR Results	Remarks
TL1	+	Band presence
TL6	+	Band presence
*TL9	+	Band presence
TL10	-	No band
*TL11	+	Band presence
SB1	-	Faint band
*SB3	+	Band presence
*SB4	+	Band presence
SB5	+	Band presence
*SB6	+	Band presence
SB7	+	Band presence
*SB8	+	Band presence
SB9	-	No band
SL1	-	No band
*SL4	+	Band presence
SL5	-	Faint band
SL6	+	Band presence
SL7	+	Band presence
*SL8	+	Band presence
SL9	+	Band presence
*C3	+	Band presence
*S3	+	Band presence

Note: only 17 out of 22 bacteria isolates were suspected having *bla*-SHV gene on the plasmid. A total of 10 suspected bacteria isolates which labelled with * were chosen to proceed to gel purification for further studies.

4.4   Gel- purified PCR Products from the Ten Chosen Bacterial Isolates
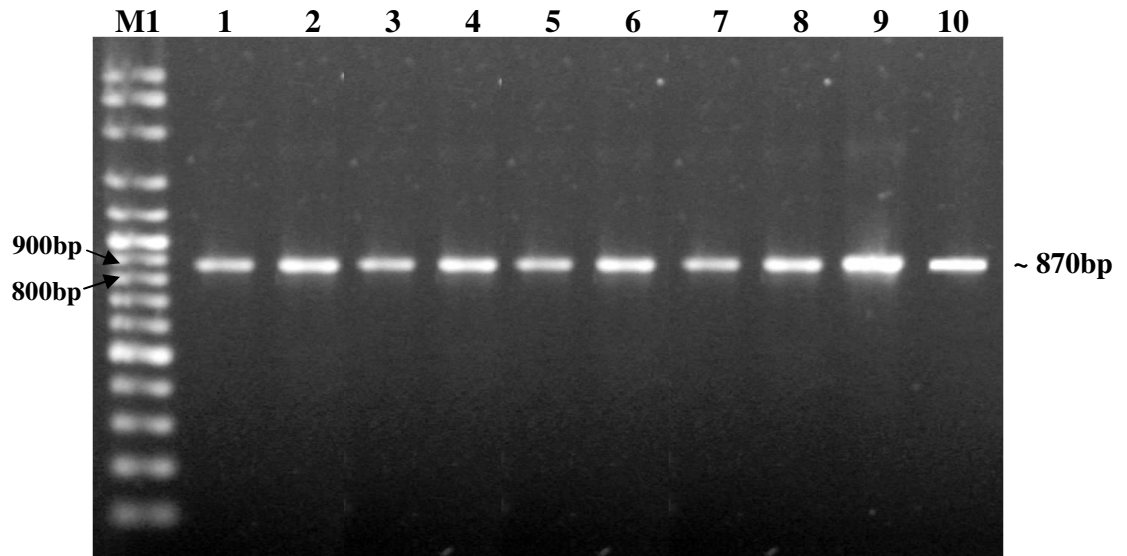
Figure 4.4 Agarose gel electrophoresis of ten purified PCR products.

Lane M1 is the 100 bp DNA Ladder (Vivantis). Lane 1 to 10 represents the gel-purified PCR product for bacterial isolates C3, S3, TL9, TL11, SL4, SL8, SB3, SB4, SB6, and SB8. The expected band size is approximately 870 bp for all of the 10 chosen isolates.

4.4.1 Purity and Concentration of the gel-purified *bla*-SHV gene fragments.

The purity and concentration of the gel-purified *bla*-SHV gene fragments were determined by spectrophotometer before proceed to ligated into vector. The absorbance readings were recorded in table 4.3.

Table 4.3 Absorbance reading taken using spectrophotometer.

Bacterial Isolates	A_{260}	A_{280}	A_{260}/A_{280}	Concentration (ng/µl)
C3	0.244	0.136	1.8	12.18
S3	0.196	0.121	1.6	9.79
TL9	0.180	0.102	1.8	8.98
TL11	0.230	0.128	1.8	11.51
SL4	0.005	0.002	2.0	15.00
SL8	0.023	0.015	1.7	47.50
SB3	0.012	0.009	1.6	20.00
SB4	0.012	0.008	1.8	22.50
SB6	0.011	0.006	1.8	24.50
SB8	0.017	0.011	1.8	35.00

