FOOD
RESEARCH

Transmission pathway of Enterobacteriaceae from reared fish and surrounding environment to water body in Batang Ai reservoir, Sarawak, Borneo

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Abstract

This study was aimed to determine the genetic distribution and characteristics of the bacterial family in the Batang Ai reservoir and their possible sources of transmission into the lake water. Surface water samples were collected from twenty-seven sampling points scattered around the Batang Ai reservoir and four fish samples were obtained from an aquaculture farm located within the water reservoir. The samples were plated on Violet Red Bile Agar (VRBA) plates for the isolation of Enterobacteriaceae. A total of 141 bacterial colonies were isolated from the culture plates and subjected to $(GTG)_5$ PCR analysis to determine the genetic similarities among the isolates. A dendrogram was plotted based on the $(GTG)_5$ PCR patterns and the representative isolates were selected for identity confirmation using 16S rRNA sequencing. Based on the sequencing, five genera of Enterobacteriaceae were identified as *Enterobacter*, *Escherichia*, *Shigella*, *Klebsiella* and *Pseudocitrobacter*, consisting of 12 identified species, namely *Enterobacter hormaechei, E. hormaechei* subsp. *xiangfangensis, E. cloacae, E. cloacae* subsp. *dissolvens*, *E. kobei, E. tabaci, Shigella boydii, S. flexneri, Pseudocitrobacter faecalis, Escherichia fergusonii, E. coli* and *Klebsiella pneumoniae*. A total of 14 antibiotics from seven anti-microbial classes including penicillins, cephems, monobactams, aminoglycosides, tetracyclines, quinolones and fluoroquinolones, and phenicols were tested against the isolates. A total of seven species within the isolates were found to have multiple antibiotic resistance (MAR) index below 0.2, suggesting those isolates were derived from low risk of antibiotics contamination sources. With the exception of ampicillin and tetracycline, all antibiotics have 25% or less bacterial species displaying resistance. Most (70%) of the bacterial species showed resistance toward ampicillin. The fish isolates demonstrated multiple antibiotic resistance indexes ranging from 0.214 to 0.5. The presence of multiple antibiotic-resistant bacteria in the water reservoir may pose the risk of antibiotic-resistant bacterial infections through water-related activities and consumption of fish harbouring antimicrobial resistance bacterial species. It was proven that *Enterobacteriaceae* were transmitted from the activities and surrounding environments to the water reservoir, thus it is proposed that a guideline for activities to be carried out in the water reservoir is created and imposed.

1. Introduction

A water reservoir is an enlarged natural or artificial lake made by humans to store a huge amount of water. It plays a huge part in the daily lives of people living nearby the water resource for different purposes including recreation, cleaning purposes, irrigation and more commonly hydroelectric generation. The water quality of the reservoir must be maintained at a certain standard and safe from water pollutants especially pollutants associated with infectious microorganisms.

Enterobacteriaceae is a family of Gram-negative bacteria naturally found in the guts of humans and animals and some species can be found naturally in the environments. Since it is present naturally in animal guts, it is often used as an indicator of faecal contamination in water bodies (Paulse *et al.*, 2012). The presence of the Enterobacteriaceae in water also indicates the presence

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of pathogens (Berg, 1978). Taxonomically, this family of bacteria is the most diverse family and most of the research work on Enterobacteriaceae in humans revolves around its epidemiology, pathogenesis and antibiotic resistance (Martinson *et al.*, 2019). In addition, the distribution and characteristics of the bacteria from the water environment and food sources have been studied (Son *et al.*, 1998; Lihan *et al.*, 1999; Sahilah *et al.*, 2003; Ng *et al.*, 2014; Lihan *et al.*, 2017; Lihan *et al.*, 2021).

Some genus within this family members have been associated with significant outbreaks of diseases such as Salmonella, Shigella and Yersinia (Dekker and Frank, 2015), while the species like uropathogenic *E. coli* has been the most commonly known to infect human usually causing complicated and uncomplicated urinary tract infections (UTIs) (Diab and Al-Turk, 2011; Percival and Williams, 2014; Flores-Mireles *et al.*, 2015). These bacteria produce toxins residing in their cell walls and are released upon cell death or the destruction of their cell walls (Percival and Williams, 2014).

In Malaysia, multiple case studies have reported on the prevalence of antibiotic resistance Enterobacteriaceae strains in healthcare settings, with a wide variation of resistant strains being identified (Al-Marzooq *et al.*, 2015; Low *et al.*, 2017; Phoon *et al.*, 2018; Gan *et al.*, 2020). Antibiotic resistance of Enterobacteriaceae towards Carbapenem has been reported in health institutions worldwide (Xu *et al.*, 2015; Duin and Doi, 2016) and it has also been reported in the clinical setting in Malaysia (Zaidah *et al.*, 2017). 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 (Lupo *et al.*, 2013). Due to this, quite a number of bacteria belonging to this family have been commonly isolated from clinical cultures, which include *Klebsiella* spp. and *Enterobacter* spp. (Van Duin and 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 and 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 (Cabral, 2010).

It has been reported that the Enterobacteriaceae become resistant to the β-lactam group of antibiotics which is the most widely used antibiotics, especially in medical settings. This is due to the extended exposure of the bacteria to antibiotics and also the prevalence of this type of antibiotic in environments contaminated with antibiotic-containing wastes (Da Silva *et al.*, 2007;

Iredell *et al.*, 2016).

Batang Ai water reservoir is located at Lubok Antu, which is about 250 km south-east of Kuching, Sarawak in Malaysian Borneo. The reservoir is surrounded by various human activities including fish farming using an open cage system, small scales paddy and pepper farming, and recreational activities (Nyanti *et al.*, 2012; Ling *et al.*, 2018). Visitors and the local community within the area are closely associated and in contact with the water of Batang Ai reservoir either through water-related recreational activities such as fishing, water sports, work-related matter for the aquaculture farm operators and farm workers, or water transportation for the tourists and locals who rely on a boat as their transportation. The lake water is also home to ecotourism attraction spots such as Batang Ai National Park and Aiman Batang Ai Resort (Sarawak Tourism Board, 2017).

