Institute of Biodiversity and Environmental Conservation

Transmission Pathway of *Enterobacteriaceae* from Reared Fish and Surrounding Environment to Water Body in Batang Ai Reservoir, Sarawak

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Transmission Pathway of *Enterobacteriaceae* from Reared Fish and Surrounding Environment to Water Body in Batang Ai Reservoir, Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Batang Ai reservoir, located in Lubok Antu district, Sarawak, is a man-made lake for the purpose of electrical power generation by Batang Ai hydroelectric station. Over time, the area is developed and now the lake is surrounded by various human activities such as farming, eco-tourism, aquaculture and recreational activities, which means increasing human exposure to the lake water. The bacterial family Enterobacteriaceae is known to be ubiquitous in nature and most studies concerning Enterobacteriaceae revolves around its epidemiology, pathogenesis and anti-microbial resistance (AMR). To date, there has been no reported study on the characteristics of Enterobacteriaceae in Batang Ai reservoir. This study aimed to determine the genetic distribution and characteristics associated with AMR of Enterobacteriaceae in Batang Ai reservoir and the possible sources of Enterobacteriaceae in the lake water. Surface water samples were collected from 27 sampling points scattered around Batang Ai reservoir while four fish samples were obtained from an aquaculture farm located near the communal jetty. The presence of human activities near each sampling point were observed and recorded. The samples were transported to the laboratory in ice box containing ice for further analysis. Preliminary selection was done by plating the samples on Violet Red Bile Agar (VRBA) plates, a selective media for Enterobacteriaceae and a total of 141 isolates were randomly selected from the culture plates. These isolates were subjected to DNA extraction by using the Boiled Cell Method with slight modifications. (GTG)5 DNA fingerprinting was carried out by using the DNA extract to determine the phylogeny of the isolates and identified by using 16S rDNA sequencing. Five genera of Enterobacteriaceae were identified which are Enterobacter, Escherichia, Shigella, Klebsiella and Pseudocitrobacter, consisting of 12 identified species of Enterobacteriaceae, namely E. hormaechei, E. hormaechei subsp. xiangfangensis, E. cloacae, E. cloacae subsp. dissolvens,
E. kobei, E. tabaci, S. boydii, S. flexneri, P. faecalis, E. fergusonii, E. coli and K. pneumoniae. Isolates belonging to the genus Enterobacter made up 29% of the total number of isolates obtained from the area, making it the most prominent genus found in Batang Ai lake. A total of 14 antibiotics from seven anti-microbial classes which are penicillins, cephems, monobactams, aminoglycosides, tetracyclines, quinolones and fluoroquinolones, and phenicols were used determine the AMR of the isolates. A total of seven species isolates were found to have MAR index below 0.2, which is considered to be the safe level of AMR in bacteria. With the exception of ampicillin and tetracycline, all antibiotics have 25% or less bacterial species displaying resistance. Most of the bacterial species showed resistance towards ampicillin which is 75%. E. fergusonii, S. flexneri and K. pneumoniae, which were isolated from water samples obtained near the aquaculture farm, communal jetty and also fish samples, have MAR index of 0.5, 0.357 and 0.214 each. It was suspected that the addition of antibiotics to the fish feed used at the aquaculture farms causes the species found in this area to develop AMR. This poses the risk of antibiotic-resistant bacterial infections towards the surrounding community through water-related activities such as traveling via water transportation and also through the consumption of reared fish harbouring aforementioned species. In conclusion, it was proven that Enterobacteriaceae are transmitted to the reservoir through aquaculture farming, littering, traditional farming and plantation sites happening in the area with aquaculture farms being the main contributor for the presence of antibiotic-resistant bacteria in the area, thus it was proposed that a guideline for activities to be carried out in the area is created and imposed.

**Keywords:** Batang Ai, Enterobacteriaceae, water-borne diseases, (GTG)$_5$ DNA fingerprinting, antibiotic resistance
Laluan Pemindahan Enterobacteriaceae dari Ikan Ternakan dan Kawasan Persekitaran ke Air di Tasik Batang Ai, Sarawak

