



Faculty of Resource Science and Technology

**Correlation between Antibiotic Resistance and The Presence of Plasmid among
Enterobacteriaceae Isolated from Rayu River Sarawak**

Tang Ping Sia

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Correlation between Antibiotic Resistance and The Presence of Plasmid in The Isolates from Rayu
River Sarawak

Tang Ping Sia (28492)

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of
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Supervisor: Dr. Samuel Lihan

Programme Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

University Malaysia Sarawak

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

Tang Ping Sia

Resource Biotechnology Programme

Department of Molecular Biotechnology

Faculty of Resource Science and Technology

University Malaysia Sarawak

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List of Abbreviations

%	Percentage
°C	Degree Celcius
DAEC	Diffusely adherent <i>E. coli</i>
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EtBr	Ethidium bromide
EPEC	Enteropathogenic <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
EMB	Eosine Methylene Blue
EIEC	Enteroinvasive <i>E. coli</i>
EAggEC	Enteraggregative <i>E. coli</i>

g	Gram
kb	Kilo-base pair
LB	Luria-Bertani
rpm	Revolutions per minute
MAR	Multiple Antibiotic Resistance
l	Microlitre
ml	Millilitre
mA	Milliampere
NA	Nutrient agar
STEC	Shiga toxinproducing <i>E. coli</i>
TBE	Tris-Borate-EDTA
V	Voltages
VTEC	Vero cytotoxin-producing <i>E. coli</i>

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Correlation between Antibiotic Resistance and The Presence of Plasmid among *Enterobacteriaceae* Isolated from Rayu River Sarawak

Tang Ping Sia

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Enterobacteriaceae are natural habitat in the human and animal's gastrointestinal tract that trigger disease by invasive action and production of toxin. To date, the occurrences of foodborne and waterborne illness due to *Enterobacteriaceae* infection has been increasing rapidly and thus raising the global awareness. Conditions become worse when most of the microorganisms become highly resistance to antibiotics. Emerging of antibiotic resistance among *Enterobacteriaceae* was hazardous and complicating the treatment of disease associated with the enteric bacteria. Thus, present study was carried out to analyse the antibiotic resistance among *Enterobacteriaceae*, presence of plasmid and correlation between the antibiotic resistance and the plasmid presence in the isolates from Rayu River, Sarawak. The antimicrobial susceptibility test shows that all of the isolates were susceptible to sulphamethoxazole trimethoprim (100%) and this was followed by norfloxacin and carbenicillin which both encountered 98.59%. About 44.48% of the isolates have MAR index higher than 0.2, implying that the areas have been highly contaminated with the application of antibiotics. The range of MAR index value obtained ranged from 0.75 to 0.5 was highly exceeds the standards of low risk contamination range. The molecular weights of plasmids detected ranged from 0.5 kb to 0.6 kb. It was rare for the occurrences of plasmid with molecular weight which was larger than 3 kb in Rayu River. Investigation showed that most of the plasmid possess more than one plasmid. Meanwhile, SPSS analysis shows that there were significant correlations between the antibiotic resistance with the presence of plasmid either in the form number or molecular weight of plasmid.

Keywords: *Enterobacteriaceae*, MAR index, number of plasmid, size of plasmid, correlation.