4.5   Detection of Transformed Bacteria Colonies by Blue-White Selection

(a)   **(b)**

(c)
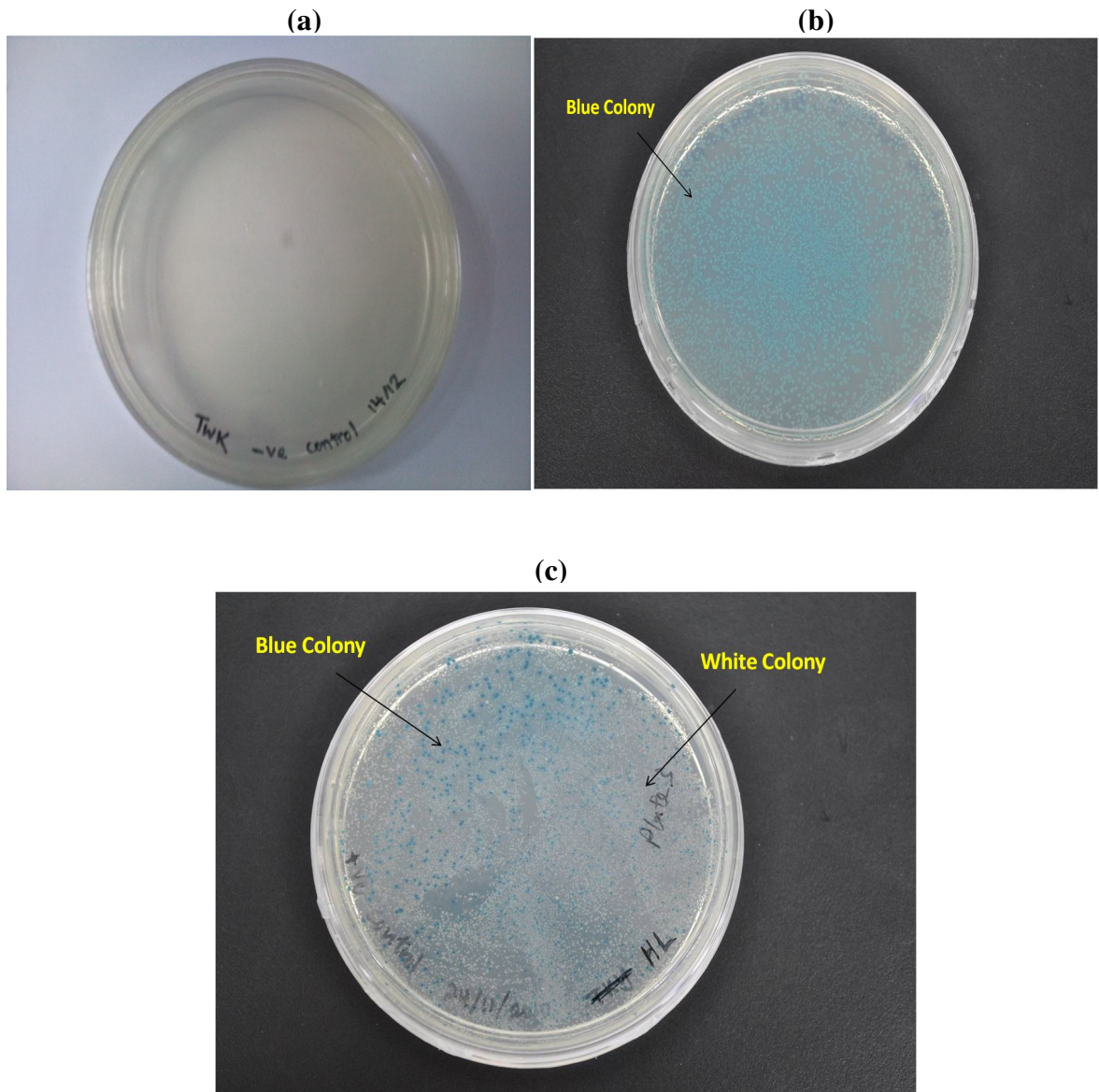
Figure 4.5 (a) Negative control plate (b) Positive control plate with a lawn of blue colonies only (c) Plate that was subjected to transformation with ligated mixture, combination of blue and white colonies were observed.

4.6   Colony PCR and Extracted Recombinant Plasmid
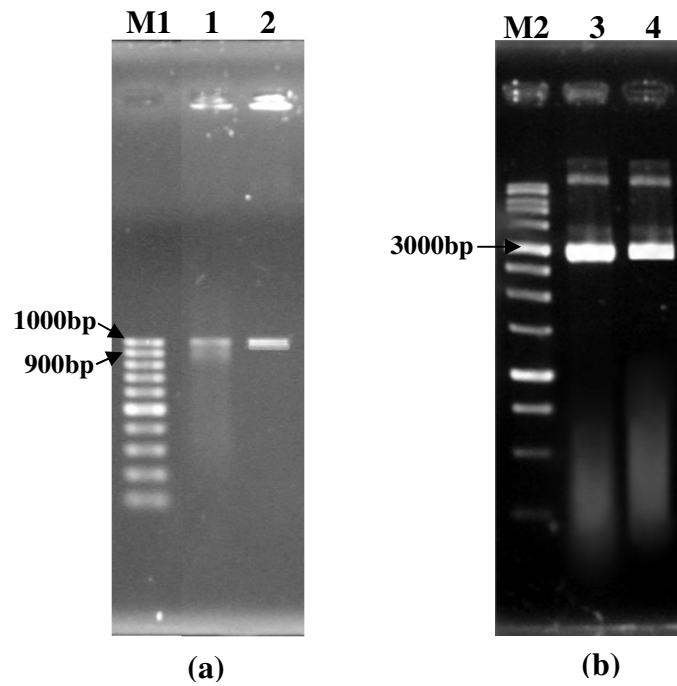
Figure 4.6 (a) The gel image of colony PCR (b) The gel image of recombinant plasmid.

Lane M1 is the 100 bp DNA Ladder (Fermentas) and lane M2 is the 1kb DNA Ladder (Fermentas). Lane 1 is the colony PCR product and lane 3 is the extracted recombinant plasmid for bacterial isolate TL9. Lane 2 is the colony PCR product and lane 4 is the extracted recombinant plasmid for bacterial isolate SB3. The expected size for colony PCR products is approximately 1000 bp while for recombinant plasmids is approximately 3000 bp.

4.7   The Sequencing Results of TL9 and SB3 Gel-purified PCR Products

(a) Bacterial isolate TL9 (868 bases)

TGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTTAGCCACCC
TGCCGCTGGCGGTACACGCCAGCCCGCAGCCGCTTGAGCAAATTAAAC
TAAGCGAAAGCCAGCTGTCGGGCCGCGTAGGCATGATAGAAATGGAT
CTGGCCAGCGGCCGCACCCTGACCGCCTGGCGCGCCGATGAACGCTTT
CCCATGATGAGCACCTTTAAAGTAGTGCTCTGCGGCGCAGTGCTGGCG
CGGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACTATCG
CCAGCAGGATCTGGTGGACTACTCGCCGGTCAGCGAAAAACACCTTGC
CGATGGCATGACGGTCGGCGAACTCTGCGCCGCCGCCATTACCATGAG
CGATAACAGCGCCGCCAATCTGCTGCTGGCCACCGTCGGCGGCCCCGC
AGGATTGACTGCCTTTTTGCGCCAGATCGGCGACAACGTCACCCGCCT
TGACCGCTGGGAAACGGAACTGAATGAGGCGCTTCCCGGCGACGCCC
GCGACACCACTACCCCGGCCAGCATGGCCGCGACCCTGCGCAAGCTG
CTGACCAGCCAGCGTCTGAGCGCCCGTTCGCAACGGCAGCTGCTGCAG
TGGATGGTGGACGATCGGGTCGCCGGACCGTTGATCCGCTCCGTGCTG
CCGGCGGGCTGGTTTATCGCCGATAAGACCGGAGCTGGCGAACGGGG
TGCGCGCGGGATTGTCGCCCTGCTTGGCCCGAATAACAAAGCAGAGC
GCATCGTGGTGATTTATCTGCGGGATACGCCGGCGAGCATGGCCGAGC
GAAATCAGCAAATCGCCGGGATCGGCGCGGCGCTGATCG**AGCACTGG
CAACGCTAACC**

*Note: The   underlined   sequences   are   the   *bla*-SHV   forward   and   reverse
complement primers sequences. (Refer Table 3.4.)

