PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF ENTEROBACTERIACEAE FROM RAYU RIVER, SARAWAK

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Phenotypic and Genotypic Characteristics of Enterobacteriaceae from Rayu River, Sarawak

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This project is submitted in partial fulfillment of the requirements for the degree of
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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 duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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List of Abbreviations

x	Times
%	Percentage
℃	Degree Celcius
µl	Microlitre
µg	Microgram
API	Analytical Profile Index
cm	Centimetre
DNA	Deoxyribonucleic acid
EMB	Eosine Methylene Blue
E. coli	*Escherichia coli*
HUS	Hemolytic Uremic Syndrome
H_2S	Hydrogen sulphite
IMViC	Indole/MR/VP/citrate
kb	kilo base pairs
LAMP	Loopamp DNA Amplification Kit
LB	Luria-Bertani
MAR	Multiple Antibiotic Resistance
MHA	Mueller-Hilton Agar
MR-VP	Methyl red and Voges-Proskauer
min	Minutes
ml	Millilitre
mm	Millimetre
NaCl	Sodium Chloride

NT	Nucleotide
PCR	Polymerase Chain Reaction
PVC	Poly Vinyl Chloride
rpm	Rotations per minute
RAPD	Random Amplified Polymorphism DNA
S.I.M	Sulphite-indole-motility
TBE	Tris/Borate/EDTA
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultra-violet
V	Volt

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ABSTRACT

Enterobacteriaceae are normal inhabitants of gastrointestinal tract of warm blooded animals and human which some of them are associated with pathologic processes in gastrointestinal tract. Waterborne and foodborne diseases has been increasing over years which raise the global concern whereby river serves as a potential transmission pathway for enterobacteriaceae to threaten human health. In this study, the enterobacteriaceae were successfully isolated by using EMB agar from samples collected from Rayu River. Gram-lysis test was carried out to differentiate gram-positive and gram-negative bacteria. The isolates were further identified by using API 20E kit and it was found that most of the isolates were *Serratia* (29.11%) and the least was *Escherichia* (1.27%). The antimicrobial susceptibility test shows that all of the isolates were susceptible to sulphamethoxazole trimethoprim (100%) while high resistant to nitrofurantoin and carbenicillin. High MAR index value obtained is ranged from 0.50 to 0.75. These values indicate that these isolates were originated from high risk sources where antibiotics are commonly used. LAMP result showed that none of the *E. coli* isolates from Rayu River consists of the virulence gene to produce Shiga toxin. Meanwhile, $(GTG)_5$-PCR fingerprinting analysis revealed that the genomes of enterobacteriaceae isolates were highly diverse and well distributed as indicated by 6 major and minor clusters in the dendrogram.

Keywords: Enterobacteriaceae, API 20E, MAR index, LAMP, $(GTG)_5$-PCR

ABSTRAK

Enterobacteriaceae ialah penghuni biasa dalam saluran pencernaan binatang berdarah panas atau manusia sementara sesetengah daripada mereka adalah berkaitan dengan proses patologi dalam saluran pencernaan. Peningkatan penyakit bawaan air dan makanan telah menimbulkan rasa prihatian masyarakat global di mana sungai berfungsi sebagai laluan penghantaran enterobacteriaceae yang berpotensi mengancamkan kesihatan manusia. Dalam kajian ini, enterobacteriaceae telah berjaya diasingkan daripada sampel dari Sungai Rayu dengan menggunakan agar EMB. Ujian gram-lisis telah dijalankan untuk membezakan bakteria gram-positif dan gram-negatif. Seterusnya, sampel telah diuji dengan menggunakan API 20E kit dan didapati bahawa kebanyakan sampel ialah Serratia (29,11%) dan paling kurang ialah Escherichia (1.27%). Ujian kerentanan antimikrob menunjukkan bahawa semua sampel bakteria kurang tahan rintangan kepada sulphamethoxazole trimethoprim (100%) manakala kebanyakan tahan rintangan kepada nitrofurantoin dan carbenicillin. Nilai MAR indeks yang tinggi diperolehi adalah di antara 0.50-0.75. Nilai-nilai ini menunjukkan bahawa sampel-sampel tersebut berasal dari sumber yang berisiko tinggi di mana antibiotik biasa digunakan. Hasilan daripada ujian LAMP menunjukkan bahawa tiada sampel bakteria E. coli dari Sungai Rayu mengandungi gen kebisaan untuk menghasilkan toksin Shiga. $(GTG)_5$-PCR analisis menunjukkan bahawa genom daripada sampel enterobacteriaceae adalah sangat kepelbagain dan luas diedarkan seperti yang ditunjukkan oleh 6 kelompok besar dan kecil dalam dendrogram yang dihasilkan.