ABSTRAK

Tasik Batang Ai yang terletak di daerah Lubok Antu, Sarawak, merupakan tasik buatan yang mengoperasikan stesen janakuasa hidroelektrik Batang Ai. Seiring masa, kawasan sekitarnya dibangunkan dan kini terdapat pelbagai aktiviti dijalankan di kawasan tersebut seperti perladangan, eko-pelancongan, penternakan akuakultur dan aktiviti rekreasi. Ini bermaksud risiko untuk manusia terdedah dengan air tasik berkenaan meningkat. Bakteria dari keluarga Enterobacteriaceae wujud secara semulajadi dalam alam sekitar dan kebanyakan kajian yang berkaitan membincangkan epidemiologi, patogenesis dan daya tahan antibiotic (DTA). Sekatakan ini, tiada laporan telah dibuat berkenaan ciri Enterobacteriaceae di kawasan Batang Ai. Kajian ini bertujuan menentukan taburan genetik dan ciri yang berkaitan dengan DTA Enterobacteriaceae di tasik Batang Ai dan kemungkinan penyumbang Enterobacteriaceae di tasik berkenaan. Sampel air diambil daripada 27 titik pensampelan di sekitar kawasan tasik Batang Ai manakala sampel ikan diperoleh daripada salah satu pusat akuakultur yang beroperasi di tasik berkenaan. Sebarang aktiviti manusia yang berdekatan dengan setiap titik pensampelan direkodkan. Sampel tersebut disimpan di dalam kotak berisi ais dan dibawa ke makmal untuk tujuan analisis. Setelah dikultur diatas media Violet Red Bile agar (VRBA), sebanyak 141 isolat dipilih secara rawak dan melalui proses ekstraksi DNA dengan menggunakan kaedah Boiled Cell yang telah diubah suai. Ekstrak DNA tersebut digunakan untuk kaedah pengesanan DNA (GTG); untuk menentukan filogeni isolat-isolat yang diperoleh dan dikenal pasti dengan menggunakan penjukan 16SrDNA. Genera yang dikenalpasti adalah Enterobacter, Escherichia, Shigella, Klebsiella dan Pseudocitrobacter, yang terdiri
daripada 12 spesies iaitu E. hormaechei, E. hormaechei subsp. xiangfangensis, E. cloacae, E. cloacae subsp. dissolvens, E. kobei, E. tabaci, S. boydii, S. flexneri, P. faecalis, E. fergusonii, E. coli dan K. pneumoniae. Sejumlah 29% daripada keseluruhan isolat merupakan ahli genus Enterobacter, sekaligus menjadikannya genus paling prominen di tasik Batang Ai. Sebanyak 14 antibiotik dari tujuh kelas antimikrobial iaitu kelas penicillin, cephem, monobactam, aminoglycoside, tetracycline, quinolone dan fluoroquinolone, dan phenicol telah digunakan untuk menentukan DTA isolat-isolat berkenaan. Sebanyak tujuh daripada 12 isolat didapati menunjukkan indeks MAR kurang daripada 0.2 yang dianggap paras selamat bagi DTA bakteria, manakala kesemua antibiotik kecuali ampicillin dan tetracycline menunjukkan maksimum jumlah spesies yang mampu melawan antibiotik berkenaan adalah 25%. Kebanyakan spesies didapati memiliki DTA terhadap ampicillin iaitu sebanyak 75%. E. fergusonii, S. flexneri dan K. pneumoniae yang diperoleh dari sampel air berdekan kawasan akuakultur, jeti dan juga sampel ikan masing-masing menunjukkan indeks MAR 0.5, 0.357 dan 0.214, dijangka kerana penggunaan antibiotik dalam makanan ikan yang digunakan di pusat akuakultur berhampiran. Ini bermakna penduduk tempatan berisiko dijangkiti bakteria tahan antibiotik (ARB) melalui aktiviti air seperti berenang dan juga memakan ikan yang dihuni bakteria berkenaan. Hasil kajian ini menunjukkan bahawa Enterobacteriaceae berpindah dari kawasan sekitar ke dalam air melalui penternakan akuakultur, pembuangan sampah, pertanian secara tradisional dan ladang-ladang di kawasan berkenaan manakala pusat akuakultur merupakan penyumbang utama kepada kewujudan ARB di tasik berkenaan, dan dicadangkan bahawa sebuah garis panduan berkenaan pelaksanaan aktiviti di kawasan tersebut diwujudkan dan dilaksanakan.

