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Isolation and Identification of Pathogenic Bacteria at Riverbank Filtration (RBF) Study Site: Lubuk Buntar, Kedah, Malaysia

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Abstract: River water is used for various purposes like as drinking water, in agriculture etc. The impact of river water pollution on human health depends mainly on the water uses, as well as the concentration of pathogen in water. Riverbank filtration (RBF) is an efficient and low-cost natural alternative filtration technology for water supply application. This technology was greatly removing many contaminants including pathogenic bacteria and viruses. However, this technology is new and not well explored in Malaysia. During the study, USM research team has embarked on the study of RBF at Lubuk Buntar, Kedah Malaysia. In order to understand the transportation of pathogenic bacteria during the filtration process, it is necessary to identify residence pathogenic bacteria in the river. Hence, this study was aimed to isolate and identify the pathogenic bacteria at the RBF study area located in Lubuk Buntar, Kedah, Malaysia. This study also conducted physicochemical test to monitor the river water quality. Analysis of 16S rDNA sequence revealed that *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* DSM 50071, *Enterobacter ludwigii* EN-149 were the origin pathogenic bacteria in the river at the RBF study site. pH for the river water was found in range 5.24-6.89 while temperature was recorded in range 30-25.31°C. BOD and COD were in range 6.8-2.0 mg/l and 31.0-8.0 mg/l respectively. The results show high value of turbidity, TSS and color which in range 179-64 mg/l, 177-68 and 74-20 PtCo respectively.

Keywords: Riverbank filtration (RBF), drinking water, pathogenic bacteria, identification, water quality

1. Introduction

Drinking water is derived from two sources, which are surface waters and groundwater. As water demand is rising continuously, the surface and groundwater resource was threatened by contamination. This issue dominates water quality concern worldwide. There are a number of threats to drinking water such as aesthetic, biological pathogen and chemicals. Heavy metals and pathogen normally found in nature in average amounts do not promote a risk to the environment [1]. However, if the levels of these materials increase, health hazard and risks correspondingly become serious. In fact, most of the studies reported pathogenic contamination of drinking water remains a significant threat even in the most developed countries. During the last decade, pathogenic bacteria abundance has increased in many surface water bodies around the world and cause of many water-borne diseases outbreaks. According to World Health Organization (WHO), most children from 3.4 million infected people die from water-related diseases each year [8]. Based on reports of United Nations Children's Fund (UNICEF 2014) assessment, 4000 children die each day because of contaminated water. Meanwhile, WHO (2010) reports that over 2.6 billion people lack access to clean water. Among them, about 2.2 million deaths annually, of which 1.4 million are in children [2].

In line with the safe drinking water demand, efficient removal of pathogen must be practically applied. Riverbank filtration (RBF) is an establish water management operation where series of abstraction wells

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were forced surface water to infiltrate prior to extraction wells [3]. RBF can be either horizontal or vertical. Most RBF systems are constructed in alluvial aquifers located along riverbanks. It has been used to provide drinking water to communities in Europe, United States and then followed with India, China, Korea Jordan and Egypt [4].

Studies have shown that RBF has be proved to remove the indigenous pathogenic bacteria in river such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Giardia*, *Cryptosporidium* and others have been reported. Including column tests, it is necessary to assess the transportation mechanism and removal of pathogen during passage through RBF system. The transportation of pathogen depends on a variety of physical, biological and geochemical parameter, such as flow rate of water, temperature, pH, sediment grain size distribution, size characteristics and others [5, 6].

To date, most of the previous studies are preferred to purchase the pathogenic bacteria for their RBF study. However, the drawback of this method is the knowledge of the bacteria transportation may be restricted as the tested pathogenic bacteria may not be existed in the study area. Thus, the isolation of located pathogenic bacteria from the study site is necessary in real study area to promote effective filtration successfully. This study was conducted to isolate and identify the potential pathogenic bacteria in river water of the RBF study area. Physicochemical test also conducted to monitor the background water quality of the river.