ABSTRAK

Enterobacteriaceae boleh didapati dalam saluran gastrousus manusia dan haiwan secara semula jadinya. Ianya boleh menyebabkan penyakit melalui tindakan invasif dan pengeluaran toksin. Setakat ini, kejadian penyakit bawaan makanan dan air yang disebabkan oleh jangkitan *Enterobacteriaceae* telah meningkat secara mendadak dan meningkatkan kesedaran global. Keadaan menjadi semakin teruk apabila kebanyakan mikroorganisma tahan rintangan kepada antibiotik. Kemunculan tahan rintangan terhadap antibiotik dalam kalangan *Enterobacteriaceae* adalah merbahaya dan merumitkan rawatan penyakit berkaitan dengan bakteria perut. Oleh itu, kajian ini telah dijalankan untuk menganalisis tahan rintangan terhadap antibiotik dalam kalangan *Enterobacteriaceae*, kehadiran plasmid serta kaitan antara tahan rintangan kepada antibiotik dan kehadiran plasmid dalam sampel bakteria daripada Sungai Rayu, Sarawak. Ujian kerentanan antibiologi menunjukkan bahawa semua sampel bakteria kurang tahan rintangan terhadap sulphamethoxazole trimethoprim (100%) dan diikuti oleh norfloxacin dan carbenicillin dengan kedua-duanya menunjukkan 98.59%. Lebih kurang 44.48% daripada sampel bakteria telah mencecah nilai indeks MAR iaitu 0.2 yang menunjukkan bahawa kawasan-kawasan tersebut tercemar dengan serius akibat daripada penggunaan antibiotik. Julat nilai indeks MAR yang diperolehi adalah di antara 0.5 hingga 0.75 iaitu melebihi piawaian julat pencemaran risiko rendah. Julat berat molekul plasmids dikesan adalah di antara 0.5 kb hingga 0.6 kb. Berat molekul plasmid yang melebihi 3 kb adalah kurang dalam Sungai Rayu. Hasil kajian menunjukkan bahawa kebanyakan sampel bakteria memiliki lebih daripada satu plasmid. Sementara itu, analisis SPSS menunjukkan bahawa terdapat hubungan yang signifikan antara tahan rintangan kepada antibiotik dengan kehadiran plasmid sama ada dari segi nombor atau berat molekul plasmid.

Kata Kunci: *Enterobacteriaceae*, indeks MAR, nombor plasmid, saiz plasmid, korelasi.

1.0 Introduction

Enterobacteriaceae are generally recognized as enteric bacteria, a common inhabitant of human's and animal's gastrointestinal tract (Rollins *et al.*, 2000). They also can be found in soil, water and plants. Although all of them are gram-negative, straight rods and facultative anaerobic bacteria but only some of them are motile. Nevertheless, most of the members of *Enterobacteriaceae* are pathogens such as *Escherichia coli*, *Salmonella*, *Yersinia pestis*, *Klebsiella* and *Shigella* can be incriminated in intestinal infection and extra-intestinal diseases. For instances, enteric infection included nosocomial infections which involved the urinary tract, surgical and wound sites, bloodstream, and pneumonias (Baron, 1996), abscesses, meningitis, pneumonia, and septicaemia. Intestinal infection triggered by *Enterobacteriaceae* was known as the major health threats in developing countries where sanitation standards are low (Jarzab *et al.*, 2011).

E. coli are universally recognized as the premier nosocomial pathogen which serve as part of the normal flora of the colon in humans and other animals and pathogenic both within and outside the gastrointestinal tract (GI) (Elshamy, n.d.). The virulent strains are located on the virulence plasmid, which serves the bacteria to colonize human tissues (Microbiology, n.d.). According to Microbiology, the frequent outbreak of pathogenic *E. coli* strain was 0157:H7, a causative agent that release Shiga-toxin in a number of recreational water outbreaks instigates diarrhoea, hemorrhagic colitis, and haemolytic uremic syndrome. In year 2000, the outbreaks of waterborne disease were affected by *E. coli* 0157:H7 that resulted in six deaths and around 2300 cases in Walkerton, Ontario Canada (Bruce-Grey-Owen Sound Health Unit, 2000). On the other hand, there was another occurrence in 2001, in which the enteric bacteria infected four young children during the bathing at a public beach in the Montreal-Centre

region (Public Health Agency of Canada, 2004). One of the properties that make *Escherichia coli* (*E. coli*) a good indicator of microorganisms for faecal contamination is that they are abundant in faeces.