Kata kunci: Batang Ai, Enterobacteriaceae, penyakit bawaan air, pengesanan DNA (GTG)5, daya tahan antibiotik
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### LIST OF ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| ARB          | Antibiotic-resistant bacteria |
| AST          | Anti-microbial susceptibility test |
| BLAST        | Basic Local Alignment Search Tool |
| CAUTI        | Catheter-associated urinary tract infection |
| CLABSI       | Central line-associated bloodstream infection |
| CLSI         | Clinical and Laboratory Standards Institute |
| CRE          | Carbenem-resistant *Enterobacteriaceae* |
| DNA          | Deoxyribonucleic acid |
| EMBA         | Eosin methylene blue agar |
| EUCAST       | European Committee on Anti-microbial Susceptibility Testing |
| ESBL         | Extended-spectrum β-lactamase |
| ESCs         | Extended-spectrum cephalosporins |
| EtBr         | Ethidium bromide |
| GPS          | Global Positioning System |
| HGT          | Horizontal gene transfer |
| ICU          | Intensive care unit |
| MAR          | Multiple Antibiotic Resistance |
| MHA          | Mueller-Hinton agar |
| MHB          | Mueller-Hinton broth |
| MIC          | Minimum inhibitory concentration |
| NB           | Nutrient broth |
| PCR          | Polymerase chain reaction |
| Abbreviation | Full Form |
|--------------|-----------|
| PBS          | Phosphate buffer saline |
| PGPR         | Plant growth promoting rhizobacteria |
| SALCRA       | Sarawak Land Consolidation and Rehabilitation Authority |
| SESCO        | Sarawak Electrical Supply Corporation Berhad |
| SSI          | Surgical site infection |
| UTI          | Urinary tract infection |
| UV           | Ultra-violet |
| VAP          | Ventilator associated pneumonia |
| VRBA         | Violet red bile agar |
| WHO          | World Health Organization |
CHAPTER 1

INTRODUCTION

1.1 Study Background

Water plays a huge part in the daily lives of worldwide population for consumption, recreation, cleaning purpose and even for research purpose. Due to this reason, water quality had always been a concern for users. Fortunately, most of the water-borne bacteria can be killed by heat treatment and removed by filtration process. Enterobacteriaceae is a family of bacteria that is commonly found in the guts of human and animals and also can be found abundantly in nature. Due to its natural habitat, it is bound to be found in the water bodies such as streams and rivers as well (Paradis et al., 2005). Some of the bacteria that usually cause gastro-intestinal infection are Escherichia, Salmonella and Shigella. Among these bacteria, E. coli is the most commonly known to infect human (Diab & Al-Turk, 2011).

Batang Ai reservoir located in Lubok Antu, Sri Aman, Sarawak, is a man-made lake which is surrounded by various human activities such as farming, eco-tourism and aquaculture. A wide range of activities taking place in this area attracts quite a huge number of visitors and workers to the area, in addition to the existing local community residing in the area. These people are constantly exposed to the water of Batang Ai reservoir either through water-related recreational activities such as fishing, work-related matter for the aquaculture farm operators and plantation workers, or water transportation for the tourists and locals who rely on boat as their transportation. This is when the bacteria would come into contact with human and could be ingested as well, either intentionally or accidentally (usually occur during rigorous water activities such as white-water rafting, kayaking and swimming).
1.2 **Problem statement**

Local community and visitors of Batang Ai reservoir are constantly exposed to the lake water in various ways. This poses them with the risk of contracting possible infections of water-borne diseases while doing activities such as fishing or even simply travelling home by means of water transportation due to accidental ingestion of the lake water. Thus, it is important to find out if there are harmful bacteria in the water and identify the possible transmission pathway of the bacteria to the water bodies to find out the source and take precaution steps to prevent bacterial infection.

1.3 **Objectives**

The study aims to determine the genetic distribution and characteristics associated with antibiotic resistance of *Enterobacteriaceae* in Batang Ai reservoir. This can be achieved through the following set of objectives:

i. To detect the presence of *Enterobacteriaceae* in the water samples taken from the water bodies and reared fish from the aquaculture centres around Batang Ai area.

ii. To identify the isolated *Enterobacteriaceae* down to species level and determine the antibiotic resistance of each species.

iii. To identify human activities that are possible sources of contamination around Batang Ai area.
2.1 Water-borne disease

Approximately 70% of the earth’s surface is covered with water, while the rest is dry land in which only 2% of the freshwater of the water bodies available are drinkable (Humayun et al., 2015). The spread of waterborne disease is quite prominent in some parts of South-East Asia. This is due to the large dependence of the communities in rural area to water bodies as their main water source to be used in daily consumption as a large part of these areas are still cut off from treated water supply. These water bodies are usually contaminated with bacteria from surrounding sources such as public latrine and poultry farms set up nearby. According to World Health Organization (WHO) in their report regarding the drinking water quality in South-East Asia in 2010, the most common form of health risk associated with the quality of drinking water is microbiological contamination. This contamination is mainly and most commonly caused by human and animal wastes, which can be by defecation near water sources, improper disposal of sewage, leakage in septic tanks, or cross contamination from sewer lines (Zaidi et al., 2004). Improper storage of drinking water at home could also be a cause for microbiological contamination.