2. Materials

2.1 RBF Study Area

In order to determine whether bank filtration will be a beneficial component of the water supply scheme in Malaysia, a through site investigation of the target site is required. A pilot project has been established by USM research team in Malaysia in the state of Kedah. This RBF study site is located in Lubuk Buntar, Kedah Darul Aman with longitude and latitude of 5° 7'37.60"N, 100°35'42.97"E. The area was near to raw water intake of SADA (Syarikat Air Darul Aman) water treatment plant (Fig. 1). Kerian River (Sungai Kerian) is the main river at the study area which is the border between Kedah and Perak and it was classified as river class II and III. According to that, this study area was chose due to the high water demand in the area and high pollution level of surface water. RBF is seen as an alternative source with very high potential to be developed as a supplementary source to meet the high public water supply demand.

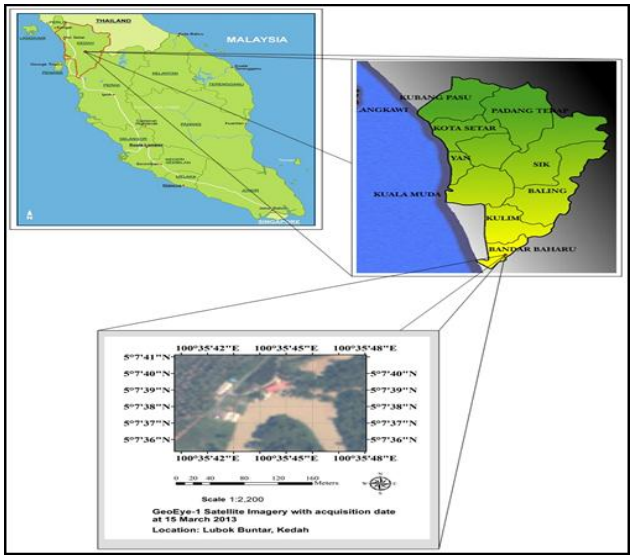


Fig. 1: Location of the RBF study area

2.2 Collection of water samples

Three samples of water from Sungai Kerian (Kerian River) were collected during July and November 2016. The samples were handled aseptically in sterilized 500ml Duran Schott bottles using standard procedure and methods prescribed by American Public Health Association [7]. For water quality monitoring, the sampling were done for one year during April 2016 until April 2017. The samples were collected in a clean polyethylene bottles and labeled properly and transported in ice box to the laboratory and was immediately stored at 4ºC in cool room prior to analysis.

3. Methods

3.1 Physicochemical characteristics test

All samples were analyzed within 8 hours after collection. The water quality assessment parameters analyzed were; pH, temperature, dissolved oxygen (DO), total dissolved solid (TDS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), turbidity, color and total suspended solid (TSS). Parameter such as temperature, pH and DO were measured using the YSI multi-parameter ORP meter (serial number: 073101344) which was done during the water sampling at the site by dipping it inside the water for a few minutes. Turbidity was done in accordance to Standard Method 2130B using Turbiditimeter. Other parameters were analyzed according to APHA 2005.

3.2 Isolation of pathogenic bacteria

Water samples for microbial analysis were analysed aseptically. Isolation of the pathogenic bacteria was performed by serial dilution. The media used to culture the bacteria was chocolate blood agar. About 100 µl for each of the dilution were transferred using micropipette onto petri dishes containing chocolate agar and labeled as 10^{-1} to 10^{-10}. By using spread plate method, the samples

were spread evenly on the agar using hockey stick. The plate was then incubated in incubator for 24 to 48 hours for the bacteria to growth until the colonies appeared in temperature of 37^{o}C. Sub-culturing was done many times to achieve pure culture. Once a bacterium has been obtained in pure culture, it has to identify. The single colony was streaked on a fresh nutrient agar (Sigma-Aldrich). Gram staining method was done to classified bacteria into gram positive and gram negative bacteria and was observed its cell morphology under microscope observation.

3.3 Biochemical Characterization of the Isolates

Biochemical test were conducted to preliminary characterization of isolated bacteria. The biochemical tests performed in the study include catalase production test, oxidase production test, indole production test, citrate utilization test, vogues-proskauer test, methyl red test. All those test were conducted as per standard Microbiological methods [8].