Emerging of the *Enterobacteriaceae* that are highly resistant to antibiotics is hazardous and can complicate the treatment of disease associated with the enteric bacteria. According to Paterson (2006), the data obtained from intensive care units in United States showed that about 20% of *Klebsiella pneumonia* and 31% of *Enterobacter spp.* infections are resistant to third-generation cephalosporins by the production of β – lactamases . Quinolone is originally used to treat the severe *E. coli* UITs and other *Enterobacteriaceae* family members (Hooper, 1998; Carson & Naber, 2004). Nevertheless, recently awareness about the resistance to the antibiotic had being increased due to the emerging of plasmid-mediated resistance to quinolones (Paterson, 2006). In Malaysia, the incidents of nosocomial caused by *Enterobacter gergoviae* had been reported in which eleven babies and nine of premature were investigated in the neonatal intensive care unit (NICU) of a general hospital in Johor Bahru (Ganeswire *et al.*, 2003).

Since the outbreaks of *E. coli* was common and threatening the human health, thus this study is carried out to study the correlation between antibiotic resistance and the presence of plasmid among *Enterobacteriaceae* isolated from water.

The specific objectives of this study are to:

- I. analyse antibiotic resistance among *Enterobacteriaceae* isolates
- II. detect the presence of plasmid in the bacteria
- III. correlate between the antibiotic resistance and the presence of plasmid

2.0 Literature Review

2.1 *Enterobacteriaceae*

Enterobacteriaceae are Gram-negative rod-shaped bacteria, and they are facultative anaerobes which have relatively simple growth requirements (McGraw-Hill Concise Dictionary of Modern Medicine, 2002). The bacteria grow readily at 35°C except *Yersinia* which grow better at 25 - 30°C (NeLI, 2007). They ferment sugars into lactic acid with the production of gas and reduce nitrates to nitrites. Most of the bacteria are motile by means of peritrichous flagella except *Shigella* and *Klebsiella* which are non-motile. *Enterobacteriaceae* are primarily saprobes and parasites which can be found in soil, water, and in plants and animals. They are part of the intestinal flora of humans and other animals whereby the disease is generally provoked by invasive action and production of toxin. Half of the nosocomial infections reported in the United States are most commonly caused by species such as *Escherichia*, *Enterobacter*, *Klebsiella*, *Proteus*, *Providentia*, and *Salmonella* spp; whereas the less pathogenic *Enterobacteriaceae* include *Citrobacter*, *Edwardsiella*, *Erwinia*, *Hafnia*, *Serratia*, *Shigella*, *Yersinia* spp (McGraw-Hill Concise Dictionary of Modern Medicine, 2002).

2.2 Common genera of the family *Enterobacteriaceae*

2.2.1 *Escherichia* species

Recently, there are six species being identified in the genus of *Escherichia* with the most common species of *E. coli* and other five less frequently encountered members namely *E.*

blattae, *E. hermannii*, *E. vulneris*, *E. fergusonii* (Farmer, 1999) and *E. albertii* (Huys *et al.*, 2003).

Escherichiae are straight, Gram-negative rods that occur singly or in pairs and can be motile or non-motile. During fermentation of d-glucose, *Escherichiae* produce strong acids and gas but they do not produce acetyl-methyl carbinol (acetoin). This species of bacteria are the inhabitants of the gastrointestinal tracts of warm-blooded animals and they do provide the microbially-derived vitamin K for the host. While many *Escherichia* are harmless commensals, yet there are several strains of *Escherichia coli* which are pathogenic, that are capable of causing urinary tract infections and gastrointestinal disease.

E. coli strains can be classified into six different main categories based on epidemiological evidence, phenotypic traits, clinical features of the disease produced and specific virulence factors (Johannes, 2005). The six categories of diarrheagenic *E. coli* which have been recognised are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAaggEC), diffusely adherent *E. coli* (DAEC) and Shiga toxin-producing *E. coli* (STEC) or Vero cytotoxin-producing *E. coli* (VTEC). Table 1 shows the strains of *Escherichia coli* that cause gastrointestinal illness (Guerrant & Thielman, 1995).