(b) Bacterial isolate SB3 (868 bases)

TGGTTATGCGTTATATTCGCCTGTGCCTTATCTCCCTGATTGCCGCCC
TGCCACTGGCGGTATTCGCCAGCCCTCAGCCGCTTGAGCAGATTAAAA
TCAGCGAAAGTCAGCTGGCGGGCCGGGTGGGCTATGTTGAAATGGAT
CTGGCCAGCGGCCGCACGCTGGCCGCCTGGCGCGCCAGTGAGCGCTTT
CCGCTGATGAGCACCTTTAAAGTGCTGCTCTGCGGCGCGGTGCTGGCC
CGGGTGGATGCCGGCGACGAACAGCTGGATCGGCGGATCCACTACCG
CCAGCAGGATCTGGTGGACTACTCCCCGGTCAGCGAAAAACACCTTGC
CGACGGGATGACCGTTGGCGAACTCTGCGCCGCCGCCATCACCATGAG
CGACAACACCGCCGGCAATCTGCTGTTGAAGATCGTCGGCGGCCCCGC
GGGATTGACCGCTTTTCTGCGCCAGATCGGTGACAACGTCACCCGTCT
TGACCGCTGGGAAACGGAACTCAATGAGGCGCTTCCCGGCGACGTGC
GCGACACCACCACCCCGGCCAGCATGGCCACCACCCTGCGCAAGCTG
CTAACCACCCCCTCTCTGAGCGCCCGTTCGCAGCAGCAGCTGCTGCAG
TGGATGGTTGACGACCGGGTGGCCGGCCCGTTGATCCGCGCCGTGCTG
CCGGCGGGCTGGTTTATCGCCGATAAAACCGGGGCCGGTGAGCGGGG
CTCACGCGGCATTGTCGCCCTGCTCGGCCCGGACGGCAAAGCGGAGC
GTATCGTGGTGATCTATCTGCGTGATACCCCGGCGACCATGGTCGAGC
GTAACCAGCAGATCGCCGGGATAGGCGCGGCGCTGATCG**AGCACTGG
CAACGCTAACC**

*Note: The underlined sequences are the *bla*-SHV forward and reverse complement primers sequences. (Refer Table 3.4.)

4.8 BlastX Alignments

(a) BlastX alignment using 868 bases from TL9 reverse complement sequence

Sequences producing significant alignments:

> ☐ ref|YP_001102238.1| G🔬 beta-lactamase [Yersinia pestis biovar Orientalis str. IP275]

ref|ZP_02226539.1| G🔬 beta-lactamase [Yersinia pestis biovar Orientalis str. IP275]

ref|ZP_02226553.1| G🔬 beta-lactamase [Yersinia pestis biovar Orientalis str. IP275]

65 more sequence titles
Length=286

GENE ID: 4927436 blaSHV-1 | beta-lactamase
[Yersinia pestis biovar Orientalis str. IP275] **(Over 10 PubMed links)**

```
 Score =  560 bits (1442),  Expect = 1e-157
 Identities = 286/286 (100%), Positives = 286/286 (100%), Gaps = 0/286 (0%)
 Frame = +3

Query  6     MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE  185
             MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE
Sbjct  1     MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE  60

Query  186   RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA  365
             RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA
Sbjct  61    RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA  120

Query  366   AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA  545
             AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA
Sbjct  121   AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA  180

Query  546   SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG  725
             SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG
Sbjct  181   SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG  240

Query  726   IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR  863
             IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR
Sbjct  241   IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR  286
```

Figure 4.7 Results obtained from BlastX alignment search tool for TL9 reverse complement sequence.

(b) BlastX alignment using 868 bases from SB3 reverse complement sequence

Sequences producing significant alignments:

```
>   gb|AAS13468.1|  penicillinase OKP-5 [Klebsiella pneumoniae]
 gb|AAZ79405.1|  beta-lactamase [Klebsiella pneumoniae]
 emb|CAP12356.2|  beta-lactamase [Klebsiella pneumoniae]
Length=286

 Score =  535 bits (1379),  Expect = 2e-150
 Identities = 285/286 (99%), Positives = 286/286 (100%), Gaps = 0/286 (0%)
 Frame = +3

Query  6     MRYIRLCLISLIAALPLAVFASPQPLEQIKISESQLAGRVGYVEMDLASGRTLAAWRASE  185
             MRY+RLCLISLIAALPLAVFASPQPLEQIKISESQLAGRVGYVEMDLASGRTLAAWRASE
Sbjct  1     MRYVRLCLISLIAALPLAVFASPQPLEQIKISESQLAGRVGYVEMDLASGRTLAAWRASE  60

Query  186   RFPLMSTFKVLLCGAVLARVDAGDEQLDRRIHYRQQDLVDYSPVSEKHLADGMTVGELCA  365
             RFPLMSTFKVLLCGAVLARVDAGDEQLDRRIHYRQQDLVDYSPVSEKHLADGMTVGELCA
Sbjct  61    RFPLMSTFKVLLCGAVLARVDAGDEQLDRRIHYRQQDLVDYSPVSEKHLADGMTVGELCA  120

Query  366   AAITMSDNTAGNLLLKIVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDVRDTTTPA  545
             AAITMSDNTAGNLLLKIVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDVRDTTTPA
Sbjct  121   AAITMSDNTAGNLLLKIVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDVRDTTTPA  180

Query  546   SMATTLRKLLTTPSLSARSQQQLLQWMVDDRVAGPLIRAVLPAGWFIADKTGAGERGSRG  725
             SMATTLRKLLTTPSLSARSQQQLLQWMVDDRVAGPLIRAVLPAGWFIADKTGAGERGSRG
Sbjct  181   SMATTLRKLLTTPSLSARSQQQLLQWMVDDRVAGPLIRAVLPAGWFIADKTGAGERGSRG  240

Query  726   IVALLGPDGKAERIVVIYLRDTPATMVERNQQIAGIGAALIEHWQR  863
             IVALLGPDGKAERIVVIYLRDTPATMVERNQQIAGIGAALIEHWQR
Sbjct  241   IVALLGPDGKAERIVVIYLRDTPATMVERNQQIAGIGAALIEHWQR  286
```

Figure 4.8 Results obtained from BlastX alignment search tool for SB3 reverse complement sequence.