3.4 Preparation of DNA extraction

Preparation of pure DNA samples is crucial for a successful cloning experiment. Pure isolated bacterial strains were inoculated in separate nutrient broth (NB) and incubated for 24 hours at 37^{o}C. 1.5ml of bacterial suspensions cultured overnight was centrifuged at 6,000xg for 2 minutes at room temperature. The supernatant was discarded and the pellet obtained is used for DNA extraction. A pure genomic DNA is prepared using GF 1 Bacterial DNA Extraction Kit. All steps were carried out according to the manufacturer's protocol and the steps taken were done at room temperature. The purity DNA samples were determined by determination of the DNA concentration using Nanodrop 2000 spectrophotometer (Fisher Scientific). The absorbance was taken at 260 nm and 280 nm which the ratio ($\geq$ 1.8) of them used to calculate the concentration.

3.5 Molecular Identification

Molecular analysis was carried out by Centre for Chemical Biology, Penang, Malaysia (CCB). The 16s rRNA gene was amplified by PCR using universal primers 16S-27F and 16S-1492R. PCR was carried out under the following conditions: 94°C (3 min), 30 cycles of 94°C (30 s), 55°C (30 s) and 72°C (1.4 min) and a cycle of final extension at 72°C (5 min). All the PCR products were subjected to electrophoresis and the gel band containing desired DNA fragment was then excised and purified for cloning and sequencing purpose. The obtained sequences were analyzed using BLAST analysis provided by National Centre for Biotechnology Information (NCBI).

4. Results and Discussion

4.1 Physicochemical Analysis

Table 1 shows the physicochemical analysis result for the monitoring program during a year. The present study was undertaken to characterize the physicochemical parameters like pH, temperature, DO, TDS, BOD, COD, turbidity, colour and TSS.

pH which is an important ecological factor that provides a crucial information about many types of geochemical equilibrium or solubility calculation that affect the flora and fauna of the aquatic system. pH for the river water was found in range 5.24-6.89. According to Drinking Water Quality Standard, pH of drinking water has to be in the range of 6.5-9.0. It is because, for pH more than the range could cause irritation and worsen the skin condition [9]. pH of water is normally influenced by geology of the catchment area and buffering capacity of water.

For temperature, it was recorded in range 30-25.31°C. In the month of April has the highest value which is 30°C due to hot weather during the investigation while during July to December the temperature was lower due to rainy season at the study area. Temperature of water is important because temperature itself influenced other water quality parameters like dissolve oxygen, pH and photosynthesis production. Increase of temperature could decrease the solubility of the oxygen and other gases in water which then could give effect to the aquatic habitat and organisms in water. Dissolved oxygen is an important parameter for the assessment of water quality and reflects the physical and biological process prevailing in the water. DO indicate the amount of oxygen that available in water. High value of DO indicates the high quality of the water and if the value of DO is low, it might because of the organic matter containing in the water because organism in the water use oxygen to breakdown and decompose the organic matter. DO at the study area was recorded in range 6.89-3.8 mg/l.

Table 1. Physicochemical analysis of river water (Sungai Kerian/Kerian River)

Water quality parameters	Unit	River Water	
		Max value	Min value
pH		6.89	5.24
DO	mg/l	6.4	3.8
TDS	mg/l	33.2	22.0
Temperature	°C	30.0	25.31
BOD	mg/l	6.8	2.0
COD	mg/l	31.0	8.0
Turbidity	NTU	179.0	64.0
Color	PtCo	74.0	20.0
TSS	mg/l	177.0	68.0