In a study conducted by Zaidi et al. in 2004 regarding the burden of infectious disease in South Asia, people in South Asia are more prone to infectious disease and they stated that lack of access to clean water, toilet facilities and proper health care as part of the main reason while diarrhoea was mentioned as one of the top fatal disease amongst children in South Asia, a fact supported by Davies et al. (2015). Within the last decade, it was reported that
publications regarding water-related health issues were increasing in terms of number and citations, meanwhile the top 10 retrieved publications were mainly in the field of environmental health or epidemiology. This shows that there is an increase of interest towards the field of health related to water-borne diseases globally (Sweileh et al., 2016). Examples of common water- and sanitation-related diseases are ascariasis (infection caused by roundworm), arsenicosis (arsenic poisoning), cholera (caused by Vibrio cholerae) and diarrhoea. Pathogenic and also virulent strains of E. coli are capable of causing extraintestinal infections in humans, some of them being urinary tract infections, neonatal meningitis and bacteremia (Ghaderpour et al., 2015). Amongst all the above-mentioned diseases, diarrhoea is one of the most prominent cases worldwide.

2.2 Enterobacteriaceae

2.2.1 Characteristics of Enterobacteriaceae

Enterobacteriaceae is a family of Gram-negative bacteria which are rod-shaped, which can be observed under the microscope. Most of the members of this bacterial family can naturally be found in animal guts ranging from human to insects and also in nature such as water, soil and plants (Paradis et al., 2005). As it is present naturally in animal guts, it is often used as an indicator for faecal contamination in water bodies (Paulse et al., 2012). Taxonomically, this is the most diverse bacterial family and most of the research concerning Enterobacteriaceae in human revolves around epidemiology, pathogenesis and antibiotic resistance (Martinson et al., 2019). According to the UK Standards for Microbiology Investigation issued in April 2015, currently, there are 53 known genera in this family with more than 170 named species. Some of the more commonly known genera that are classified under the family Enterobacteriaceae are Cronobacter, Enterobacter, Escherichia,
Klebsiella, Salmonella, Shigella and Yersinia. Out of these 53 genera, 26 of them are associated with bacterial infections in human.

Members of this family are important causes of community- and hospital-acquired infections, of which the more serious cases are usually treated by using extended-spectrum cephalosporins (ESCs) (Lupo et al., 2013). Due to this, quite a number of bacteria that belongs to this family has been commonly isolated from clinical cultures, which includes Klebsiella spp. and Enterobacter spp. (Van Duin & Doi, 2017). The most important model organism for Enterobacteriaceae is Escherichia coli. This organism is found to be able to survive in subsurface water for more than 40 days and can further move to underground water (Diab & Al-Turk, 2011), which causes it to spread easily via water. The presence of multiple flagella distributed around this organism also contributes to its motility (Lihan et al., 2017). Figure 2.1 shows the general structure and physical characteristics of Enterobacteriaceae.

![General structure of Enterobacteriaceae](image)

**Figure 2.1:** General structure of Enterobacteriaceae

(Source: Medical Microbiology: an introduction to infectious disease)
Some of the members of Enterobacteriaceae family are resistant to certain types of antibiotics such as β-lactam. This type of bacteria is known to produce extended-spectrum β-lactamase (ESBL) or also called as ESBL-producing bacteria. This is quite a concern as the β-lactam is used widely to cure infections caused by bacteria and about half of commercially-available antibiotics worldwide belonged to the category β-lactam. ESBL-producing Enterobacteriaceae are also prevalent worldwide, which adds to the concern (Abdallah et al., 2015). Some strains are also resistant to carbapenem, or also known as carbapenem-resistant Enterobacteriaceae (CRE). The presence of these ESBL producers in the microflora of human intestine is quite a concern as this could cause drug-resistant genes to be horizontally transferred from commensal flora to enteric pathogens (De Boeck et al., 2012).

2.2.2 Manifestation of disease

Bacteria from the family Enterobacteriaceae are known to be opportunistic pathogens from clinical point of view. Enterobacter sakazakii, for instance, is widely associated with bacterial infections in infants. These infections include meningitis, urinary tract infection (UTI), and bacteremia (Farmer et al., 1980). Most commonly, E. sakazakii infected the infants through consumption of contaminated formula powder (Bowen & Braden, 2006). Infants which are premature, immunocompromised, and/or with low birth weight are at a particular risk of being infected. Clinical infections in neonatal intensive care unit (ICU) are usually associated with contaminated milk formula (Iversen & Forsythe, 2003; Drudy et al., 2006). Some of the more well-known diseases or infections caused by Enterobacteriaceae are shigellosis, nosocomial infections in healthcare settings and salmonellosis.
Shigellosis, which is a potentially deadly diarrheal disease, is caused by the genera *Shigella*. Depending on the severity of the infection, the symptoms of this disease range from mild intestinal discomfort to death. It rarely occurs amongst infants, and most cases of shigellosis infection amongst infants are reported in developing countries with poor level of sanitary practice which results in the infants exposed to infection while being bottle-fed (Sawardekar, 2004; Kania et al., 2016). It is listed as one of the main bacterial disease which is transmitted through drinking water (Cabral, 2010). In a study conducted in 2006 in six Asian countries, it was reported that children below five years old are more susceptible towards shigellosis compared to older children and adults, with the burden of shigellosis being 13.2 per 1000 population in children below five years old compared to 2.1 per 1000 population in all ages per year (von Seidlein et al., 2006).