4.9 BlastN Alignments

(a) BlastN alignment using 868 bases from TL9 reverse complement sequence

> gb|FJ668799.1| Klebsiella pneumoniae strain 7 plasmid beta-lactamase SHV-1 (blaSHV)
gene, complete cds
Length=937

```
 Score = 1659 bits (863),  Expect = 0.0
 Identities = 865/866 (99%), Gaps = 0/866 (0%)
 Strand=Plus/Plus

Query  1    TGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTTAGCCACCCTGCCGCTGGCGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72   TGGTTATGCGTTATATTCGCCTGTGTATTATCTCCCTGTTAGCCACCCTGCCGCTGGCGG  131

Query  61   TACACGCCAGCCCGCAGCCGCTTGAGCAAATTAAACTAAGCGAAAGCCAGCTGTCGGGCC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  132  TACACGCCAGCCCGCAGCCGCTTGAGCAAATTAAACTAAGCGAAAGCCAGCTGTCGGGCC  191

Query  121  GCGTAGGCATGATAGAAATGGATCTGGCCAGCGGCCGCACCCTGACCGCCTGGCGCGCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  192  GCGTAGGCATGATAGAAATGGATCTGGCCAGCGGCCGCACCCTGACCGCCTGGCGCGCCG  251

Query  181  ATGAACGCTTTCCCATGATGAGCACCTTTAAAGTAGTGCTCTGCGGCGCAGTGCTGGCGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252  ATGAACGCTTTCCCATGATGAGCACCTTTAAAGTAGTGCTCTGCGGCGCAGTGCTGGCGC  311

Query  241  GGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACTATCGCCAGCAGGATCTGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  312  GGGTGGATGCCGGTGACGAACAGCTGGAGCGAAAGATCCACTATCGCCAGCAGGATCTGG  371

Query  301  TGGACTACTCGCCGGTCAGCGAAAAACACCTTGCCGATGGCATGACGGTCGGCGAACTCT  360
            |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct  372  TGGACTACTCGCCGGTCAGCGAAAAACACCTTGCCGACGGCATGACGGTCGGCGAACTCT  431

Query  361  GCGCCGCCGCCATTACCATGAGCGATAACAGCGCCGCCAATCTGCTGCTGGCCACCGTCG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  432  GCGCCGCCGCCATTACCATGAGCGATAACAGCGCCGCCAATCTGCTGCTGGCCACCGTCG  491

Query  421  GCGGCCCCGCAGGATTGACTGCCTTTTTTGCGCCAGATCGGCGACAACGTCACCCGCCTTG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  492  GCGGCCCCGCAGGATTGACTGCCTTTTTTGCGCCAGATCGGCGACAACGTCACCCGCCTTG  551

Query  481  ACCGCTGGGAAACGGAACTGAATGAGGCGCTTCCCGGCGACGCCCGCGACACCACTACCC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  552  ACCGCTGGGAAACGGAACTGAATGAGGCGCTTCCCGGCGACGCCCGCGACACCACTACCC  611

Query  541  CGGCCAGCATGGCCGCGACCCTGCGCAAGCTGCTGACCAGCCAGCGTCTGAGCGCCCGTT  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  612  CGGCCAGCATGGCCGCGACCCTGCGCAAGCTGCTGACCAGCCAGCGTCTGAGCGCCCGTT  671

Query  601  CGCAACGGCAGCTGCTGCAGTGGATGGTGGACGATCGGGTCGCCGGACCGTTGATCCGCT  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  672  CGCAACGGCAGCTGCTGCAGTGGATGGTGGACGATCGGGTCGCCGGACCGTTGATCCGCT  731

Query  661  CCGTGCTGCCGGCGGGCTGGTTTATCGCCGATAAGACCGGAGCTGGCGAACGGGGTGCGC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  732  CCGTGCTGCCGGCGGGCTGGTTTATCGCCGATAAGACCGGAGCTGGCGAACGGGGTGCGC  791

Query  721  GCGGGATTGTCGCCCTGCTTGGCCCGAATAACAAAGCAGAGCGCATCGTGGTGATTTATC  780
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           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  792  GCGGGATTGTCGCCCTGCTTGGCCCGAATAACAAAGCAGAGCGCATCGTGGTGATTTATC  851

Query  781  TGCGGGATACGCCGGCGAGCATGGCCGAGCGAAATCAGCAAATCGCCGGGATCGGCGCGG  840
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  852  TGCGGGATACGCCGGCGAGCATGGCCGAGCGAAATCAGCAAATCGCCGGGATCGGCGCGG  911

Query  841  CGCTGATCGAGCACTGGCAACGCTAA    866
           ||||||||||||||||||||||||||
Sbjct  912  CGCTGATCGAGCACTGGCAACGCTAA    937
```

Figure 4.9 Results obtained from BlastN alignment search tool for TL9 reverse complement sequence.