Table 1: *Escherichia coli* strains that cause gastrointestinal illness

Strain	Type of diarrhea	Virulence factors	Genetic coding
Enteroinvasive (EIEC)	Acute dysenteric	Cell invasion and intracellular multiplication	Plasmid and chromosomal
Diffusely adherent (DAEC)	Watery, in children	Both fimbrial and non- fimbrial adhesins	Plasmid and chromosomal
Enteroaggregative (EAEC)	Persistent	Aggregative adherence and heat-stable enterotoxin	Plasmid
Enterotoxigenic (ETEC)	Acute watery	Adherence and heat- labile toxins	Plasmid and chromosomal
Enteropathogenic (EPEC)	Acute and/or persistent	Localized and attaching and effacing adherence	Plasmid and chromosomal
Enterohemorrhagic (EHEC)	Bloody, with or without sequelae including haemolytic uremic syndrome	Attaching and effacing adherence, enterohemolysin, and Shiga-like toxins	Phage, plasmid, and chromosomal

Note. From “Foodborne infections and intoxications” by P. M. Fratamico *et al.*, 2006, New York: Elsevier.

Enteropathogenic *E. coli* (EPEC) referred to strains include certain O:H serotypes associated with illness. The pathogenic characteristics of EPEC are due to its ability to cause attaching and effacing (A/E) histopathology and its inability to produce Shiga toxins (Kaper, 1996). EPEC strains secrete EspA, B, D and Tir proteins used in attaching and effacing activity through type III apparatus. EPEC induces tyrosine phosphorylation; *in vitro* release of intracellular calcium; phospholipase C activity; reactions that induce host cell proteins to initiate cytoskeletal rearrangement; and bacterial uptake (Rosenshine *et al.*, 1992; Manjarrez-Hernandez *et al.*, 1996).

Enterotoxigenic *E. coli* (ETEC) strains are the main cause of diarrhoea in humans and domestic animals by the adherence and colonization of the intestinal mucosa, and they produce enterotoxins which are heat-labile (LT) or heat-stable (ST) (Cohen & Giannella, 1995). The major adherence factors in human strains include colonization factors antigens

(CFAs) which comprise of CFAs I, II and IV, and they are found in diarrhoea-causing ETEC only (Salyers & Whitt, 1994).

Enteroinvasive *E. coli* (EIEC) strains are biochemically and genetically related to *Shigella*, which capable of invading and multiplying in the intestinal epithelial cells. EIEC move directly through the cytoplasm and the movement is mediated by the attachment of cellular actin to one pole of the bacterial cell (Nataro & Kaper, 1998).

Enteroaggregative *E. coli* (EAaggEC) are characterized by aggregative adherence (AA) pattern to Hep-2 cells *in vitro* whereby the AA pattern is plasmid-mediated (Nataro *et al.*, 1985) and was suspected to be a putative agent of diarrheal disease (Vial *et al.*, 1988). A three-stage model has been proposed for EAEC pathogenesis; stage I is the adherence of EAaggEC to the intestinal mucosa and mucous layer; stage II involves the production of mucus, leading to a thick EAEC-encrusted biofilm on the mucosal surface; and stage III involves the elaboration of cytotoxin(s) resulting in intestinal secretion and damage to the intestinal mucosa (Nataro *et al.*, 1998; Okeke & Nataro, 2001). The inability of EAaggEC-infected patients in repairing the damage on the intestinal mucosa will lead to persistent diarrhoea.

Diffusely adherent *E. coli* (DAEC) are characterized by the diffuse adherence (DA) pattern of *E. coli* strains to HEp-2 cells (Scaletsky *et al.*, 1984; Nataro *et al.*, 1985). The role of DAEC in diarrhoea remains unclear. A fimbriae designated F1845 is responsible for the diffusion of Hep-2 cell adhesion by diarrheic *E. coli* isolates (Bilge *et al.*, 1989). Meanwhile, another adhesin designated AIDA-I has also been found to be associated with DA of *E. coli* of serotype O126:H27 (Benz & Schmidt, 1989).

