

The distribution and characteristics of bacteria in recreational river water of a community resort in Baram, Sarawak, Malaysian Borneo

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Abstract

Enterobacteriaceae is a large family within the Gram-negative bacteria that primarily inhabits in the gastrointestinal tract of human and animals. The bacteria within this group are readily survived in the environment with some species found living free in the water where energy sources are scarce, making them ideal indicators for faecal contamination of the river water. Some species within the family have been used as indicator for the presence of pathogenic bacteria whilst on the other hand some species have been directly associated with various diseases in human and animals. The main aim of this research study was to determine the distribution and characteristics of the *Enterobacteriaceae* in water samples collected from river and waterfalls within a community resort. The health risk associated with the bacteria was analysed with regard to their susceptibility to antibiotics. Samples were collected from surface water and water falling down directly from waterfalls of river within the community resort. The samples collected were plated onto Eosine Methylene Blue agar (EMBA) for the isolation of the *Enterobacteriaceae*. Bacterial colonies growing on the agar were randomly picked, purified, stocked and then identified using API 20E identification kit. DNA fingerprinting using (GTG)5-PCR was utilised to determine their genetic profiles before the isolates were grouped into a dendrogram using RAPDistance software package. The level of antibiotic susceptibility of the bacteria isolates was analysed using disc diffusion technique. This study confirmed the presence of *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pantoea* and *Serratia* in the water samples with their single and multiple antibiotic resistance and susceptible characteristics. The dendrogram presented in this study shows genetic similarities and differences among the strains, suggesting while there is a potential for single distribution of a clone, there is also possibility of the distribution of different strains within species in the water environment. Therefore, awareness on the potential risk associated with genetically diverse intermediate and resistant enteric bacteria in the recreational water should be communicated to the public especially communities within the study area.

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Introduction

The *Enterobacteriaceae* group of bacteria consists of the harmless symbionts and pathogens of human and environmental species such as commonly found bacteria *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp. and many others (Cabral, 2010). The coliforms are among the group of indicator bacteria that have been used to measure of water quality. Besides, they are among the major contributions to the contaminants of surface and recreational waters in developing countries (Cabral, 2010).

Diseases-causing *Enterobacteriaceae* have

the capability to invade their host in many ways because they possess some important characteristics like motility, colonization factors, endotoxin and enterotoxin (Guentzel, 1996; Peterson, 1996). Some species are motile because they possess several flagella distributed around their perimeter. Apart from that, the enterobacterial colonization factors consist of hairy appendages which can make them bind tightly to their host. The endotoxins are the cell membrane constituent of Gram-negative bacteria including *Enterobacteriaceae*, which trigger infected individuals to have high fever (Peterson, 1996).

Pollution of water especially with faeces

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from wild animals and human can lead to health problems associated with the presence of pathogenic microorganisms (Whitlock *et al.*, 2002; Cabral, 2010). Other than that, water normally accommodates free-living infectious microbes (Daggett *et al.*, 1982). Available reports suggests that the most common health problem associated with exposure to faecally polluted water is acute gastrointestinal illness, like self-recovery gastroenteritis, which may just be temporary and may not be documented. Young children have been reported to contract more illness from recreational water when they play in the shallow water and in muddy area where the bacteria are concentrated and may swallow more volume of water while swimming (Ackman *et al.*, 1997; Denno *et al.*, 2009; Valderama *et al.*, 2009).