Suspended solid (SS) is one of the important parameter for water quality because high value of SS can

cause high turbidity in water. The value of TSS is correlated with turbidity. The readings of TSS and turbidity during the monitoring were in range 177-68 mg/l and 179-64 mg/l respectively. Turbidity value was exceeded Drinking Water Quality Standard which is the permissible limit is 5 mg/l. The higher value was happened during the rainy season due to the increasing of the river water flow rate and also the runoff from heavy rains because runoff can introduce large amount of solids from land surface into the water. A high turbidity can interfere with the disinfection process and can provide a medium for microbial growth. It may also indicate the presence of microbes Turbidity of water could affect the color of water [10]. The reading of color was in range 74-20 PtCo. BOD indicates the microbial pollutant in the river water while COD represents the oxygen required for chemical oxidation of organic pollutant both degradable and non-biodegradable matter. BOD and COD were in range 6.8-2.0 mg/l and 31.0-8.0 mg/l respectively. Heavy rainfall during July to December was found to the reason for reduction of BOD during those months.

4.2 Isolation of pathogenic bacteria

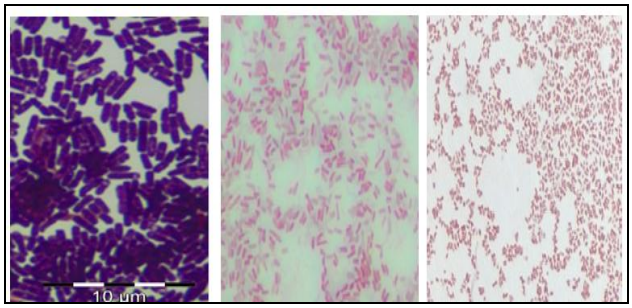
In present study 3 types of colonies were selected from the isolation process. The isolates of the 3 types of bacteria were chose based on their abundance colony on the chocolate agar. The isolated bacteria were first identified through conventional morphological and cultural characteristics identification method. The bacteria were named as A, B and C. Table 2 shows the morphological and cultural characteristics of selected bacteria. From the table it shown that bacterium A is a gram positive and the rest are gram negative. They have rod shape. Gram staining image were shown in Fig. 2. For cultural characteristics, the bacteria colours were opaque, blue-green and lack of sheen. After this the isolated bacteria were identified by biochemical identification method. The results of biochemical analysis of bacterial strains are given in Table 3.

Table 2. Morphological and cultural characteristics of bacteria isolates from river water samples

Bacteria Sample	Gram staining	Shapes	Elevation	Color	Odour
A	+ve	Rod	Flat to raised	Opaque	Mousy smell
B	-ve	Rod	Raised	Blue-green	Fruity
C	-ve	Rod	Convex	Lack of sheen	Fecal odour

Table 3. Biochemical characteristics of isolated bacteria

Bacteria sample	Biochemical Identification Test					
	Catalase	Oxidase	Indole	Citrate	Voges Proskauer	Methyl Red
A	-	-	-	+	+	-
B	+	+	-	+	+	-
C	-	-	-	+	+	-



Bacteria A Bacteria B Bacteria C

Fig. 2. Gram staining image for the bacteria

3.3 Molecular Identification

The molecular identification techniques are accurate, rapid and sensitive for the study of specific pathogenic bacteria. These tools can be used to analyse the water sample accurately [11]. DNA extraction of the selected bacteria was conducted then the DNA samples were proceeding for PCR test to amplify the DNA which was done by CCB. Amplified PCR products were sent for subsequent sequencing by DNA sequencer (Applied Biosystem). These complete sequences were then probed using NCBI BLAST program. The sequence retrieved from NCBI data base giving the closet match in pair wise. BLAST were identified the bacteria strain.

Basic Local Alignment Search Tool (BLAST) were identified the bacteria strain. Table 4 shows the first top hits (best matches) for the types of bacteria. This sequence alignment shows how well the query sequence match with the database sequence. From the tables, the max score is the score for the longest matching sequence meanwhile total score is the sum of the sequences that match. From Table 4, *Bacillus cereus* and *Pseudomonas aeruginosa* show the highest percent of identify which is 100% compared to other hits from the database while 98% for *Enterobacter sp*. The percent of identity give the meaning that percent of similarity between the query and the subject sequences over the length of the coverage are. Smaller E Value means the higher chance that the similarity is real.