***Kata kunci**: Enterobacteriaceae, API 20E, MAR indek, LAMP, $(GTG)_5$-PCR*

1.0 Introduction

Enterobacteriaceae are enteric bacteria which can be found in soil, water, plants and animals. They are Gram-negative, straight rods and facultatively anaerobic bacteria with some of them are motile. Nevertheless, some of the enterobacteriaceae are associated with pathologic processes in gastrointestinal tract of human and other animals (Janda & Abbott, 2006). Besides, enterobacteriaceae also causing extraintestinal disease including blood-borne infections, respiratory and urinary tract infections, infectious processes of wounds or surgical sites and disease that involve the ear, eye, nose and throat. Pathogenic enterobacteriaceae such as *E. coli* with serotype O157:H7 which carry Shiga toxins is able to cause disease such as bloody diarrhea. Moreover, *E. coli* which is a fecal coliform indication on water caused food borne and waterborne diseases have been associated with outbreak world widely. Animal's gastrointestinal tract is the primary reservoir for enterobactriaceae which also have been known as the source of enterobacteriaceae.

Rayu River stream has the potential to be contaminated with feces of animals which are infected with enterobacteriaceae. In addition, Rayu River is an essential water source for villagers and village poultry and livestock. Moreover, human activities such as bathing, washing and recreation activities are carried out at the river. These serve as transmission vehicle for the entrobacteriaceae to infect and threaten human and now become a public concern. Various treatments had been applied to cure health problems on human or animal which caused by enterobacteriaceae. One of the treatments is by giving out antibiotics to patients. However, after a prolonged usage of antibiotics, it is possible that a novel bacterial strain which resist antibiotic will develop. When the antibiotic resistance bacteria discharge from the animal, it will transferred its antibiotic resistance gene to other bacteria vertically and horizontally. Hence, the emergence of food-borne

pathogens which are resistant towards the antibiotic will become pandemic among community. As a result, one aim of this study will focus on whether the enterobacteriaceae isolated from Rayu River are resistant to antibiotics. Besides, there is interest in knowing the exact species diversities of enterobacteriaceae and the presence of pathogenic *E. coli* in Rayu River. Furthermore, there is also interest about whether the enterobacteriaceae isolated from Rayu River exist in large genetic diversity and genetic related among the same species and between each species.

The objectives of this study are to:

- Isolate enterobacteriaceae from Rayu River, Sarawak.
- Identify the species of the enterobacteriaceae.
- Determine the antibiotic resistance among the enterobacteriaceae isolates.
- Detect the presence of shiga-toxin gene among *E. coli* species.
- Analyze the genetic diversity of the enterobacteriaceae among different samples from different sampling locations.

2.0 Literature Review

2.1 Rayu River (Sungai Rayu), Sarawak

Rayu River also called Sungai Rayu is a stream in the region of Sarawak with an average elevation of 1 meter above sea level. The area is mildly densely populated with 308 people per km^2. The Rayu River's water is clear and is known as rich in fish fauna (Doi *et al*., 2001). Rayu River trail run through 2 famous recreation parks in Sarawak which are Kubah National Park and Matang Wildlife Centre. Kubah National Park is a tropical rainforest national park in the Kuching Division of Sarawak which has an edge for its rich and rarely seen wildlife. It covers an area of 2230 hectares and is dominated by a sandstone plateau that juts out to a height of 450 meters. Among the peaks here are Gunung Serapi, Gunung Selang and Gunung Sendok. Matang Wildlife Centre is a wildlife centre at the western corner of Kubah National Park. It covers an area of 179 hectares of lowland forest. The forest promotes breeding and rehabilitation of uncommon animals such as the Red-Haired Apes (Orang utan) originating from Borneo and Sumatra and is a popular picnic spot for locals, especially during weekends. There is also a long house (Rumah Juqad) situated along Rayu River bank whereby human activities, recreational activities and daily activities such as road construction, logging activities, bathing and washing are carried out at or near the Rayu River stream. In addition, water source for poultry and livestock come from Rayu River stream (Sungai Rayu, n.d.).

2.2 Enterobacteriaceae

Enterobacteriaceae are Gram-negative, straight rods bacteria with some of which are motile. Most species grow well at 37 ℃, although some species grow better at 25-30 ℃. They are facultatively anaerobic, oxidase-ngative and catalase-positive (except for *Shigella dysenteriae* type 1). They are distributed worldwide and normally can be found in soil,

water, plants and animals. Enterobacteriaceae can be further categorized into 8 genera which are *Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Enterobacter, Serratia* and *Yersinia* (Health Protection Agency, 2010).