In recent years, the increased usage of antibiotics has led to the phenomenon of antibiotic resistance among bacteria including enteric bacteria (Chitanand *et al.*, 2010; Apun *et al.*, 2011; Ng *et al.*, 2014). Bacteria with highest frequency of resistance towards commonly use antibiotics have been isolated from hospitals and its environments such as hospital effluents, other sources like sewage and waste water (Chandrasekaran *et al.*, 1998; Moges *et al.*, 2014). The presence of multiple antibiotic resistant bacteria in various water environments have been reported in Malaysia (Son *et al.*, 1998; Samuel *et al.*, 2011; Abdullahi *et al.*, 2013; Ng *et al.*, 2014; Lesley *et al.*, 2016). It has also been reported that the multiple resistant bacteria are also presence in recreational water which constitute a direct threat to those associated with the water through potential transfer of resistance to human and animal strains (Pathaka *et al.*, 1993; Alonso *et al.*, 2001; Yin *et al.*, 2013; Zhu *et al.*, 2013). Therefore, the monitoring on the presence of indicator bacteria for the presence of pathogenic bacteria and their level of susceptibility towards certain antibiotics commonly used in the clinical and agricultural settings is important to make sure treatment associated with water borne diseases still remain effective.

Tenyok Rimba resort, a community resort located within Tenyok Rimba forest reserve within the area of Long Bedian village in Baram, Fourth Division, Sarawak, Malaysian Borneo is famous for its tourist attraction for its beautiful waterfalls, clear river, virgin forest with variety of wild animals including birds. The resort sits on the edge of the reserve, with a clear river called Tenyok river running by, close to the chalets within the resort. The river has been used for recreational activities like fishing, bathing, swimming, picnicking and many other outing activities. Although the river plays essentials role in

the life of people in the community, many individuals are not aware about the potential risk associated with microorganisms including bacteria that may have potential to cause waterborne diseases.

Therefore, the aim of this research study was to investigate the occurrence of indicator microorganisms within the family of *Enterobacteriaceae* in the recreational water within the community resort area and also to determine to which extend the bacteria may pose risk with regards to the bacterial characteristics.

Materials and Method

Sample collection

Water samples (3 samples from each of the locations) were collected with sterile 500 ml Scott's bottles from Tenyok River, Nyang waterfall and Nawan waterfall which are located within the Tenyok Rimba community resort in Long Bedian, Fourth Division, Sarawak, Malaysian Borneo. The water samples collected were kept at 4°C in an icebox-containing ice during the transportation.

Bacterial isolation

Approximately 100 µl of the water samples were direct plated on EMBA and incubated at room temperature for 24 hr. The EMBA plates showing the growth of bacteria were sealed with parafilm to prevent contamination and the plates were transported to University laboratory for further analysis. Upon arrival in the laboratory, bacterial colonies were randomly selected, purified and subjected to biochemical tests as a preliminary test including triple sugar iron, Kligler iron agar and oxidase reagent before they were subjected to identification using API kits. The readily known *Enterobacteriaceae* isolates were further analysed with (GTG)5-PCR fingerprinting.

Analytical profile index (API) 20E test system

Nineteen bacterial isolates were selected for the API 20E kit (BioMerieux) identification. It was conducted following to the manufacturer's instructions and reports described elsewhere (Butler *et al.*, 1975; Aldridge *et al.*, 1978; Barry and Badal, 1979). Briefly, the test began with the inoculation of NaCl suspension of pure culture into each well of the API 20E test strip then followed by incubation of the test strip at 37°C for 24 hours. After the incubation period, the appropriate reagent was added to the |IND|, |VP| and |TDA| well. The result of the test was analysed through on-line database, APIwebTM.

DNA extraction

Bacterial DNA was extracted by boiling cell method (Freschi *et al.*, 2005). Briefly, 2.0 ml of the bacterial solution were transferred into a microcentrifuge tube, centrifuged at 10,000 rpm for 5 min and then the pellet was collected. This step was repeated once again for rapid cell collection. The pellet collected was re-suspended with 500 µl of sterile distilled water. The mixture was homogenized by using vortex until no clump was observed. The microcentrifuge tube was then transferred into a boiling water bath at 100°C for 10 min and immediately cooled in ice for 5 min. Lastly, the mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was stored in a new sterile 0.6 ml tube and kept at 4°C for (GTG)5-PCR analysis.