Up to this day, some villages located in the upstream area of rivers tributaries which flow to the Batang Ai reservoir can only be reached by boat as there is still no proper road connecting the villages to other areas (Sarawak Tourism Board, 2017; Sarawak Forestry, 2020). This increases the exposure of the locals to the lake water, risking them contracting bacterial infection via accidental ingestion or by physical contact. This study is aimed to determine the genetic distribution and antibiotic resistance of Enterobacteriaceae from the Batang Ai reservoir and human activities that may contribute to the possible sources of contamination around the Batang Ai area.

2. Materials and methods

2.1 Sample collections and sampling sites

Water samples were collected from twenty-seven points within the Batang Ai lake by using 5 mL centrifuge tubes. Samples of fish were purchased from one of the aquaculture farms operating on a floating platform at the lake. Locations of sampling points are indicated by the blue bubble markers as shown in Figure 1 and Figure 2. Land uses and human activities taking place surrounding the sampling points were also observed and recorded. All collected samples were transported to the laboratory in ice box-containing ice. Upon reaching the laboratory, the water and the fish samples were analysed for the presence of Enterobacteriaceae bacteria within 24 hrs.

2.2 Sample processing

A volume of 100 µL of the water samples was plated directly on the VRBA plates and incubated at 29°C for 24 hrs. For the fish samples, prior to plating on VRBA
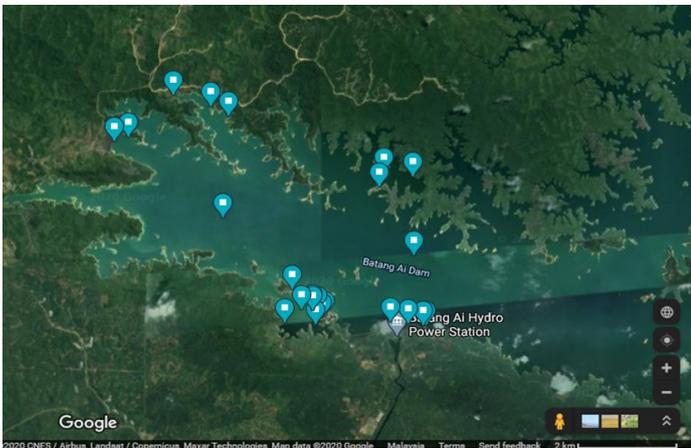
Figure 1. Locations of sampling points within the Batang Ai area (source of map: google map).

Figure 2. Close-up of sampling area around the communal and tourism jetties. Blue markers with white squares mark the sampling points around the area (source of map: google map).

plates, approximately 1 g of fish intestine was weighed and vortexed in 9 mL of phosphate buffer saline (PBS) solution. A volume of 100 µL of the fish intestine mixture was plated on the VRBA plates and incubated at 29°C for 24 hrs. After incubation, the plates were observed for bacterial growth. For each plate, 3 to 5 different bacterial colonies were chosen at random, purified and stocked in slant agar and glycerol (20%). The isolates were Gram-stained and only those isolates observed as rod-shaped and pink-coloured under the microscope were selected.

2.3 DNA fingerprinting and dendrogram construction

Prior to the $(GTG)_5$-PCR analysis, isolates were subcultured into 5 mL of nutrient broth and then incubated at 29°C for 24 hrs. A volume of 2 mL of the cultured broth was used for DNA extraction utilising the boiled cell method (Kathleen *et al*., 2014). The total volume for PCR content was fixed at 25 µL consisting of the following components; 5.0 µL of 5X Green GoTaq® flexi buffer (5 µ/µL), 3.0 µL of 25mM $MgCl_2$, 2.5 µL of 2mM dNTPs, 0.5 µL of 10mM $(GTG)_5$ primer, 8.7 µL of ddH$_2$0, 5.0 µL of DNA extract and 0.3 µL of GoTaq® flexi polymerase (5U/µL). The PCR was run according to the following cycles; one initial denaturation at 95°C for 2 mins, 30 repeated cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and an extension at 72°C for 1 min. A final extension cycle at 72°C for 5 mins was also included. The PCR products were resolved in 1.2% (w/v) agarose gel stained with 1 µL of ethidium bromide (EtBr) and electrophoresed at 400 mA, 90 V for 75 mins. The gel was then visualised under a UV transilluminator (Maestrogen, Taiwan). A dendrogram was constructed by using software GelJ v2.0 with bands obtained from the visualised gel.

2.4 The 16S rRNA PCR

The 16S rRNA PCR was carried out in a total volume of 30 µL consisting of the following components; 15 µl of 5X ExTen mastermix, 1.2 µL of
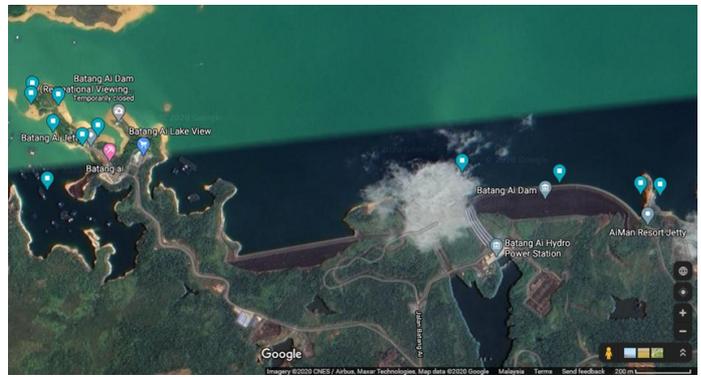
10mM 27F primer, 1.2 µL of 10mM 519R primer, 3.6 ddH$_2$O, 9 µl of DNA extract. The PCR was run according to the following cycles, one initial denaturation at 95°C for 10 mins, 26 repeated cycles of denaturation at 95°C for 0.5 min, annealing at 55°C for 1.5 mins and extension at 72°C for 1.5 mins. A final extension cycle at 72°C for 10 mins was also included. The PCR products were resolved in 0.8% (w/v) agarose gel stained with 1 µL of ethidium bromide (EtBr) and electrophoresed at 400 mA, 90 V for 1 hr. The gel was then visualised under a UV transilluminator (Maestrogen, Taiwan) to check for the presence of a band.