Table 4. First top hits (Best matches) of significance alignment for bacteria

Bacteria	Description	Max Score	Total score	Query Cover	E Value	Ident	Accession
A	*Bacillus cereus* ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	2545	2545	100%	0.0	100%	NR_074540.1
B	*Pseudomonas aeruginosa* strain DSM 50071 16S ribosomal RNA gene, partial sequence	2518	2518	100%	0.0	100%	NR_117678.1
C	*Enterobacter sp.* 16S ribosomal RNA gene, complete sequence	2462	2462	98%	0.0	99%	NR_042349.1

Results in Table 5 listed the identified bacteria from Kerian River water. According to the 16S rDNA sequencing and blasting have shown that the identified pathogenic bacteria include *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* DSM 50071, *Enterobacter sp.*

Table 5. Identified bacteria in Kerian River water

Bacteria	Identified Bacteria
A	*Bacillus cereus* ATCC 14579
B	*Pseudomonas aeruginosa* DSM 50071
C	*Enterobacter ludwigii* EN-149

Bacillus cereus is a Gram-positive, rod-shaped, aerobic, facultatively anaerobic, motile, beta hemolytic bacterium commonly found in soil and food. Water is an environment where spores and vegetative cells of *Bacillus spp*. are expected to be present. Spores present in water may pass through simple drinking water treatment, such as filtration and disinfection (chlorination), and then unexpectedly introduce *Bacillus spp.* into food products [12].

The pathogenicity of *B. cereus*, whether intestinal or non-intestinal, is intimately associated with tissue-destructive/reactive exoenzyme production. In addition to food poisoning, *B. cereus* causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals [13]. While for *Pseudomonas aeruginosa*, the organism was a gram-negative bacterium, one or more polar flagella providing motility, aerobic, non-spore-pore forming, catalase positive and Oxidase positive.

It is an opportunistic pathogen, meaning that it exploits break in host defense to initiate an infection. It has become increasingly recognized as an immerging opportunistic pathogen of clinical relevance. *P. aeruginosa* is the type species of the genus Pseudomonas [14]. Its occurrence in drinking water is probably related more to its ability to colonize biofilms in plumbing fixtures (i.e., faucets, showerheads, etc.) than its presence in the distribution system or treated drinking water. *P. aeruginosa* can survive in deionized or distilled water.

It can cause endocarditis, osteomyelitis, pneumonia, urinary tract infections, gastrointestinal infections, and meningitis, and is a leading cause of septicemia. *P. aeruginosa* is a major cause of folliculitis and ear infections acquired by exposure to recreational waters containing the bacterium [15]. It causes urinary tract infection, respiratory system infection, dermatitis, soft tissue infection, bacteremia bone and joint infection gastrointestinal infection and a variety of systemic infection particularly in a patient with a severe burn, in cancer and AIDS patient who are immunocompromised [16].

Enterobacter is a genus of common gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria of the family *Enterobacteriaceae* [17]. Several strains of these bacteria are pathogenic and cause opportunistic infections in immunocompromised (usually hospitalized) hosts and in those who are on mechanical ventilation. The urinary and respiratory tracts are the most common sites of infection [18]. It does not belong to the *fecal coliforms* (or thermotolerant coliforms) group of bacteria, unlike *Escherichia coli*, because it is incapable of growth at 44.5 °C in the presence of bile salts. *Enterobacter spp*. has emerged as pathogens responsible for hospital-acquired infections.

5. Summary

The study clearly indicates that Sungai Kerian is contaminated by bacteria those are present in all sort of environmental conditions that allows human involvement. According to the 16S rDNA sequencing and blasting have shown that the identified pathogenic bacteria include *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* DSM 50071, *Enterobacter ludwigii* EN-149. For the water quality monitoring, Sungai Kerian (Kerian River) shows that it has high turbidity, TSS and color especially during the rainy season. pH for the river water was found in range 5.24-6.89 while temperature was recorded in range 30-25.31°C. BOD and COD were in range 6.8-2.0 mg/l and 31.0-8.0 mg/l respectively.The results from this study will be initiated for the study of bacterial transport during the RBF process.

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