(GTG)5-PCR analysis

(GTG)5-PCR fingerprinting was carried out as described by Matsheka *et al.* (2006) with minor modifications. A final volume of 25 µl PCR mixture consisting 5 µl of 5×Green GoTaq® Flexi buffer, 3 µl of 25 mM MgCl₂, 0.5 µl of 10 mM dNTPs, 0.5 µl of 25 µM (GTG)5 primer (5'-GTG GTG GTG GTG GTG-3'), 10.7 µl of sterile distilled water, 5 µl of DNA template and 0.3 µl of GoTaq® Flexi DNA polymerase. (GTG)5-PCR amplification was performed with an initial denaturation at 95°C for 2 min, 30 amplification cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final polymerization step of 72°C for 5 min. The PCR products were electrophoresed on 1.2% agarose gel in 1×TBE buffer at 90V with 400 mA for 75 min. An 1 kb DNA ladder (Fermentas) was included as a DNA size marker. The electrophoresed agarose gel was stained with ethidium bromide and then visualised on an UV transilluminator.

DNA fragment analysis

The DNA fingerprint profiles obtained from the (GTG)5-PCR were analysed using the RAPDistance package (version 1.04). The scoring was done based on the banding patterns obtained using binary data format. The presence and absence of band was scored "1" and "0", respectively. Dice formulation was used to determine the genetic distances between the banding profiles (Nei & Li, 1979). From the calculated genetic distances, a dendrogram of neighbour joining tree was constructed.

Antibiotics susceptibility tests

Antibiotics susceptibility test was conducted using disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2012) on

Mueller Hinton agar (MHA). Antibiotic discs (Oxoid, England) were used in this testing with *E. coli* ATCC 25922 as the positive control. Antibiotic discs used in the testing were meropenem (10 µg), imipenem (10 µg), piperacillin (100 µg), ampicillin (10 µg), tobramycin (10 µg), amikacin (30 µg), nitrofurantoin (300 µg), ceftazidime (30 µg) and nalidixic acid (30 µg). Briefly, a sterile cotton bud was dipped into an overnight grown bacterial suspension and then swabbed evenly on the surface of the MHA. The agar plate was allowed to dry for 5 min before the antibiotic discs were placed on the MHA plate. The plate was incubated at 30°C ± 1°C for 24 hours. After the incubation, the diameter of inhibition zone on the MHA was analysed and interpreted as susceptible (S), intermediate (I) or resistance (R) based on the Clinical and Laboratory Standards Institute (CLSI, 2012), antimicrobial susceptibility testing standards M02-A11.

Results and Discussion

The presence and distribution of *Enterobacteriaceae* in the recreational river within Tenyok Rimba resort and to which extent the bacteria may pose risk to those who are in contact with the water were analysed. Water samples were collected from Tenyok river, Nyang waterfall and Nawan waterfall within the resort, which are among the famous spots of attractions for recreation activities among tourists and locals.

In this study, API 20E test system confirmed the identity of a number of different bacterial genus and species within the *Enterobacteriaceae* family presence in the water. Table 1 shows the bacterial species and their characteristics isolated from the water samples collected from recreational river and waterfalls within the community resort. The API kit confirmed the presence of *Enterobacter*, *Klebsiella*, *Citrobacter*, *Pantoea* and *Serratia* in the water samples.

Some members in the *Klebsiella* genus are opportunistic pathogens where they primarily attack immunocompromised individuals who suffered from other chronic diseases such as diabetes or chronic pulmonary obstruction (Podschun and Ullmann, 1998). Besides that, *Klebsiella* also accounts for most of the urinary tract infections in the world (Podschun and Ullmann, 1998). *Klebsiella pneumoniae* has been reported to be among the most common human pathogens grouped under the family of *Enterobacteriaceae* (Guentzel, 1996). This bacterium is responsible for a wide range of community-acquired diseases and majority of the illnesses caused

Table 1. Bacterial species and their characteristics from the water samples collected from recreational river and waterfalls within the community resort