Nosocomial infections are infections that occurs in medical settings within 48 hours of hospital admission, three days after discharge, or 30 days after an operation. It happens as often as one infection in 10 patients and is responsible for approximately 5000 deaths every year (Inweregbeu et al., 2005). The most common types of nosocomial infections are catheter-associated urinary tract infections (CAUTI), central line-associated bloodstream infections (CLABSI), surgical site infections (SSI) and ventilator associated pneumonia (VAP). UTI is a very common bacterial infection which affects approximately 150 million people worldwide every year (Stamm & Norrby, 2001). It is caused by a huge range of pathogens, but the most common are *E. coli, Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* (Flores-Mireles et al., 2015). According to WHO, about 15% of hospitalized patients are infected by nosocomial infections (Khan et al., 2017). These infections are often caused by genus *Enterobacter*, mainly *Enterobacter aerogenes* and *Enterobacter cloacae*. Out of all *Enterobacter* sp. isolates obtained from
clinical samples, *E. cloacae* is the most commonly isolated species. It is a very well-known nosocomial pathogen which causes bacteremia, endocarditis, septic arthritis, osteomyelitis, skin/soft tissue infection and urinary-tract infections (Fata et al., 1996). Meanwhile in a study conducted in a teaching hospital in China in 2013 and 2015, three most commonly isolated nosocomial pathogens are *Staphylococcus aureus*, *K. pneumoniae* and *Pseudomonas aeruginosa*. This finding is found to be similar with reports from other countries (Wang et al., 2019).

Salmonellosis, a form of food poisoning, is another very common bacterial infection caused by *Enterobacteriaceae*, specifically by the genus *Salmonella*. The onset symptoms of salmonellosis are acute fever, abdominal pain, nausea, diarrhoea and even vomiting. These symptoms can present in the infected individual within six to 72 hours after the ingestion of *Salmonella*. According to WHO, as of 2018, *Salmonella* is one of the four main global causes for diarrheal diseases. It usually spreads via consumption of contaminated food. However cross-contamination may also occur when a cooked food is handled using contaminated utensils. It can also be transmitted from person to person and from animal to person (Queensland Government, n. d.). Salmonellosis can cause a huge lose in the livestock and poultry industry through decreased production and also the death of young animals (Joseph et al., 1988).

As of date, no record of major outbreak of disease caused by *Enterobacteriaceae* happening in Batang Ai area in the past can be found. However, it is still necessary to find possible sources of *Enterobacteriaceae* contamination in the area to possibly contain it as the lake water is mainly used for transportation by the locals and tourists visiting the area, and also major fish rearing in aquaculture farms for food (SALCRA, 2012).
2.3 Batang Ai, Lubok Antu, Sarawak

Batang Ai is located in district Sri Aman, subdistrict Lubok Antu, approximately 275 km from Kuching with a population size of 27,984 as of 2010 and an area of $3,142.55 \text{ km}^2$ (Majlis Daerah Lubok Antu, 2017; Sarawak Government, 2017). It is home to ecotourism attraction spots such as Batang Ai National Park and Aiman Batang Ai Resort (Sarawak Tourism Board, 2017). The potential of Batang Ai area as an up-and-coming hotspot for ecotourism has been acknowledged by multiple parties and various efforts had been taken to develop Batang Ai such as construction of hotel and basic facilities and also various activities to attract more visitors, such as Pesta Batang Ai which is held yearly. It was also gazetted as a national park in 1991 as an effort to conserve and protect the unique population of wildlife in the area which includes orang utan and wild hornbill. To further protect the area, only a small area of Batang Ai national park is open to visitors while other parts are off limits (Sarawak Tourism Board, 2017; Sarawak Forestry, n. d.; Endemic Guides, 2020). Figure 2.2 shows the map of Batang Ai area.

![Map of Batang Ai area](Source: Google map)

**Figure 2.2:** Map of Batang Ai area

(Source: Google map)