Shiga toxin-producing *E. coli* (STEC) or Vero cytotoxin-producing *E. coli* (VTEC) strains are defined by their ability to produce bacteriophage-mediated cytotoxins, *Stx1* or VT1 and *Stx2* or VT2. Levine (1987) stated that the term *enterohemorrhagic E. coli* (EHEC) is used to refer to strains that have similar clinical and pathogenic features with the prototype organism *E. coli* O157:H7. Stxs/VTs act to inhibit protein synthesis through depurination of adenine which in turn inhibiting the elongation factor 1 (EF-1)-dependent aminoacyl-tRNA binding to 60S ribosomal subunits (Endo *et al.*, 1988).

2.2.2 *Salmonella* species

Salmonella is a gram-negative facultative rod-shaped microbe under the family of *Enterobacteriaceae*. This microorganism had been identified as the origin of the infection of salmonellosis (Todar, 2012). Infection of salmonellosis can become severe when the infection spread from the intestines to blood stream and other parts of body (Centers for Disease Control and Prevention, 2013). The genus of *Salmonella* encompass of five species: *Salmonella arizonae*, *Salmonella choleraesuis*, *Salmonella enteritidis*, *Salmonella typhi* and *Salmonella typhimurium* (Euzéby, n.d.). *Salmonella* can be found either in intestinal tracts of warm or cool blooded animals. For instance, subspecies of *Salmonella*, *S. enteric* include I = *enterica*, II = *salamae*, IIIa = *arizonae*, IIIb = *diarizonae*, IV = *houtenae* and VI = *indica* (Brenner *et al.*, 2000).

The outbreak of foodborne disease due to the infection of *Salmonella* in United States is common. These kind of enteric bacteria can be transmitted by food, animals and their environment such as contaminated eggs, poultry, meat, reptiles, amphibians, pet food and treats. The *Salmonella* family comprise of more than 2300 serotypes of bacteria that had been identified (United States Department of agriculture, n.d.).

2.2.3 *Shigella* species

Shigellosis is a disease caused by *Shigella* in humans and other primates (Dupont, 2000), which associated with bloody diarrhoea and other gastrointestinal symptoms (Dupont, 2000; Todar, 2012). There are four species of *Shigella*, namely *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei* that differentiated based on their biochemical traits, an ability to ferment D-mannitol and antigenic properties (Marler Clark, 2013).

2.2.4 *Klebsiella* species

Klebsiellae are gram-negative bacteria with a prominent polysaccharide capsule that are capable in resisting host defence mechanisms (Medscape, 2013). This genus expresses pathogenic antigens such as lipopolysaccharide (O antigen) and capsular polysaccharide (K antigen) on their surface (Medscape, 2013). There are seven species of *Klebsiella* being identified, namely *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*, *Klebsiella oxytoca*, *Klebsiella planticola*, *Klebsiella terrigena* and *Klebsiella ornithinolytica*.

2.2.5 *Proteus* species

There are five named species in the genus *Proteus* that are *P. mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxofaciens*, and *P. hauseri*; and three unnamed genomospecies (O'Hara, *et al.*, 2000). *Proteaeae* are widespread in the environment and they are mostly found as part of normal flora in the human gastrointestinal tract (O'Hara, *et al.*, 2000; Medscape, 2013).

2.2.6 *Enterobacter* species

Members of the genus *Enterobacter*, mainly *E. cloacae* and *E. aerogenes* are important nosocomial pathogens of various diseases such as bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections and septic arthritis (Medscape, 2013); they can also cause opportunistic infections in immunocompromised patients (Public Health England, 2013). According to Reissbrodt and Rabsch (1988), all the strains of *E. cloacae*, *E. aerogenes*, *E. gergoviae*, *E. sakazaki* and *E. agglomerans* produced enterochelin under iron-scarcity conditions to solubilise and import the required iron.