Bacterial code	Bacterial species	Antimicrobial resistant profile	Antibiotic intermediate profile ¹
Tenyok (c)	<i>Enterobacter sakazakii</i>	- ¹	CAZ, N, NA
Tenyok (d)	<i>Enterobacter cloacae</i>	N	-
Tenyok 1 (b)	<i>Enterobacter asburiae</i>	AMP, N, PRL	IPM
Nyang (a)	<i>Enterobacter cloacae</i>	-	AK, N, IPM, TOB
Nyang (c)	<i>Serratia marcescens</i>	N	AMP, IPM
Nyang (d)	<i>Serratia marcescens</i>	N	AMP, IPM
Nyang 1 (b)	<i>Klebsiella pneumoniae</i> ssp. <i>ozaenae</i>	-	IPM
Nawan (a)	<i>Enterobacter aerogenes</i>	AMP	IPM, TOB
Nawan (b)	<i>Serratia marcescens</i>	N	AMP, IPM
Nawan (c)	<i>Citrobacter youngae</i>	N	AMP, PRL
Nawan 1 (a)	<i>Serratia marcescens</i>	N	IPM
Nawan 1 (b)	<i>Serratia marcescens</i>	N	AK, AMP, IPM, TOB
Nawan 1 (c)	<i>Enterobacter cloacae</i>	-	N, IPM
Nawan 1 (d)	<i>Enterobacter cloacae</i>	AMP, N	AK, IPM, TOB
Nawan 1 (e)	<i>Enterobacter sakazakii</i>	AMP	-
Nawan 2 (a)	<i>Klebsiella pneumoniae</i> ssp. <i>pneumoniae</i>	AMP, PRL, TOB	AK, N, MEM
Nawan 2 (b)	<i>Klebsiella pneumoniae</i> ssp. <i>ozaenae</i>	AMP	N, IPM
Nawan 2 (e)	<i>Pantoea</i> spp.	-	IPM, TOB
Nawan 2 (f)	<i>Enterobacter cloacae</i>	N	IPM

¹The isolate showed no resistant/intermediate characteristic against any of the antibiotics tested.

Antibiotics concentration (µg/ml) tested: Amikacin (AK, 30), Ampicillin (AMP, 10), Ceftazidime (CAZ, 30), Nitrofurantoin (N, 300), Imipenem (IPM, 10), Meropenem (MEM, 10), Nalidixic acid (NA, 30), Piperacillin (PRL, 100), Tobramycin (TOB, 10).

by the bacterium can be treated with beta-lactams group of antibiotics (Stock, 2014). For example, it is a well-known pathogen associated with bacterial pneumonia, which usually occurred particularly in chronic alcoholism. Most of *Klebsiella* infections are normally associated with hospitalization.

Serratia marcescens was also among the Enterobacteriaceae isolated from the water in this study. Although *S. marcescens* has been isolated from various habitats including soil, plant and water, it has been most commonly isolated from patient during nosocomial outbreak (Grimont and Grimont, 2006). The healthy people does not often become susceptible to *Serratia* infection, however hospitalized patient is commonly infected. At present, only *S. marcescens* is associated with hospital-acquired disease in human although other species have been occasionally isolated from patient samples (Farmer *et al.*, 1985; Grimont and Grimont, 2006). *Serratia* infections include a wide range of diseases similar to other genus within the Enterobacteriaceae from respiratory tract infection and colonization to septicaemia in patient (Cabrera, 1969; Altemeier *et al.*, 1969; von Graevenitz, 1980).

Enterobacter has been associated with various

diseases ranging from respiratory tract, urinary tract, skin and soft-tissue infections to bacteremia (Parija, 2009). *Enterobacter* infections can impose prolonged hospitalization, and need higher dose and appropriate antimicrobial drugs for its treatment (Fraser and Sinave, 2015). *Enterobacter sakazakii* has been implicated with neonatal sepsis with meningitis (Hunter, 2008). The major species within the genus are *Enterobacter aerogenes*, *Enterobacter cloacae* and *Enterobacter agglomerans* (Maki *et al.*, 1976).