2.5 DNA purification and sequencing analysis

After the product of 16S rRNA-PCR was viewed under the UV transilluminator, the visible DNA band was cut and transferred into a clean 2 mL centrifuge tube by using a sterile blade and forceps. For the DNA purification, briefly, a total of 300 µL of QG buffer was transferred into the tube and placed in a 50°C water bath for 10 mins to melt the agarose gel. After the agarose gel has melted completely, 100 µL of isopropanol was added to the mixture and was set aside for about 1 min. The solution was transferred into the spin column and centrifuged at 10,000 rpm for 1 min. The eluent in the collection tube was discarded. Another 500 µL of QG buffer was added into the spin column before centrifugation at 10,000 rpm for 1 min. This is to ensure that any traces of agarose gel and EtBr were removed completely from the sample. A volume of 750 µL of PE buffer was added into the spin column and set aside. After 3 mins, the mixture was centrifuged at 10,000 rpm for 1 min and the eluent in the collection tube was discarded. This step was repeated twice to ensure that the PE buffer was completely removed from the spin column. The spin-column was then transferred to a 1.5 mL centrifuge tube. A volume of 50 µL of EB buffer was added into the spin column and centrifuged at 10,000 rpm for 1 min. The eluent containing the purified PCR product was then kept at -20°C until further use. A

volume of 5 µL of the product obtained was electrophoresed in 0.8% (w/v) agarose gel at 90 V, 400 mA for 1 hr then visualised under the UV transilluminator. The products that show bands on agarose gel were sent for DNA sequencing by Apical Scientific Sdn. Bhd. (Malaysia).

2.6 Antimicrobial susceptibility test

The antimicrobial susceptibility test (AST) was carried out based on the protocols stated in CLSI (2018) by using the agar disc-diffusion method on Mueller-Hinton (MH) agar. Briefly, suspensions of bacterial cultures were diluted to get 0.5 McFarland standard and then swabbed on the MH agar plates by using sterile cotton swabs. After plating, antimicrobial agent-impregnated discs were placed on the plates by using sterilised forceps.

Each plate was placed with five antimicrobial discs, each containing different antimicrobial agents. After incubation at 29°C for 24 hrs, the plates were observed for halo zones which were then measured and recorded. These measurements were compared with the minimal inhibitory concentration (MIC) table for Enterobacteriaceae found in CLSI (2018). *Escherichia coli* ATCC 25922 was used for antibiotic quality control. Table 1 shows the group of antimicrobial agents used in this study. The anti-microbial agents were chosen to take into consideration the commonly used anti-microbial agents in treating bacterial infections.

2.7 Human activities surrounding Batang Ai

Human activities taking place near each of the sampling points were observed and noted. The correlation between these activities and the presence of Enterobacteriaceae near these activities was observed afterwards. The activities and GPS points for each

activity were recorded, while the photographic evidence for the said activities was taken.

3. Results

3.1 Sample collection and bacterial isolation

Water samples were taken from 27 sampling points scattered around the area of Batang Ai lake while the fish samples were obtained from an aquaculture farm operating at the lake. The coordinates for each sampling point are shown in Table 5. After incubation of the samples on Violet Red Bile Agar (VRBA) plates, three to five isolates were randomly picked from each of the plates. The isolates were seen as purple, indicating that those isolates were lactose fermenters. Figure 3 shows an example of bacterial growth on the VRBA plates.
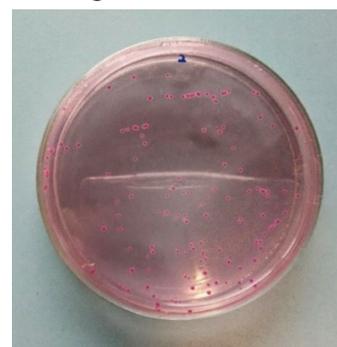

Figure 3. Growth of *Enterobacteriaceae* on VRBA plate showing the colonies of bacterial isolates obtained from sample BA2

3.2 Gram staining

Gram-staining was carried out to select Gram-negative bacterial isolates for further testing, where only Gram-negative isolates were further tested. This was done by selecting only the isolates that appeared rod-shaped and pink in colour when stained and observed under the microscope.

Table 1. Anti-microbial agents used for antimicrobial susceptibility testing

Antibiotic group	Anti-microbial agent	Code	Concentration (µg)
Penicillin	Ampicillin	AMP	10
	Piperacillin	PRL	10
Cephem	Ceftazidime	CAZ	30
	Cefotaxime	CTX	30
Carbapenem	Imipenem	IMP	10
	Meropenem	MEM	10
Aminoglycoside	Gentamycin	CN	10
	Tobramycin	TOB	10
	Amikacin	AK	30
	Streptomycin	S	10
Tetracycline	Tetracycline	TE	30
	Doxycycline	DO	30
Quinolones and fluoroquinolones	Ciprofloxacin	CIP	5
Phenicol	Chloramphenicol	C	30

3.3 (GTG)₅ DNA fingerprinting

Figure 4 shows the band images obtained from agarose gel electrophoresis (AGE) of isolates obtained from the water samples. The lanes are marked with the codes for the corresponding isolates. The size of the DNA bands produced after the PCR amplification ranged from approximately 250 bp to 2500 bp. The DNA bands obtained were used to construct the dendrogram using software GelJ version 2.0. Figure 5 shows the dendrogram constructed using DNA bands obtained from (GTG)₅ PCR. As some isolates failed to produce DNA bands after going through PCR and AGE, those isolates were not included in the dendrogram.

At 50% similarity level, isolates group under the same cluster are considered as belonging to the same family, while at 60% similarity level, isolates under the same cluster are considered as strains belonging to the same species (Baron, 1996; Paradis *et al*., 2005). Based on the dendrogram generated, the lowest percentage of relatedness amongst the isolates is 50%, which indicates

all of the isolates belong to the same family. The isolates were grouped into 12 clusters with each cluster having at least 60% genetic similarity level among the members. Two clusters were found to have isolates obtained from both water and fish samples which are Cluster 11 and Cluster 12. From each cluster, one isolate was selected to represent the cluster for 16S rRNA sequencing. The largest cluster is Cluster 12 with 63 isolates belonging to the group while the smallest clusters are Cluster 1, Cluster 2, Cluster 5, and Cluster 8 with one isolate in each group. The clustering among all of the isolates is shown in Table 2.