(b) BlastN alignment using 868 bases from SB3 reverse complement sequence

> gb|AY512506.1| Klebsiella pneumoniae penicillinase OKP-5 gene, complete cds
Length=929

```
 Score = 1654 bits (860),  Expect = 0.0
 Identities = 864/866 (99%), Gaps = 0/866 (0%)
 Strand=Plus/Plus

Query  2     GGTTATGCGTTATATTCGCCTGTGCCTTATCTCCCTGATTGCCGCCCTGCCACTGGCGGT  61
             |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GGTTATGCGTTATGTTCGCCTGTGCCTTATCTCCCTGATTGCCGCCCTGCCACTGGCGGT  123

Query  62    ATTCGCCAGCCCTCAGCCGCTTGAGCAGATTAAAATCAGCGAAAGTCAGCTGGCGGGCCG  121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   ATTCGCCAGCCCTCAGCCGCTTGAGCAGATTAAAATCAGCGAAAGTCAGCTGGCGGGCCG  183

Query  122   GGTGGGCTATGTTGAAATGGATCTGGCCAGCGGCCGCACGCTGGCCGCCTGGCGCGCCAG  181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   GGTGGGCTATGTTGAAATGGATCTGGCCAGCGGCCGCACGCTGGCCGCCTGGCGCGCCAG  243

Query  182   TGAGCGCTTTCCGCTGATGAGCACCTTTAAAGTGCTGCTCTGCGGCGCGGTGCTGGCCCG  241
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   TGAGCGCTTTCCGCTGATGAGCACCTTTAAAGTGCTGCTCTGCGGCGCGGTGCTGGCCCG  303

Query  242   GGTGGATGCCGGCGACGAACAGCTGGATCGGCGGATCCACTACCGCCAGCAGGATCTGGT  301
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   GGTGGATGCCGGCGACGAACAGCTGGATCGGCGGATCCACTACCGCCAGCAGGATCTGGT  363

Query  302   GGACTACTCCCCGGTCAGCGAAAAACACCTTGCCGACGGGATGACCGTTGGCGAACTCTG  361
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   GGACTACTCCCCGGTCAGCGAAAAACACCTTGCCGACGGGATGACCGTTGGCGAACTCTG  423

Query  362   CGCCGCCGCCATCACCATGAGCGACAACACCGCCGGCAATCTGCTGTTGAAGATCGTCGG  421
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424   CGCCGCCGCCATCACCATGAGCGACAACACCGCCGGCAATCTGCTGTTGAAGATCGTCGG  483

Query  422   CGGCCCCGCGGGATTGACCGCTTTTCTGCGCCAGATCGGTGACAACGTCACCCGTCTTGA  481
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   CGGCCCCGCGGGATTGACCGCTTTTCTGCGCCAGATCGGTGACAACGTCACCCGTCTTGA  543

Query  482   CCGCTGGGAAACGGAACTCAATGAGGCGCTTCCCGGCGACGTGCGCGACACCACCACCCC  541
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  544   CCGCTGGGAAACGGAACTCAATGAGGCGCTTCCCGGCGACGTGCGCGACACCACCACCCC  603

Query  542   GGCCAGCATGGCCACCACCCTGCGCAAGCTGCTAACCACCCCCTCTCTGAGCGCCCGTTC  601
             ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  604   GGCCAGCATGGCCACCACCCTGCGCAAGCTTCTAACCACCCCCTCTCTGAGCGCCCGTTC  663

Query  602   GCAGCAGCAGCTGCTGCAGTGGATGGTTGACGACCGGGTGGCCGGCCCGTTGATCCGCGC  661
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  664   GCAGCAGCAGCTGCTGCAGTGGATGGTTGACGACCGGGTGGCCGGCCCGTTGATCCGCGC  723

Query  662   CGTGCTGCCGGCGGGCTGGTTTATCGCCGATAAAACCGGGGCCGGTGAGCGGGGCTCACG  721
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  724   CGTGCTGCCGGCGGGCTGGTTTATCGCCGATAAAACCGGGGCCGGTGAGCGGGGCTCACG  783

Query  722   CGGCATTGTCGCCCTGCTCGGCCCCGGACGGCAAAGCGGAGCGTATCGTGGTGATCTATCT  781
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  784   CGGCATTGTCGCCCTGCTCGGCCCCGGACGGCAAAGCGGAGCGTATCGTGGTGATCTATCT  843

Query  782   GCGTGATACCCCGGCGACCATGGTCGAGCGTAACCAGCAGATCGCCGGGATAGGCGCGGC  841
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  844   GCGTGATACCCCGGCGACCATGGTCGAGCGTAACCAGCAGATCGCCGGGATAGGCGCGGC  903

Query  842   GCTGATCGAGCACTGGCAACGCTAAC  867
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              ||||||||||||||||||||||||||
Sbjct  904  GCTGATCGAGCACTGGCAACGCTAAC  929
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Figure 4.10 Results obtained from BlastN alignment search tool for SB3 reverse complement sequence.

CHAPTER 5

DISCUSSION

5.1   Molecular Characterization of Bacterial Isolates for *bla*-SHV Gene

5.1.1   Sources of Bacterial Strains and Preliminary Screening for *bla*-SHV Gene

A total of 22 bacterial strains were used in this study. They were isolated from different types of vegetables obtained from Tesco, Kampar. Twenty bacterial strains were isolated from bean sprout and lettuce while the other two strains labelled as C3 and S3 were isolated from carrot and spring onion. All bacterial strains were subjected to various biochemical tests and antibiotic susceptibility disc tests in order to identify the characteristics of the bacteria and also to detect the resistancy of bacteria towards different type of antibiotics (Eo, 2010; Ng, 2011). Among 22 bacterial strains, only four isolates labelled as TL9, SB3, C3 and S3 were chosen to proceed to API 20E kit tests for presumptive identification of the bacteria species. The test results illustrated that all of the four bacterial isolates were correspond to *Klebsiella pneumoniae* (Eo, 2010; Ng, 2011).

The fast boil methodology were performed to extract the total DNA of 22 bacterial strains and subjected to PCR amplification using *bla*-SHV specific primers. This preliminary screening showed that all of the bacteria strains do

carry homologous *bla*-SHV gene based on the fragment size of approximately 870bp which identified through agarose gel image (Eo, 2010; Ng, 2011).

5.1.2 Plasmid DNA Extraction Using Alkaline Lysis Method

Although homologous *bla*-SHV gene was detected among the bacterial isolates, the location of this gene was not determined since the template used for PCR amplification was total DNA which composed of bacteria chromosomal DNA along with plasmid DNA. In order to find out the location of this gene, both of them must be separated for further studies. The solution to this problem is to extract pure plasmid DNA only from the bacteria isolates and proceed to PCR amplification again using the *bla*-SHV specific primers. Since the template used in PCR amplification was plasmid DNA, the presence of *bla*-SHV gene on the plasmid would be amplified and could be identified through agarose gel electrophoresis. There are a number of methods for extracting plasmid DNA from bacterial isolates, for example cesium chloride – ethidium bromide centrifugation (Clewell & Helinski, 1969), rapid-boiling method (Holmes & Quigley, 1981) and also alkaline lysis method (Birnboim & Doly, 1979). Alkaline lysis was chosen for extraction process as it is relatively easy to be performed and commonly used by researchers to determine plasmid borne *bla*-SHV. (Howard et al, 2002).