2.2.1Common genera of the family Enterobacteriaceae

2.2.1.1 *Escherichia* species

There are six species and the most common is *Escherichia coli* which contains numerous serotypes. A number of strains of *E. coli* contain virulence factors which are associated with specific diseases. One of the known *E. coli* strains that are pathogenic is the serotype O157:H7 which carry Shiga toxins encoded by *stx*1 and *stx*2 genes (Ferens *et al*., 2006). This toxin lead to gastrointestinal disease such as bloody diarrhea and it will lead to hemolytic uremic syndrome (HUS).

2.2.1.2 *Salmonella* species

Serotypes of *Salmonella* and *Arizona* are now considered to belong to two species which are *Salmonella bongori*, (formerly subspecies V) and *Salmonella enterica* which comprises six subspecies: I =*enterica*, II = *salamae*, IIIa = *arizonae*, IIIb = *diarizonae*, IV = *houtenae*, and VI = *indica*. Most serotypes are motile except *Salmonella typhi* which produce gas from glucose. Most *Salmonella* species produce hydrogen sulphide. However, *Salmonella paratyphi A* is normally hydrogen sulphide negative and *S. typhi* is a weak producer.

2.2.1.3 *Shigella* species

There are four *Shigella* species which are *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei*. All *Shigella* species are non-motile. *Shigella* species are highly infective, particularly *S. dysenteriae* (Health Protection Agency, 2010).

2.2.1.4 *Klebsiella* species

The genus *Klebsiella* contains five species and four subspecies. Four species, previously named *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis* and *Klebsiella aerogenes* are now classed as subspecies of *K. pneumoniae*. *K. pneumoniae* subspecies *aerogenes* is the most frequently isolated species. All grow readily on ordinary media, are non-motile and are capsulated. *Klebsiella* species inhabit in soil, water, sewage and plant. They can severely cause pneumonia among human (Podshun & Ullmann, 1998). Some strains of *Klebsiella* spp. have evolved to be resistant towards certain antibiotics due to the widespread use of antibiotic within the environments.

2.2.1.5 *Proteus* species

There are four species of *Proteus* whereby three can cause diseases. All strains are urease positive and motile. They may swarm on blood agar, producing concentric zones or an even film. They are resistant to polymyxin B and colistin. *Proteus* species can resemble non-motile salmonella biochemically, and can agglutinate in polyvalent *Salmonella* antisera.

2.2.1.6 *Enterobacter* species

There are eleven species, but only eight have been isolated from clinical material. They grow readily on ordinary agar, ferment glucose with the production of acid and gas, and are motile by peritrichous flagella. Some strains with a K antigen possess a capsule.

2.2.1.7 *Serratia* species

The genus *Serratia* contains ten species but only two are commonly isolated from clinical materials which are *Serratia liquefaciens* and *Serratia marcescens*. Most of the species are

motile. Members of this genus characteristically produce three enzymes which are lipase, DNase and gelatinase. They are also resistant to polymyxin B and colistin and this resistance may be heterogeneous, leading to a target-zone appearance.

2.2.1.8 *Yersinia* species

The genus *Yersinia* contains eleven species and grows readily on ordinary media. Three of the *Yersinia* spp. which are *Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are known pathogens of man and animals.

2.3 Gastrointestinal disease and waterborne disease

According to Janda & Abbott (2006), enterobacteriaceae are associated with gastrointestinal tract of human and other animals and infection of gut as a result of pathologic processes. Besides, enterobacteriaceae also play an important role in extraintestinal disease including blood-borne infections, respiratory and urinary tract infections, infectious processes of wounds or surgical sites and disease that involve the ear, eye, nose and throat. Pathogenic enterobacteriaceae such as *E. coli* able to cause disease such as bloody diarrhea (HUS) and can be transmitted to human by various transmission modes. Enterobacteriaceae such as *E. coli*, fecal coliform indication on water play a role in waterborne disease that outbreak world widely. As reported by Hazen (1988), there are over 2 billion people or half of the world's population has suffered from diseases due to drinking polluted waters. There are also more than 250 million new cases of waterborne disease reposted each year globally which results in more than 10 million deaths. Nearly 75% of these waterborne disease cases occur in tropical and subtropical areas. The high mortality rates indicate the contamination of nature water in tropical areas has raised the

public concern on the water quality of drinking water and the transmission of waterborne disease.