3.4 Bacterial identification

The sequence obtained from the product of 16S PCR were compared with highly similar DNA sequences in the GenBank database by using BLAST (Basic Local Alignment Search Tool) program in the NCBI database. The result shows that the 12 bacterial isolate representatives have at least a 98.39% similarity level consisting of different strains belonging to the family
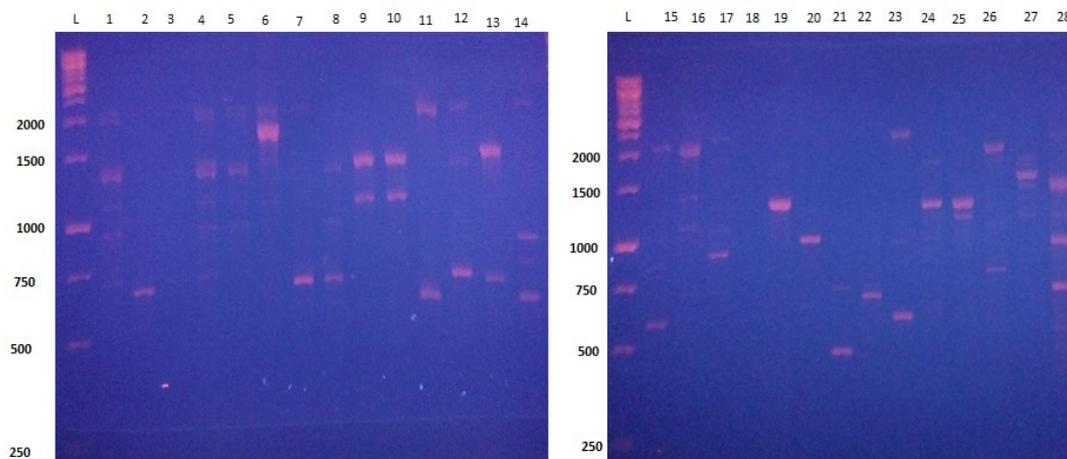

Figure 4. Representative DNA profile images of bacterial isolates (isolates number 1 to 28) obtained from water samples. Numbering on each lane denotes the code for isolates loaded in the respective lanes. Lanes labelled "L" are loaded with 1 kb molecular ladder.

Table 2. Grouping of all the isolates tested in this study

| Cluster	Related isolates (≥ 60% similarity)	Sample from which isolates were obtained	Isolates chosen for 16S DNA sequencing
1	6	BA1	6
2	19	BA4	19
3	25, 27	BA5	27
4	16, 17, 57, 59	BA3, BA4, BA14	17
5	29	BA5	29
6	4, 5	BA1	5
7	34, 70, 81, 112	BA7, BA22, BA18, BA19	34
8	12	BA13	12
9	10, 26, 78, 107	BA2, BA5, BA21	107
10	24, 38, 68, 82, 116	BA6, BA17, BA23	82
11	40, 49, f10	BA6, BA8, A4	f10
12	1, 8, 11, 13, 14, 15, 20, 21, 23, 28, 30, 31, 35, 36, 37, 39, 41, 44, 45, 46, 47, 48, 50, 51, 53, 62, 63, 66, 69, 75, 77, 80, 83, 86, 87, 89, 90, 94, 96, 98, 99, 100, 101, 102, 103, 106, 109, 113, 120, 121, 123, 128, 127, f1, f3, f4, f5, f6, f7, f8, f9, f12, f14	BA1, BA2, BA3, BA4, BA5, BA6, BA7, BA8, BA9, BA10, B12, BA13, BA16, BA17, BA24, BA25, BA26, BA27, A1, A2, A3, A4	127
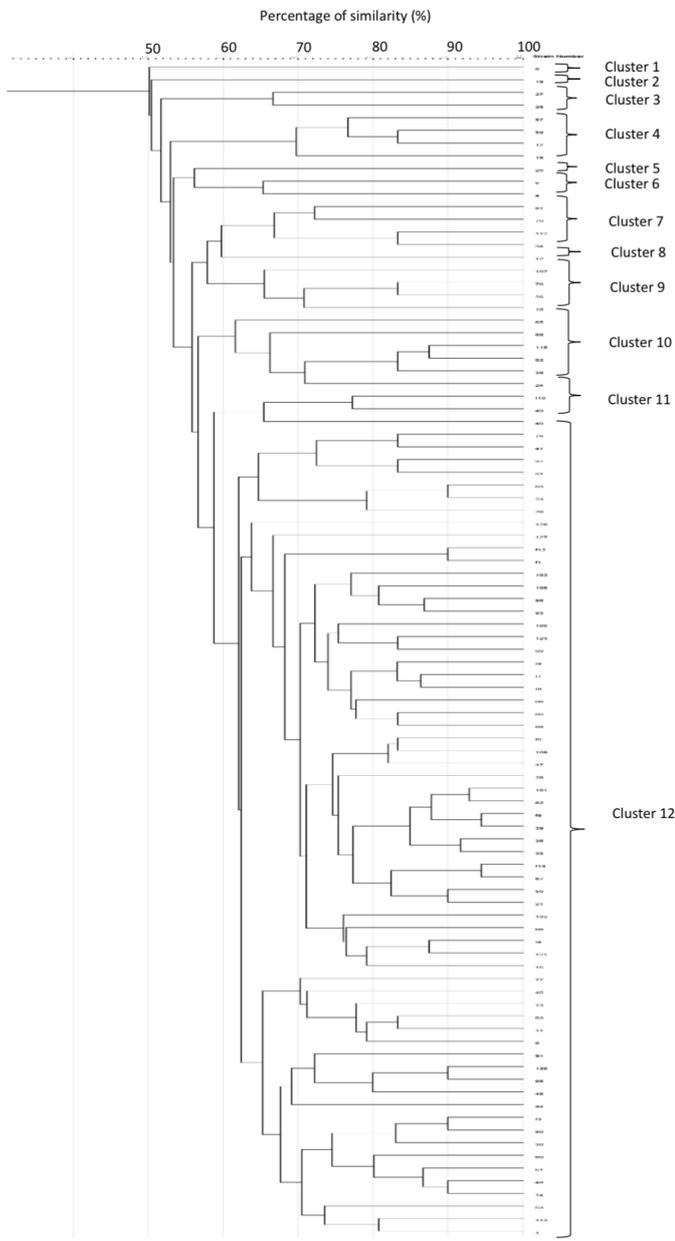
Figure 5. Dendrogram constructed using DNA bands obtained from (GTG)$_5$ PCR

Enterobacteriaceae. The identity of the isolates is shown in Table 3. The five genus of bacteria within the family of Enterobacteriaceae were identified in this study, which was *Escherichia*, *Enterobacter*, *Shigella*, *Pseudocitrobacter* and *Klebsiella*.