5.1.3 Observation of the Extracted Plasmid DNA after Gel Electrophoresis

Based on Figure 4.1 and 4.2, it showed that 18 out of 22 bacterial isolates do carries plasmids while the other four isolates SB1, SB9, SL1 and SL5 do not

seemed to contain any plasmids. The 18 plasmid-borne bacterial isolates were labelled as TL1, TL6, TL9, TL10, TL11, SB3, SB4, SB5, SB6, SB7, SB8, SL4, SL6, SL7, SL8, SL9, C3 and S3. The size of plasmids for each isolate is different since they were from different strains of bacteria. It was assumed that the presence of few bands in gel which varies in size for isolates TL1, TL9, SB3, SB5, SL6 and SL9 could be due to the fact that they have multiple plasmids in the cell.

The presence of multiple bands in Figure 4.1 & 4.2 could also be due to the various conformations of the plasmids since they are not linear in shape naturally. Generally, native plasmids should be in the form of covalently closed circular supercoiled molecules. If one of the strands was nicked due to pressure, they would favour to adopt a more relaxed form of plasmid known as open circular. A linearized form of plasmid could be detected when both of DNA strands were nicked by enzymes or other chemical agents. These three different conformations of plasmid have different mobility rate but most of the time the open circular plasmids move slower compared to others when all of them have equal mass because their size is bigger and hard to migrate through the gel pores. So, the number of bands appeared in the gel do not directly demonstrated the number of plasmids and it could be due to the different conformations of a plasmid only. Since alkaline lysis was performed using conventional methods instead of extraction kit, the appearance of high intensity of smear at the bottom part of the gel was due to contamination by RNA or protein that is not being discarded off completely during the extraction process.

5.1.4 PCR Screening for Plasmid-borne *bla*-SHV Gene

All extracted plasmids were subjected to PCR amplification using *bla*-SHV specific primers. Parameters such as time and temperature for denaturing, annealing and extension steps were experimentally optimized to obtain the desired gene fragments with appropriate size. In the end, the PCR cycles were reduced from 35 to 30 cycles while the annealing temperature was decreased to 50°C.

Based on Figure 4.3 (a) and (b), distinct band size of approximately 870 bp was detected for those 17 bacterial alkaline lysis extracts. Sample labelled as TL10 (lane 4) was the only plasmid-carrying isolate that does not show any band. The *bla*-SHV gene is not amplified in the 4 non-plasmid-carrying isolates (lane 4, 6, 14 and 16) as no distinct band was observed from the gel. The distinct band with expected size is an indication that *bla*-SHV gene is present on the plasmid since the region of interest were amplified using specific primers that hybridize to the coding region only. Since no band was observed for TL 10 isolate, this suggests that not all plasmid-carrying bacteria isolates have *bla*-SHV encoding genes. A number of bands other than expected size were detected in PCR products of the SB3, SB5 and SL4 isolates (lane 7, 9 and 15). This might be caused by unspecific binding of the primers to regions other than *bla*-SHV gene. It can be improved by increasing the temperature until one distinct band is being observed on the gel.

The purpose of including SB1, SB9, SL1 and SL5 isolates which did not carry any plasmids in PCR screening were to test whether they have *bla*-SHV gene to

be amplified. This is because they might have low copy number of plasmid which was not able to be detected or their bands might overlap with the high intensity of smearing appeared in Figure 4.1 and 4.2. The undetectable or low copy number of plasmids might contain the *bla*-SHV gene and thus being amplified through PCR reaction. Since no distinct bands were found among those 4 isolates based on Figure 4.3(a) and (b), hence it can be confirmed that no plasmid is present in their cells. On the other hand, this could serve as an indication that the alkaline lysis methodology were performing well for extracting plasmid only but not chromosomal DNA as *bla*-SHV gene could be found in chromosome and being amplified. The *bla*-SHV gene was found in the chromosome of the 4 isolates through preliminary PCR screening performed in earlier phase by Ng (2011).

5.1.5   Purification of PCR Gene Fragments from Agarose Gel

The amplified *bla*-SHV gene fragments of bacterial isolates C3, S3, TL9, TL11, SL4, SL8, SB3, SB4, SB6, and SB8 were successfully purified using the Gel Extraction Kit (Fermentas). The 10 purified gene fragments were within the expected size, approximately 870 bp and only one intense distinct band was observed showing that the purification procedures were successful. The absorbance and concentration readings for the 10 chosen bacterial isolates were illustrated in Table 4.3. The yields of purified gene fragments were relatively high for SL8 and SB8 with 47.50 ng/µl and 35.00 ng/µl, respectively. The concentrations of other isolates such as SB3, SB4 and SB6 were within the range of 20.00 ng/µl to 24.50 ng/µl while isolates SL4, C3, and TL11 have

concentrations between the range of 12.18 ng/µl to 15.00 ng/µl. The concentrations for S3 and TL9 were the lowest compared to other isolates, each having 9.79 ng/µl and 8.98 ng/µl.

The purity of DNA could be indicated by comparing the absorbance ratio of 260 nm and 280 nm. Generally, a pure DNA would have an A_{260}/A_{280} ratio of 1.7-2.0 after evaluation by spectrophotometer. A ratio that is less than 1.7 signifies protein contamination while a ratio more than 2.0 shows RNA contamination (Sambrook and Russell, 2001). There are total eight isolates having ratio of within the range of 1.7-2.0 and were categorized as pure DNA gene fragments, these isolates include C3, TL9, TL11, SL4, SL8, SB4, SB6 and SB8. The gene fragments of isolates S3 and SB3 were suspected having protein contamination as their ratio is 1.6, a slight lower compared to purity range.