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

Shigatoxigenic group of *E. coli* (STEC) also known as enterohemorrhagic *E. coli* (EHEC). One of the known pathogenic STEC is the strains with the serotype O157:H7 which carry Shiga toxins encoded by *stx*1 and *stx*2 genes (Ferens *et al*., 2006). In addition, STEC is a vero toxin-producing *E. coli* (VTEC) (Al-Darahi *et al*., 2008). The site of action of Shiga toxin is usually at the lining of the blood vessels of digestive tract, as a result cause hemorrhagic colitis (HC). The HC causes gastrointestinal disease such as bloody diarrhea and it will further lead to hemolytic uremic syndrome (HUS) result in kidney failure. Another enteric pathogen that also produces Shiga toxins is *Shigella dysenteriae*.

2.5 Analytical Profile Index (API) 20E identification kit

The API 20E identification kit is used for identification of enteric bacteria (bioMérieux, Inc., USA) which provides an easy way to inoculate and read tests relevant to members of the Family Enterobacteriaceae and associated organisms. API 20E kit enables the determination of 20 different biochemical characteristics done on one strip. With the API 20E kit, most enteric bacilli can be identified to the species level within 18 to 24 hours after primary isolation; a few isolates require additional tests which can be completed after 1 or 2 additional days (Barry & Badal, 1979). The reliability of this system is very high, and mostly use in many food and clinical laboratories.

2.6 Antibiotic resistance

Antibiotic resistance is the ability of bacteria to resist certain drugs which will indirectly increase the pathogenicity of the bacteria (Pietro, 2002). Due to the transferable of resistance gene that occurs increasingly among the bacteria within the environment, the pathogenicity of the bacteria species also increase gradually. Besides, antibiotic resistance gene can be transferred to wide varieties of bacteria species either from the gram negative donor to the gram positive acceptor or the same group among the same species. There are 4 fundamental methods for antimicrobial susceptibility test for bacteria which are the disc diffusion, agar dilution, broth dilution and gradient diffusion method. Disc diffusion method as proposed by Kirby-Bauer and Stokes (1977) is the most commonly used method and it is used commonly for testing pathogenic enterobacteriaceae. Bacterial strains are cultured in Mueller-Hinton agar to check for their susceptibility against antibiotic discs.

2.7 Loopamp DNA Amplification Kit (LAMP)

Loopamp DNA Amplification Kit (LAMP) also known as Loop Mediated Isothermal Amplification is a novel gene amplification method using a single enzyme under isothermal reaction conditions. There are 4 kinds of primers with high specificity and high amplification efficiency which recognize 6 distinct regions on the target are used. With its high efficiency, only a shorter time requires to produce tremendous amount of amplified products which makes simple detection possible. In this study, the Loopamp DNA Amplification Kit is used to target Shiga toxin genes *stx*1 and *stx*2 gene on *E. coli* (Hara-Kudo *et al*., 2007). Amplification of the target DNA can be conducted by simply incubating specimen and all reagents provided at a constant temperature (60-65℃) for a fixed period of time (1 hour for standard). By adding Fluorescent Detection Reagent to the amplification reagent of Loopamp DNA Amplification Kit before use, the amplification

reaction result can be visually detected by using ultraviolet (UV) lamp. Since PCR machine and electrophoresis is not required, the entire process is completed within one reaction tube, which greatly reduces the possibility of cross-contamination frequently associated with nucleic acid amplification technologies.

2.8 Molecular Typing Methods on Enterobacteriaceae

2.8.1 $(GTG)_5$-PCR

Repetitive-PCR methods including $(GTG)_5$-PCR fingerprinting is the easy and rapid perform molecular typing tools that have been applied in taxonomical studies dealing with various bacterial groups such as enterobacteriaceae. Besides that, it is a powerful typing method to divulge genomic diversity of bacteria. The primer used in this PCR is the repetitive GTG nucleotide primer. Besides that, cluster analysis of the $(GTG)_5$-PCR is considered as a powerful genotypic tool for tracking the sources of *E. coli* contamination of drinking water at the source and is found to be the most robust molecular tool for differentiation of *E. coli* populations in aquatic environments (Mohapatra & Mazumder, 2008). This technique is selected to determine the genetic differences or genetic diversity and clonal genetic relatedness among enterobacteriaceae isolated from water and sediment samples from Rayu River.

2.8.2 Other molecular typing methods

There are various PCR-based profiling techniques which have been applied to observe the genetic diversity and epidemiology relationship of enterobacteriaceae. Among them are amplified restriction fragment length polymorphism (RFLP), plasmid profiling, pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), and multiplex-PCR (Sahilah *et al*., 2010; Radu *et al*., 2001).