3.5 Antimicrobial susceptibility test

In this study, 14 antimicrobial agents were selected for the anti-microbial susceptibility test (AST). The selection of these antimicrobial agents was made based on the guidelines provided by CLSI (2018). The multiple antibiotic resistance (MAR) index for each of the isolates was calculated following the interpretation of Krumperman *et al.* (1983). The formula for the calculation of MAR index a/b, in which a = number of anti-microbial agents a particular isolate is resistant to, and b = total number of antimicrobial agents used for testing.

The result of the AST of the bacterial isolates, whether they are resistant, intermediate or sensitive to the antibiotics tested, and the multiple antibiotic-resistant indexing is shown in Table 4. *Escherichia fergusonii* showed the highest MAR value which is 0.500. Out of the 12 species, five species showed MAR values higher than 0.200 which are *E. hormaechei* (0.256), *E. kobei* (0.256), *E. fergusonii* (0.500), *K. pneumoniae* (0.214), and *S. flexneri* (0.357).

Figure 6 shows the percentage of bacterial species resistant, intermediate and susceptible to the selected antimicrobial agents tested. Out of 14 antimicrobial agents tested, ampicillin has the highest number of bacterial species being resistant to it, which is 75% (9 out of 12 species), while imipenem and amikacin both have zero per cent resistance, respectively, in all the 12 tested isolates. Aside from ampicillin and streptomycin, 12 other antimicrobial agents had more than six isolates showing an intermediate level of resistance towards each of the anti-microbial agents.
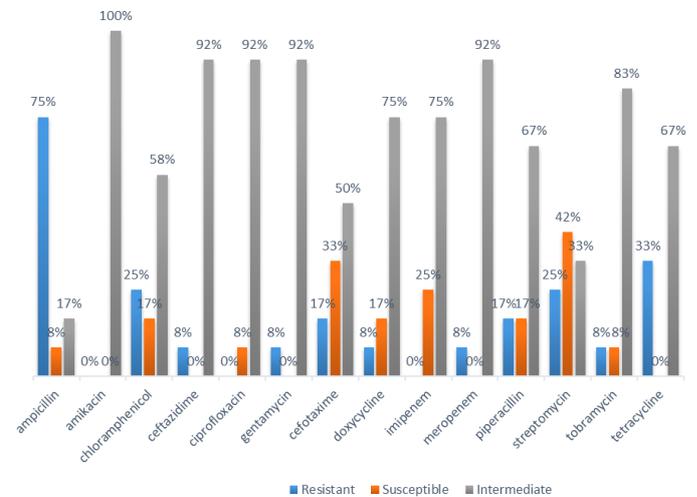

Figure 6. Graph showing the percentage of bacterial species resistant, intermediate and susceptible to the selected antimicrobial agents.

3.6 Human activities surrounding Batang Ai

Human activities taking place near each of the sampling points were observed and recorded. The correlation between these activities and the presence of Enterobacteriaceae near those activities was observed afterwards. The human activities and GPS points for each activity are listed in Table 5.

4. Discussion

Batang Ai reservoir is one of the oldest man-made water reservoirs in Sarawak, Malaysian Borneo. It was built mainly for hydroelectric power generation. In recent years, there has been an increase in human activities associated with the reservoir such as aquaculture which adopts the open cage system for

Table 3. Bacterial identities of the representative isolates of the Enterobacteriaceae

Representative isolate no.	Cluster	Bacterial identity	Similarity percentage (%)
6	1	*Enterobacter hormaechei* subsp. *xiangfangensis*	99.58
19	2	*Enterobacter kobei*	98.78
27	3	*Shigella boydii*	98.58
17	4	*Pseudocitrobacter faecalis*	98.60
29	5	*Escherichia fergusonii*	98.40
5	6	*Enterobacter cloacae* subsp. *dissolvens*	99.60
34	7	*Escherichia coli*	98.78
12	8	*Enterobacter hormaechei*	98.95
107	9	*Klebsiella pneumoniae*	98.39
82	10	*Enterobacter tabaci*	98.59
f10	11	*Shigella flexneri*	99.59
127	12	*Enterobacter cloacae*	99.18

Table 4. The AST of the bacterial isolates and multiple antibiotic resistant indexing

Bacterial isolate	Antimicrobial agents														MAR index
	AMP	AK	C	CAZ	CIP	CN	CTX	DO	IMP	MEM	PRL	S	TOB	TE	
Enterobacter cloacae	R	S	S	S	S	S	I	S	I	S	I	S	S	S	0.071
Enterobacter cloacae subsp. *dissolvens*	R	S	S	S	S	S	I	S	S	S	S	I	S	S	0.071
Enterobacter hormaechei	I	S	I	R	S	S	I	S	S	S	S	R	R	R	0.256
Enterobacter hormaechei subsp. *xiangfangensis*	R	S	S	S	S	S	I	S	S	S	S	I	S	S	0.071
Enterobacter kobei	R	S	I	S	S	S	R	S	I	S	S	R	S	R	0.256
Enterobacter tabaci	R	S	S	S	S	S	S	S	S	S	S	S	S	S	0.071
Escherichia fergusonii	R	S	R	S	S	R	R	I	S	R	R	R	S	S	0.500
Escherichia coli	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0.000
Klebsiella pneumoniae	R	S	R	S	I	S	S	I	S	S	I	I	I	R	0.214
Pseudocitrobacter faecalis	R	S	S	S	S	S	S	S	S	S	S	I	S	S	0.071
Shigella boydii	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0.000
Shigella flexneri	R	S	R	S	S	S	S	R	I	S	R	I	S	R	0.357

Key: AMP, ampicillin (10 µg); AK, amikacin (30 µg); C: chloramphenicol (30 µg); CAZ: ceftazidime (30 µg); CIP: ciprofloxacin (5 µg); CN: gentamycin (10 µg); CTX: cefotaxime (30 µg); DO: doxycycline (30 µg); IMP: imipenem (10 µg); MEM: meropenem (10 µg); PRL: piperacillin (10 µg); S: streptomycin (10 µg); TOB: tobramycin (10 µg); TE: tetracycline (30 µg).