5.1.6   Transformation

Six purified gene fragments such as C3, S3, TL9, TL11, SB3 and SB4 were chosen to proceed to transformation process in order to determine the type of *bla*-SHV gene present in those isolates. All of them were ligated into pGEM®-T Easy vector system with the help of T4 ligase and ligation buffer. These ligation processes require overnight incubation at 4°C.

5.1.7 Colony PCR

In this study, the purified *bla*-SHV gene fragments from two different bacterial isolates labelled as TL9 and SB3 were successfully inserted into pGEM-T Easy vector and transformed using *E. coli* strain JM109 while the other four fragments, C3, S3, TL11 and SB4 failed to undergo transformation. The transformed white colonies carries the recombinant plasmids from 2 different bacteria populations were chosen using toothpick before proceeding to colony PCR using T7 forward primer and SP6 reverse primers.

Based on Figure 4.6 (a), the sizes of colony PCR products for TL9 and SB3 were around 1000 bp which correspond to the expected size of approximately 1000 bp. The colony PCR product covers gene fragment which has the size of 870 bp and also the T7 and SP6 promoter region along with multiple cloning region which have the size of 140 bp. Therefore, it can be assumed that the whole purified gene fragment was ligated into plasmid and the colony PCR conditions works well to amplify the whole fragment region. Colony PCR was performed to quickly screen for plasmid inserts directly from *E. coli* bacteria colonies and to determine the insert size and orientation in the vector. This screening is important as not all the white colonies have the complete fragment size inserted into the plasmid. It is possible for a small piece of fragment DNA to be ligated into the vector's multiple cloning sites and hence disrupt the expression of β-galactosidase that is needed to cleave the X-gal forming blue colonies.

5.1.8   Recombinant Plasmid Extraction

Since the transformed colonies of TL9 and SB3 were confirmed to carry the ideal size of gene fragments on plasmid through colony PCR, both of them were used for recombinant plasmid extraction using Plasmid DNA Purification Kit. The size and purity of purified recombinant plasmid were determined using agarose gel electrophoresis and nano-spectrophotometer respectively. Based on Figure 4.6(b), both recombinant plasmids carrying TL9 and SB3 gene-fragments having size of approximately 3000 bp.

There were a few bands with some smearing appeared on the gel image indicated that the recombinant plasmid might have different conformation, each with different sizes. The differences in recombinant plasmid size might due to the plasmid's conformation, they tend to achieve closed circular supercoiled state compared to relax stated judged by band intensity. The closed-circular supercoiled plasmids are able to move easily between gel pores during the electrophoresis process and appeared the furthest from loading well most of the time. So, the plasmid would appear as smaller size compared to expected size. The TL9 and SB3 recombinant plasmid with purity around 1.8 to 2.0 were outsource for sequencing to determine the identity of the inserted gene fragments.

5.1.9   BlastX and BlastN Alignment Search

In this study, two purified recombinant plasmid inserted with TL9 and SB3 gene fragments were sequenced by 1st Base Laboratories Sdn. Bhd. Both the forward

and reverse sequence of gene fragments were sequenced using the T7 and SP6 primers. The DNA sequences were aligned using BlastX and BlastN software that can be accessed through the NCBI main page.

The BlastX alignment search for TL9 reverse complement sequence showed high similarity with beta-lactamase SHV-1 produced by *Yersinia pestis* biovar Orientalis str. The alignment showed 100% identities for a total of 286 amino acids residues and has score hits of 560 with low E-value of 1e-157. A total of 868 nucleotide sequences were used for this alignment with query coverage of 98%. The BlastX alignment search for SB3 reverse complement sequence showed high similarity with penicillinase OKP-5 produced by *Klebsiella pneumoniae*. The alignment showed 99% of identities for 285 out of 286 amino acid residues. It has score with 535 hits and E-value of 2e-150. The query coverage is 98% and a total of 868 nucleotide sequences were used for the alignment.

The BlastN alignment search for TL9 reverse complement sequence showed 99% identity with *Klebsiella pneumoniae* strain 7 plasmid beta-lactamase SHV-1 and no gaps were found in this search. For SB3 reverse complement sequence, it showed 99% identity with *Klebsiella pneumoniae* penicillinase *OKP*-5 gene after the BlastN alignment search was performed. From the electropherogram, it demonstrated that both of them have distinctive and high peaks for the overall sequences which signified the sequences are reliable for further interpretation.

5.2   Prevalence of *Klebisella pneumoniae* Carrying *bla*-SHV Gene

The SHV family of β-lactamases appear to have been derived from *Klebisella* spp. SHV-1 was the progenitor for SHV class of enzymes which generally found in chromosome of *K. pneumoniae* (Chaves et al, 2001) and it confers resistance to broad-spectrum penicillins and cephalosporins (Livermore, 1995). In 1983, three strains of *K. pneumoniae* and one *Serratia macrcescens* isolated in West Germany were demonstrated to cause transferable resistance to cefotaxime and to other new cephalosporin (Knothe et al, 1983). This new plasmidic β-lactamase known as SHV-2 derived from a mutation in SHV-1 which marked the emergence of extended-spectrum β-lactamases (ESBLs). Mutation happens due to replacement of glycine by serine at the amino acid 238 resulted in an enchance affinity of the SHV-2 β-lactamase for oxyimino cephalosporins compared to SHV-1. The substitution confers a large increase in resistance to cefotaxime and a small increase in resistance to ceftazidime (Kliebe et al, 1985).