The presence of pathogenic bacteria along with their antibiotic resistance characteristic associated with recreational water has been a major public health concern (Klare *et al.*, 1995; Kummerer, 2004; Baquero *et al.*, 2008; Martinez, 2008; Zhang *et al.*, 2009). The issue of antibiotic resistant bacteria is becoming more serious in aquatic environments and there have been many studies conducted to investigate the presence of bacteria along with their antibiotic resistance characteristics in river water (Mudryk *et al.*, 1998; Ash *et al.*, 2002; Ram *et al.*, 2008; Yin *et al.*, 2013). It has been reported that the sources of faecal indicator bacteria to river consist of both human waste (Edberg *et al.*, 2000; Whitlock *et al.*, 2002; Ram *et al.*, 2008)

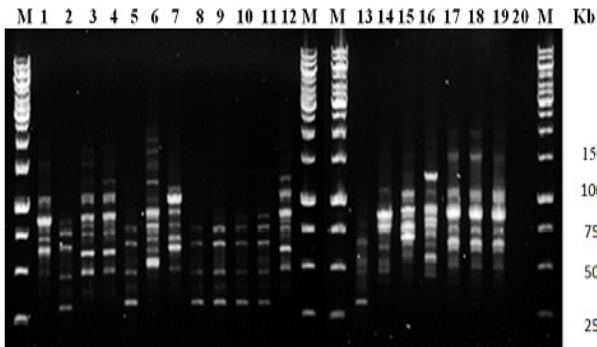


Figure 1. Genomic DNA profiles of the isolates from the water samples. Lane M, GeneRuler™ 1kb DNA ladder (Fermentas, USA); Lane 1, Nawan 1(e); Lane 2, Nawan 1(b); Lane 3, Nawan 1(d); Lane 4, Nawan 1(c); Lane 5, Nawan 1(a); Lane 6, Nyang 1(b); Lane 7, Nyang (a); Lane 8, Nyang (c); Lane 9, Nyang (d); Lane 10, Nawan (b); Lane 11, Nawan (c); Lane 12, Nawan (a); Lane 13, Tenyok 1(b); Lane 14, Nawan 2(b); Lane 15, Nawan 2(a); Lane 16, Nawan 2(e); Lane 17, Nawan 2(f); Lane 18, Tenyok (d); Lane 19, Tenyok (c).

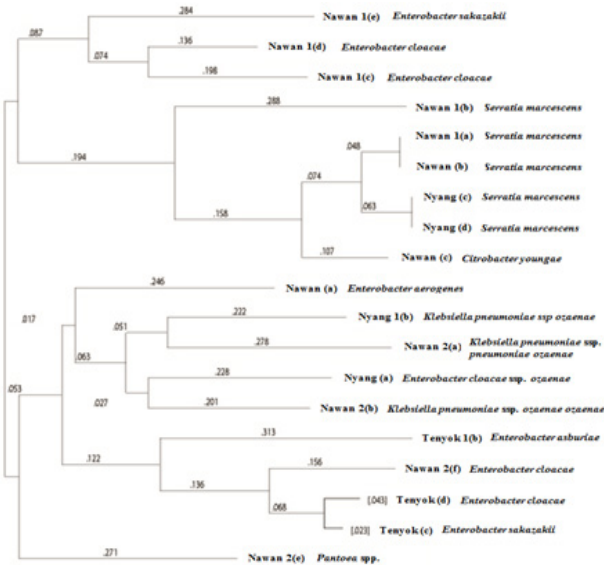


Figure 2. Dendrogram showing the genetic distributions of the isolates from the water

and animal waste from wildlife (Shellenbarger *et al.*, 2008) and domestic (Whitlock *et al.*, 2002). During the day of the sampling of this study, there were no recreational activities going on in the recreational area, suggesting the possibility of fecal from wild animals, organic matter and soil run off from the jungle could contribute to the contamination of the water.