Table 5. Human activities with GPS coordinates observed within the surrounding of Batang Ai

No	GPS point	Human activity
1	N 1.149528° E 111.876333°	Batang Ai dam
2	N 1.149139° E 111.879000°	Aiman Batang Ai Resort jetty
3	N 1.185167° E 111.822444°	Nyato dam
4	N 1.193361° E 111.804944°	Nanga Tiga village jetty
5	N 1.194278° E 111.833333°	Nanga Tiga pepper farm
6	N 1.176250° E 111.871083°	Abandoned aquaculture farm
7	N 1.178306° E 111.877222°	Cleared paddy field
8	N 1.152028° E 111.859750°	Littering
9	N 1.150722° E 111.860556°	Communal jetty
10	N 1.152222° E 111.856778°	Active aquaculture farm
11	N 1.149694° E 111.853667°	Paddy field

culturing tilapia (*Oreochromis* sp.) (Ling *et al*., 2012; Ling, Lee and Nyanti *et al*., 2013). Many studies related to water quality have been conducted in the Batang Ai reservoir but these studies did not include the microbiological water quality parameter despite the increasing human activities associated with the water reservoir (Nyanti *et al*., 2012; Ling *et al*., 2012; Ling *et al*., 2013; Ling, Lee and Nyanti *et al*., 2013). Smallholder paddy, rubber and pepper farms can be seen on the surrounding land area of the reservoir. Farmers may apply nutrients to their farms in the form of chemical fertilizer and animal manure which will enter the lake through soil erosion and runoff and contaminate the lake or water reservoir. In addition, eroded soil particles with attached nutrients will accumulate as sediment in the lake and serve as a source of available nutrients for the microbes and other living organisms in the lake. Poor water quality threatens the use of the water for human usage and furthermore, poor water quality has been linked to many waterborne disease outbreaks.

In this study, the presence, distribution and characteristics of Enterobacteriaceae were determined. A phylogenetic tree that shows the degree of genetic relatedness amongst the organisms was constructed. The phylogenetic confirms the presence of 12 main clusters with at least 60% genetic similarity. Each cluster was assumed as representing one species, which means that there were 12 different species isolated from both the fish and water samples. From each cluster, one single strain was selected to represent the cluster for further identification using 16S rRNA sequencing.

From the dendrogram generated, it was found that two of the 12 clusters consisted of isolates obtained from both water and fish samples. Upon observation, the sampling locations in which the water samples for these two clusters were obtained were located fairly close to the active aquaculture farms operating in the reservoir. This shows that the bacteria contained in the guts of the reared fish are related to the bacteria found in the water surrounding the aquaculture area. Based on the 16S rRNA sequencing result, these bacteria were identified as *S. flexneri* and *E. cloacae*.

Various studies have shown that depending on the location, the species and quantity of microbes present in the area vary (Obioma *et al*., 2017). In this study, some species were found constantly present in multiple sampling points within the water reservoir. Enterobacteriaceae are generally motile, due to most of the members of the family possessing several flagella distributed around the organism which allows them to travel and populate a considerably large area (Octavia and Lan, 2014). The most prevalent genera of

Enterobacteriaceae in Batang Ai lake were found to be the genera of *Enterobacter* making up 80.22% of the population, followed by *Shigella* (5.49%), *Escherichia* (5.49%), *Klebsiella* (4.40%) and *Pseudocitrobacter* (4.40%).

Enterobacter is pathogenic species which are widely present in nature and is also recognized as clinically significant as a human opportunistic pathogen despite *Enterobacter* spp. not being a primary pathogen for humans (Haryani *et al*., 2008, Liu *et al*., 2013; Davin-Regli and Pagès, 2015). *Enterobacter cloacae* have been also highly associated with plants. Several earlier studies regarding *E. cloacae* isolates obtained from plant origins have shown that it commonly colonizes plants such as rice, cucumber and corn. Some *E. cloacae* strains were also recognized as plant growth-promoting rhizobacteria (PGPR) (Liu *et al*., 2013). The above-mentioned crops are commonly planted alternately in paddy fields by local farmers in rural areas of Sarawak, including the land area of Batang Ai reservoir. Correspondingly, near the Batang Ai reservoir where paddy and pepper farms are found, this species was also detected. Other members of the genus *Enterobacter* which are *E. hormaechei*, *E. hormaechei* subspecies *xiangfangensis*, *E. kobei* and *E. tabaci* were also isolated from sampling points scattered all over the reservoir.

The most common member of the family that has been used as an indicator of faecal contamination in water is *E. coli* (Tallon *et al*., 2005). From this recent study, it was found that *E. coli* was isolated from samples obtained from the sampling points located in the area where the jetty for ecotourism is situated and also the area where a hill paddy is located. *E. fergusonii* and *K. pneumoniae* were also detected in the same area. Two species from the genus *Shigella* were identified as *S. boydii* and *S. flexneri*. Two prominent human activities spotted near the sampling points from which the isolates were obtained were an active floating aquaculture farm at the lake and a hill paddy farm. One isolate of *S. flexneri* was obtained from a fish sample. Species belonging to this genus are non-motile, which explains the absence of members of the genus *Shigella* in further areas.

Shigella sp. is known as one of the four leading causes of pathogens for shigellosis amongst toddlers, pre-teens and adults, while infections in infants are quite rare with *S. flexneri* being one of the most common causes for infections in infants and *S. boydii* being the least common causing strain (Sawardekar, 2004; Akter *et al*., 2019). It is also an important human pathogen known to cause diarrhoea and bacillary dysentery, which makes it a clinically important pathogen (Feng *et al*., 2003). In

developing countries, *S. flexneri* and *S. boydii* are considered to have epidemiological importance due to the burden of their infection on the population (Yousefi *et al*., 2018).

Pseudocitrobacter faecalis were found to be present in two separate areas in the reservoir. *Pseudocitrobacter* has been described by Kampfer *et al.* (2013) as a new genus. Although it has been isolated from faecal samples of patients the clinical importance of this genus is still unclear (Morales-López *et al*., 2019).