Based on this study, it was demonstrated that most of the Enterobacteriaceae carry *bla*-SHV gene on their plasmids. It is prevalence in *Klebsiella pneumoniae* as 4 bacterial isolates labelled as C3, S3, TL9 and SB3 were identified as *Klebsiella pneumoniae* after API 20E kit tests were performed (Eo, 2010; Ng, 2011). TL9 bacterial isolates are suspected carrying *bla*SHV-1 gene while SB3 bacterial isolates might carry penicillinase *OKP*-5 gene based on the BlastX and BlastN results. The *bla*SHV-1 gene are categorised as non-ESBL gene which are normally found in *Klebisella pneumoniae* (Mendonca et al, 2009). Penicillinase

OKP-5 is a specific type of beta-lactamase which evolved from a common ancestor over years along with other two families of chromosomal beta-lactamase gene, *bla*-SHV and *bla*-LEN (Haeggman, Lofdahl, Paauw, Verhoef & Brisse, 2004). The TL9 bacterial isolates showed ESBL producing traits while SB3 did not exhibit this trait after both of them were subjected to phenotypic confirmatory tests for ESBL production (Ng, 2011). In this test, ESBL producers would demonstrate a ≥ 5mm zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone or when tested alone with Cefotaxime/Clavulanate and Ceftazidime/Clavualanate discs (Al-Jasser, 2006).

It is currently unknown whether the genes encoding non-ESBLs in ESBL-positive strains are located on the chromosome or on plasmids (Howard et al, 2002). This study revealed that the non-ESBL gene such as *bla*SHV-1 is found in plasmids rather than chromosome. The plasmid preparation using alkaline lysis method should contain pure plasmid DNA only and it was certain that *bla*SHV-1 gene were being amplified from plasmid rather than chromosome. If the gene was amplified from small quantity of chromosome DNA, the band intensity would not as bright as those bands that were amplified with large quantities of plasmid DNA prepared from alkaline lysis. The determination of the location of non-ESBL gene is significant in order to select those phenotypically ESBL-positive *K. pneumoniae* strains. *Klebsiella pneumoniae* isolates that lack of plasmid-borne *bla*-SHV gene are unable to evolve to become phenotypically ESBL positive strain (Hammond, Harris, Bell, Turnidge & Giffard, 2008). This might due to

enhanced expression of plasmid-borne *bla*-SHV affected by copy number and also the promoter activity (Turner et al, 2009).

It is important to understand the location of non-ESBL gene presence in bacteria and how this contributed to the emergence of bacteria which able to synthesize the multidrug-resistant ESBL. The amount of time require to invent and produce new antibodies which are efficient in treating those patients infected with *Klebsiella* spp. that carries ESBL-encoding gene would be shorten if the enzyme mechanism or pathway were fully explored and understand. Both improvements in antibiotic and infection control strategies are needed before dealing to serious infections caused by multidrug-resistant Enterobacteriaceae especially *Klebsiella* spp. as the plasmid containing genes which encode ESBL are easily transferable among bacteria species.

5.3   Future Studies

Transformation could be conducted for bacterial isolates that were not able being transformed for sequencing, so that more information could be gathered to reconfirm the location and type of non-ESBL or ESBL gene in phenotypically ESBL positive bacteria strains other than *Klebsiella pneumoniae* since only two bacterial isolates TL9 and SB3 were managed sent for sequencing in this study. Plasmid fingerprinting and characterizing can be performed by digesting the desired plasmid with an appropriate restriction enzyme to produce linear fragments which can be precisely sized on an agarose gel since the plasmid size

was not determined in this study. With this excised plasmid fragments, Southern

blot can be carry out to determine the exact location of the *bla*-SHV gene using

DNA probe which complementary to the gene sequences.

CHAPTER 6

CONCLUSION

In this study, 18 out of 22 bacterial isolates from family of Enterobacteriaceae shown to contain plasmid after extraction using alkaline lysis methodology. Among the plasmid carriers, *bla*-SHV gene was suspected to present in 17 bacterial isolates after PCR amplification was conducted using forward and reverse *bla*-SHV specific primers. PCR products corresponding to the expected size of *bla*-SHV gene of six bacterial isolates were ligated and proceed to transformation. Recombinant plasmid containing gene fragment from TL9 and SB3 were then subjected to colony PCR to access the success of the ligation performed. The desired recombinant plasmids were subsequently extracted before out-sourced for sequencing.

The sequencing results of TL9 and SB3 gene fragments were aligned using BlastX and BlastN software available in NCBI website. The BlastN alignment results indicated that both of TL9 and SB3 shown high similarities with gene fragment of *Klebsiella pneumoniae*. The PCR fragment of TL9 was identified as non-ESBL *bla*SHV-1 gene while SB3 amplified fragment was identified as penicillinase *OKP*-5 gene. TL9 bacterial isolate demonstrated that gene encoding non-ESBLs such as *bla*-SHV-1 are located on plasmids in ESBL producing strains. The ESBLs associated infections might become increasingly complex to

treat as the bacteria are able to confer resistance to different types of antibiotic since the plasmid which carries resistance gene are transferable among bacteria species. A full understanding about the genotype and phenotype of ESBL producing bacteria would allow clinical microbiology laboratories to diagnose them easily and came out with appropriate antimicrobial agent as treatment or preventions.

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The promoter and multiple cloning sequence of the pGEM®-T Easy vector
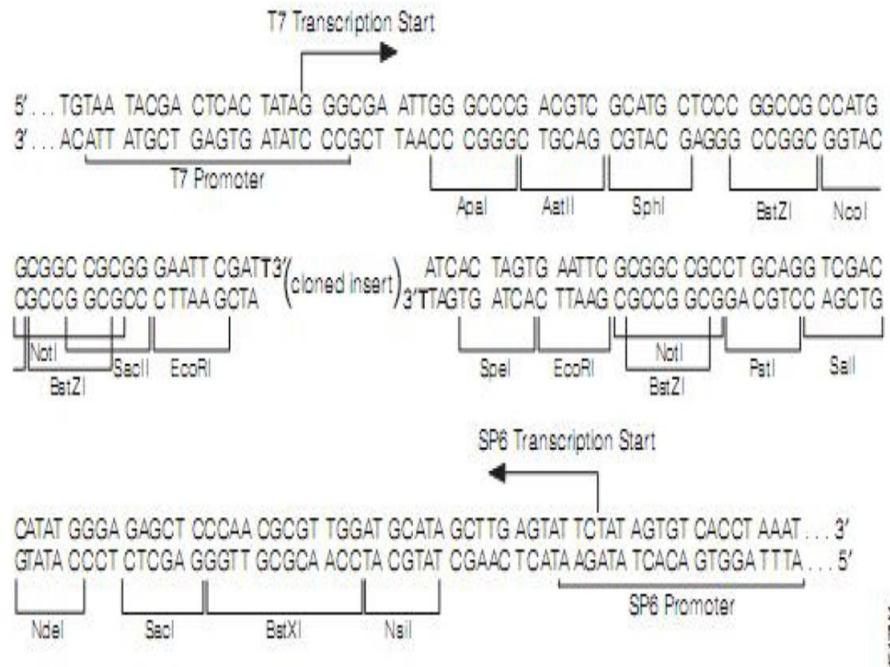
pGEM®-T Easy Vector Map and Sequence Reference Points
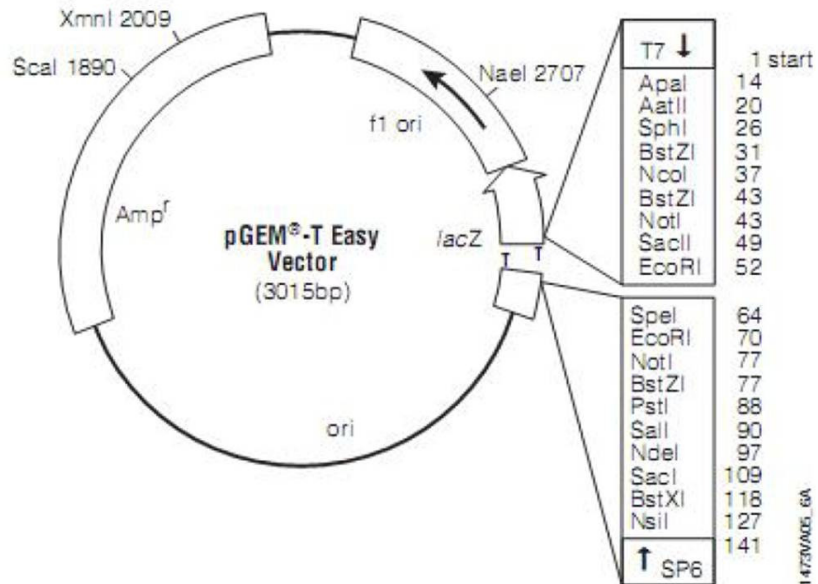
pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3