2.2.7 *Serratia* species

The genus *Serratia* are opportunistic gram-negative bacteria with *Serratia marcescens* appears to be the primary pathogenic species (Mahlen, 2011). Some strains of *S. marcescens* produce nondiffusible red pigment, prodigiosin (Williams & Qadri, 1980), which might be toxic to protozoa (Groscop & Brent, 1964).

2.2.8 *Yersinia* species

Yersinia species are facultative anaerobes, relatively slow growers and they display their biochemical characteristics most reliably at temperatures between 25 and 32°C (Anonymous, 1997). There are eleven species in the genus of *Yersinia*, with three species namely, *Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* that are pathogenic in human and animals (Wolters Kluwer Health, 2013).

2.3 Outbreak of *Enterobacteriaceae* Disease

The outbreaks of gastrointestinal disease which caused by Shiga toxin-producing *Escherichia coli* (STEC) has posed a great impact to public health (Griffin *et al.*, 1994; Fukushima *et al.*, 1997). In 1999, Centers for Disease Control and Prevention (CDC) reported that about 73 480 people per year in the USA were infected with *E. coli* 0157:H7 with 61 of these cases were fatal (Mead *et al.*, 1999). Most of the illness cases related with *E. coli* 0157:H7 are due to food-borne infection (Armstrong *et al.*, 1996). However, there is also the spread of disease through direct contact with animals and manure at petting zoos and dairy farms (Heuvelink *et al.*, 2002; Duffy, 2003). Among the *E. coli* 0157:H7 food-borne outbreaks in 1999, one-third of the cases were attributable to beef with the most common medium was the undercooked ground beef (Griffin *et al.*, 2000). The main source of the bacteria in ground beef is bovine faeces, which contaminates carcasses before evisceration (Elder *et al.*, 2000; Nou *et al.*, 2003).

Among the genera of *Salmonella*, *Salmonella enterica serotypes typhi* and *paratyphi* are the host for the occurrence of typhoid and paratyphoid fever respectively. In year 2000, there was serious epidemic of typhoid fever in which there were about 21.7 million people infected and caused 217000 deaths (Crump *et al.*, 2004). In the same year, there were approximately 5.4 million illnesses triggered by *S. Paratyhi* (Crump *et al.*, 2004).

2.4 Shigatoxigenic Group of *E. coli* (STEC)

Shiga toxin (*Stx*)-producing *Escherichia coli* (STEC) are food-borne pathogens which are a part of gastrointestinal microbiota with two immunologically distinct *Stxs* found in *E. coli* that are, *Stx1* and *Stx2* (Ferens *et al.*, 2006). The pathogenic of *E. coli* can be categorised into those strains that causing inner intestinal tract disease and the infection at extra-intestinal sites (Kaper *et al.*, 2004). Currently, it has been identified that STEC strains belonging to a diverse range of serotypes are responsible in causing serious human disease. Among the many serotypes, O157:H7 strains are found to be the type most commonly associated with large outbreaks (Hockin & Lior, 1987; Smith & Scotland, 1988; Melton-Celsa *et al.*, 2012). The verotoxigenic *E. coli* strains can cause hemorrhagic colitis (HC) and life threatening sequelae, haemolytic uremic syndrome (HUS) (Karmali *et al.*, 1983; Riley *et al.*, 1983; Cray *et al.*, 1996; Elder *et al.*, 2000).

The pathogenesis of *E. coli* (STEC) consists of a series of multistep process which involve a complex interaction between a range of bacterial and host factors. Firstly, the survived STEC organisms in the harsh environment of the stomach will colonize the intestinal by competing with other gut microorganisms. Then the *Stx* produced will be absorbed by the intestinal epithelium and translocated into the bloodstream, allowing the deliverance of the toxics to the specific toxin receptors on target cell surfaces which thereafter induce local and systemic effects (James & Adrienne, 1998).

One of the virulence factors of STEC strains is mainly due to their adherence ability to the intestinal epithelial cells and the colonization of the human gut. The infectious dose for STEC strains, O111:H⁻ and O157:H7 are estimated to be 1 to 100 Colony-Forming Unit (CFU)