As observed in this study, several human activities were found to be quite prominent around Batang Ai reservoir including fish cage culture, small-scale hill paddy and pepper farming, rubber planting and tourism activities. It was noted that the paddy and pepper farms are scattered around Batang Ai and these agriculture activities are mostly situated very close to the water body. The community living within the Batang Ai reservoir practice the traditional method of paddy and pepper farming, which means they will clear a small land area for planting and these areas are usually hill slopes. Clearing of the land area is normally done by cutting down trees, drying and burning. The cleared area will lead to the exposure of soil surface or soil erosion. The farmers are known to use fertilizers to improve the growth of the crops. The common way for fertilizers to work, both organic and inorganic, is by supplying nutrients to the plants and at the same time, it also supplies nutrients to the soil, which improves the bacterial growth in the soil (Ojo *et al.*, 2015). Farmers in this area are regularly using fertilizers on the crops, which possibly affects the abundance of soil microbes in the area. Aside from paddy, local farmers in the area also plant cucumber, corn and other leafy vegetables in their paddy fields by rotation while waiting for the paddy to ripen and be ready for harvest. *Enterobacter cloacae* have been found to colonize these types of plants which means it is abundantly present in the soil where these crops are planted. In addition, the soil has been reported to be the natural habitat for Enterobacteriaceae, thus when runoff water from the exposed soil leaches into the water body nearby, it becomes a transmission medium for Enterobacteriaceae from the soil to the water body (Mulamattathil *et al*., 2014).

Small huts were a common fixture observed in each field and the farming area, while no proper latrine was seen in any of these fields. These huts are used by the farmers to rest after working on their field and also as a place to sleep in whenever they decide to spend a few days in the field without going home. With no proper toilet around, it is a common practice for these farmers and workers to relieve themselves near the water body

instead. This unhygienic practice is common in parts of South Asia that lack access to toilet facilities and is also a matter of mentality of the rural population (Zaidi *et al*., 2004).

Tourism and recreational activities are also quite prominent in the area. One of the two jetties in the area is for those who wish to go to a resort called Aiman Resort, located approximately 30 mins away from the jetty via boat. Another jetty in the area is a communal jetty which is mainly used by the locals when they travel by boat. The area where the communal jetty is located is also made into a recreational spot by the local developer with a few observatory huts placed around the area where visitors can sit and enjoy the surroundings. This means the constant presence of humans in both jetty areas, which leads to heavy littering by irresponsible visitors. Toilet facilities were also spotted near both jetties, along with a number of coffee shops. These toilets were built at a short distance from the water body which poses the risk of the water body nearby being contaminated with sewage waste. Areas that are suspected to be contaminated with these sewage wastes were found to harbour strains of *E. coli*, *K. pneumoniae*, *E. tabaci* and *E. cloacae*. Correspondingly, although *E. coli* is named as the most suitable indicator for faecal contamination or faecal coliform, these strains are usually known to be present when an area is found to be contaminated with sewage waste. The presence of these strains in the area could be an indicator of sewage contamination (WHO, 2001; Tallon *et al*., 2005; Price and Wildeboer, 2017).

Aquaculture farm is found to have the largest impact on the transmission of Enterobacteriaceae to their surrounding area. There are a few active aquaculture farms present at Batang Ai reservoir still operating during the time of the sampling. All of the farms adopt the open-cage system, where the fish are kept inside the cages and prevent them from escaping into the lake by nets. This means the fish are in direct contact with the lake water. Due to this, the faecal waste of the reared fish is discarded directly into the lake. As Enterobacteriaceae is naturally present in the guts of animals, the faecal waste of the fish becomes the transmission medium for the bacteria from the fish gut into the surrounding water environment. The underwater current further facilitates the spread of the microbe from the original contamination point in addition to the motility of the bacteria itself.

Multiple antibiotic resistance index (MARI) was used to determine the level of antibiotic resistance among the bacteria isolated in this study. The higher the value of MARI for an isolate, the more resistant the bacteria is to multiple antimicrobial agents. Any

bacterium with an index value of 0.2 and above is considered to originate from high-risk sources of contamination (Krumperman, 1983). In this study, it was found that the highest value of MARI obtained was 0.5, which was for *E. fergusonii* isolated from a sampling point located near the active aquaculture farm. Meanwhile, five isolates were found to have a MARI value higher than 0.2. These isolates are *E. hormaechei* with a MARI value of 0.256, *E. kobei* (0.256), *E. fergusonii* (0.500), *K. pneumoniae* (0.214) and *S. flexneri* (0.357). These isolates were obtained from sampling points located near both abandoned aquaculture farms and also from active fish open cage systems. This finding reflects the possibility that the points where these isolates were obtained were contaminated with antibiotics (Kathleen *et al.*, 2014; Sandhu *et al.*, 2016). The finding of this study also coincides with the reports stating that the antibiotic resistance profile of gut microbiota of animals is influenced by the proximity to human activities (Radhouani *et al.*, 2014).

Tetracyclines and fluoroquinolones can persist in the environment for a longer period of time which allows native bacteria to develop resistance to them and will lead to resistance selection (Gardea *et al.*, 2016). In this study, tetracycline (TE) and doxycycline (DO) were selected to represent the tetracycline family while ciprofloxacin (CIP) was selected to represent the fluoroquinolone family. Out of all the tested isolates, *S. flexneri* was found to be resistant towards DO, while four strains were found to be resistant towards TE which were *E. hormaechei*, *E. kobei*, *K. pneumoniae* and *S. flexneri*. All four strains have MAR index value exceeding 0.200. No strain was found to be resistant to CIP. Ampicillin (AMP) has the highest number of resistant strains with nine out of 12 (75%) strains being resistant towards AMP while the lowest resistance was observed towards amikacin (AK) and CIP with zero resistant strain (0%).

One of the concerns regarding antibiotic resistance in bacteria is resistance against carbapenems. The antibiotics in the B lactam group, used in treating serious infections caused by Enterobacteriaceae, and carbapenems are considered a crucial therapeutic option (Haldorsen *et al.*, 2018). In this study, two antibiotics from the class carbapenem were used which are imipenem and meropenem. In the family Enterobacteriaceae, resistance to carbapenems is rare (Aubron *et al.*, 2005). Based on the results obtained from this study, out of 12 identified isolates, only one isolate was found to be resistant to meropenem which is *E. fergusonii*. This isolate represents the cluster which consists of isolates obtained from a sampling point located near an active aquaculture farm.