In this study, the genetic distribution of the *Enterobacteriaceae* was analysed using (GTG)5-PCR. Figure 1 shows the DNA profiles of the bacterial isolates after gel electrophoresis where the bands produced were diversified in terms of the size and thickness of the bands, reflecting the genomic heterogeneity among the isolates within genus and species. The DNA bands were analysed further to

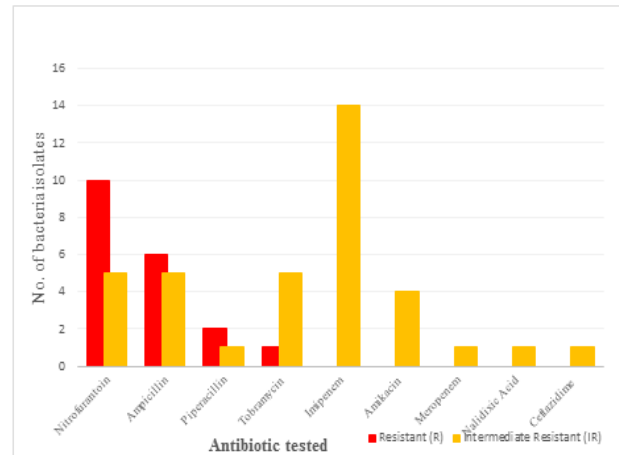


Figure 3. Bar graph showing the susceptibility of the isolates from the water

group the isolates in a dendrogram which clearly separated the bacterial into different groups (Figure 2), clearly showing the distribution of the bacterial from one site to other sites within the study area. For example, *S. marcescens* from Nyang and Nawan waterfalls were grouped in the same cluster in the dendrogram, this suggested that the contamination was originated from one unknown source. On the other hand, isolates of *Enterobacter cloacae* from Nawan waterfall were grouped into different clusters suggesting diverse strains, instead of a single strain of *Enterobacter cloacae* circulating in the recreational water in the area. The profiles of the DNA obtained in this study could be useful for tracing the bacteria in epidemiological studies.

Table 1 shows the antibiotics susceptibility of all the isolates towards the antibiotics tested. Figure 3 shows the bar chart of the number of isolates showing resistant and intermediate towards the antibiotics tested. In this study, among the bacteria analysed, *Klebsiella* spp. exhibits resistant or intermediate to at least one of the antibiotics tested. *Klebsiella* is often showing low susceptibility to multiple antibiotics and it has been proven that plasmids are the source of the resistance determinants in resistant isolates (Hudson *et al.*, 2014). It has been reported that *Klebsiella* spp. can produce extended-spectrum beta-lactamases (ESBL) which cause them to be resistant to many groups of antibiotics such as aminoglycosides, tetracyclines, fluoroquinolones, trimethoprim/sulfamethoxazole and chloramphenicol (Nathisuwan *et al.*, 2001).

In general, the level of resistance of the isolates in this study was relatively low compared with studies reported elsewhere (Alhaj *et al.*, 2007; Chitanand *et al.*, 2010; Ng *et al.*, 2014). The isolated bacteria in this study showed resistant towards nitrofurantoin (52.6%), ampicillin (31.6%), piperacillin (10.5%),

tobramycin (5.3%). Intermediate resistant was exhibited by at least 5.3% of the isolates against all the antibiotics tested (Figure 3). The explanation for the relative low resistance is because the isolates were taken from environmental water where the source may not be exposed to the contamination from domestic or industrial sources. It is hypothesized that faecal from wild animals, organic matter and soil run off from the jungle could contribute to the contamination of the water in the study area since the resort is quite far from the village and also agricultural activities.

Conclusion

The presence of diverse species of *Enterobacteriaceae* in the water samples along with some pathogenic groups with antibiotic resistant and intermediate characteristics may pose public health risk especially to those who are in direct contact with the water during recreational activities. It is recommended that the communities should be aware about the potential health hazard associated with microorganisms in the recreational water.

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