3.0 Materials and Methods

3.1 Sample collection/ sampling procedures

Samples were collected from various locations in Rayu River, Sarawak. Sediment and water samples were collected at campsite 1 (CS1), campsite 2 (CS2), upper campsite 1 (UCS1), upper campsite 2 (UCS2), middle upper campsite 1 (MUCS1), lower campsite 1 (LCS1) and lower campsite 2 (LCS2). Ten samples of sediment and water were collected from each campsite. At each campsite, 50 ml of water sample was collected using sterile container at approximately 50 cm under water surface. For sediment collection, 5-10 cm of the sediment was collected by using a pre-sterile PVC pipe. Aseptic technique was applied during sampling. All the samples were collected and then kept under cold temperature in a sterile ice box which containing ice. The bottles were labeled base on the different sampling sites and the date of sample collection. All the samples were taken to the Microbiology Laboratory, UNIMAS for enterobacteriaceae isolation and further study was carried out.

3.2 Sample processing

The samples were processed within 1 day period after sample collection to optimize the growth and avoid death of the microorganisms. The samples were immediately processed once been taken into the laboratory. Sample processing was carried out by making serial dilution followed by spread plate methods. About 1 ml of water sample or 1g of sediment sample was diluted with 9ml of saline solution (0.85% NaCl). Serial dilution procedure was carried out using autoclaved test tube at dilution factors of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} for water samples and 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} for sediment samples in a saline solution (0.85% NaCl). After that, 100µl of the serial dilutions from 10^{-2} till 10^{-5} for water samples and 10^{-2} till 10^{-4} for sediment samples were spread plated onto Eosine Methylene Blue

(EMB) agar and incubated overnight at 28 ℃. Prior to bacterial isolation step, an overnight culture was done and this step was to promote the growth of bacteria from the samples. Colonies grown on the EMB agar was counted. Dilution factor that can yield 30-300 colonies was used for the enterobacteriaceae isolation for this study.

3.3 Samples preservation and working stocks preparation

About 5-10 pure colonies were isolated from the selected dilution of EMB plates and were transferred into a prepared glycerol stocks. The glycerol stocks were stored at -20 ℃ for long term preservation or storage. Prior to use, the bacteria were then revived in Luria-Bertani (LB) broth. Approximately 100 µl of each glycerol stocks was transferred into LB broth by using micropipette and incubated 24 hours at 28 ℃ for enrichment. After incubation, bacteria culture was transferred onto a new EMB agar by streaking method. The plate was incubated for 24 hours at 28 ℃. One pure single colony was obtained from each incubated EMB plate and streaked onto Luria-Bertani (LB) slant agar. The LB slant agars with the bacteria culture were stored at 4 ℃. Every time before any test were to be carried out, colonies from slant agar were streaked on a new LB agar plate and incubated overnight at 28 ℃, after that a single colony was selected and inoculated into LB broth culture. Then, broth culture were incubated overnight at 28 ℃ and tests were carried out using samples from the broth.

3.4 Identification of enterobacteriaceae

Phenotypic characteristics of enterobacteriaceae were indentified phenotypically by using gram-lysis test method, conventional biochemical tests and Analytical Profile Index (API) 20E identification kit.

3.4.1 Gram-lysis test/ "String test"

Gram-lysis test or "String" test method was carried out to distinguish between two major groups of bacteria which are the gram positive bacteria and gram negative bacteria. About 10 µl of 0.5M sodium hydroxide solution was placed onto a clean, glass slide. Small amount of bacteria colony was taken from a purity plate by using a sterile loop. The sample was mixed into the NaOH on the slide for up to one minute. At intervals, carefully raise the loop from the mixture to check for the presence of a 'string' between the loop and the mixture. A positive result was characterized by the presence of the DNA string which indicated that the bacteria are Gram-negative. A negative result was characterized by the formation of a cell suspension with no string which indicated that the bacteria are Gram-positive.

3.4.2 Biochemical tests

Conventional biochemical tests were carried out in order to ensure the growth of enterobacteriaceae and characterized the physiological properties of the enterobacteriaceae. An IMViC tests which consisted of Indole test, Methyl red test, Voges-Proskauer test, and the Citrate utilization test were conducted. Incubation of the inoculated agars and broth were needed for at least 24-48 hours for biochemical reactions to occur. A consistent reactions and characteristics shown by the colonies were compared with *E.coli* strain ATCC 25922 as a reference (Johnson *et al*., 2003).