In aquaculture farming, antibiotics are often used as

therapeutics, prophylactics, and metaphylactics (Shariff *et al.*, 2000). This approach is often used in the mass rearing of aquatic livestock such as fish and prawns as a means to reduce the stress exerted on the livestock. Aside from that, antibiotics are also used as a growth promoter, which in turn would contribute to a shorter period between each harvest and ultimately increase the total yield of the farm and therefore increase the revenue. To administer the antibiotics, fish feeds are commonly mixed with antibiotics (Ghaderpour *et al.*, 2015; Goncalves *et al.*, 2018). Antibiotics have been applied to treat and prevent bacterial infection in the reared fish (Miranda *et al.*, 2018). Another alternative way to administer the antibiotics is to directly apply the antibiotics into the water. However, this approach is not practical in aquaculture farms that adopt an open cage system because the antibiotics will be diluted and will have less effect on bacterial diseases. According to the farmers at Batang Ai lake, the common approach used by the farm operators is by mixing the fish feed with antibiotics. However, the operators did not disclose the exact dosage of antibiotics per kg of fish feed used. Constant exposure to antibiotics may cause the bacteria that colonize the guts of fish reared in these farms to develop resistance to antibiotics and these bacteria are continually discharged to the surroundings. In Asian countries, a wide range of antibiotics is approved to be used in the aquaculture industry, while in Malaysia, the Southeast Asian Fisheries Development Centre of Aquaculture Department reported that the types of antibiotics used are mainly sulphonamides, tetracyclines, virginiamycin, oxolinic acid and chloramphenicol (FAO, 2005).

Batang Ai lake is surrounded by various water-related activities including fishing and leisure kayaking. It is also the main port for water transportation used by the local community and also by visiting tourists. Through such activities, there is a feasible risk of them contracting water-borne bacteria through direct contact or accidental ingestion of the lake water. *K. pneumoniae* strains were isolated from points located near a communal jetty within the area. *K. pneumoniae* is a known human pathogen that naturally resides in the gastrointestinal tract, and is pretty harmless. However, once it manages to gain access to other cells, it can cause severe infection (Paczosa and Mecsas, 2016). Next to *E. coli*, it is the most common gram-negative bacterial species to cause infections. The infection spectrum of *K. pneumoniae* is pretty wide, including bloodstream infections, urinary tract infections and pyogenic liver abscesses. It is also associated with community-acquired pneumonia in Africa and Asia (Ko *et al.*, 2002; Vading *et al.*, 2018). In recent years, *K. pneumoniae* had been gaining recognition as an emerging major pathogen due

to the increasing emergence of antibiotic-resistant strains worldwide (Piperaki *et al.,* 2017). In this study, *K. pneumoniae* strains were isolated from a water body located near the communal jetty. The communal jetty is the main entry point for the locals who wish to return to their villages which cannot be accessed via road. This means local communities of all ages are exposed to the risk of being infected by *K. pneumoniae* as they prepare to journey home by using the longboat.

Batang Ai is also very well known for its red tilapia which are reared and supplied by the aquaculture farms operating at the lake. Batang Ai aquaculture farms are the main suppliers for red lake tilapia in Sarawak. According to the information given by one of the aquaculture farm workers, one of the main purchasers for Batang Ai red lake tilapia is a home-grown fast-food chain, Sugarbun Restaurant. According to Schamburg *et al.* (2014), one of the possible transmission pathways of bacteria to humans is the raw meat and fish trade in which the livestock is improperly handled. This gives rise to the risk of contracting the foodborne infection by coming in contact with or consuming improperly prepared meat. There have been numerous publications on outbreaks associated with the consumption of raw produce contaminated with Enterobacteriaceae (Al-Kharousi *et al.*, 2016). From the guts of fish obtained from the aquaculture farm, *S. flexneri* and *E. cloacae* were isolated.

Shigella flexneri is one of the main causing strains of shigellosis, a disease whose symptoms range from mild intestinal discomfort to death, depending on the severity of infection. The incubation period for this disease is approximately one to four days, starting with the manifestation of fever, loss of appetite, fatigue and malaise by the infected individual. *Shigella flexneri* can survive in water for at least six months at room temperature which contributes to its high survival and transmission rate through water (Cabral, 2010). On human skin, it can survive up to one hour which means that an individual can also be infected if no proper hygiene practice is observed while handling the fish harbouring this species. As it can survive in a human's acidic gut, it only requires as little as 100 to successfully infect a healthy adult. The bloody stool which is usually expelled by an infected individual is due to the inflammatory response of the host which destroys the colonic epithelial layer (Jennison and Verma, 2004).

Genus *Enterobacter* is found to be the most prominent in the Batang Ai reservoir. Members of this genus were isolated from almost all the sampling points throughout the study sites and also all the fish samples, showing the high prevalence of this genus in the Batang

Ai reservoir. The most prevalent species, *E. cloacae*, is part of the group of well-known pathogens which cause nosocomial infections in hospital settings. Similar to *K. pneumoniae*, it is a natural inhabitant of a healthy human gut microflora which is harmless in its normal habitat, but once it is outside of its natural setting, it will take advantage of its serum resistance and invade the eukaryotic cells. This proves that *E. cloacae* are an opportunistic pathogen (Keller *et al.*, 1997). It also can latch itself onto eukaryotic cells, which improves its survival rate outside of the gut microflora. Since *E. cloacae* were isolated from most of the areas in which human activities are abundant, it has a very high chance to infect unsuspecting individuals or use humans as a transmission medium.

4. Conclusion

The findings of this study suggested that there is possible transmission of genetically diverse antibiotic-resistant Enterobacteriaceae, as a result of human activities within the surrounding environment, to the water reservoir of Batang Ai dam. Further research should be carried out to study antibiotic usage and the impact of antibiotic usage in aquaculture farms and their surrounding environment and also its impact on the health of the local community and visitors of Batang Ai. Based on visual observation at the study site, a number of prominent human activities were present around the area which may contribute significantly to the pollution of the lake water which in turn posed a health risk for the local community and visitors. It is proposed that a proper guideline on the execution of activities in the Batang Ai area and the guideline may include proper standards on farming, operating of the aquaculture farms, proper sewage disposal, and also the type of recreational activities allowed in the area.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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