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FULL PAPER

The characterization of Enterobacteriaceae and Pseudomonadaceae isolated from natural salt licks in Sarawak Borneo

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Abstract

Salt lick sites are popular hot spots for hunting among the rural community because many animals are attracted to the sites for mineral licking. The animals roaming freely within the salt lick sites may contribute to the transmission of bacterial pathogens, especially through faecal contamination of the salt lick environments. This study aimed to isolate, identify and characterise bacteria from water and soil of salt lick sites and to determine if there is evidence of the transmission of bacteria between two salt lick sites located within the vicinity of Semait river in the NCR (native customary rights) land area of Long Selaan village, Upper Baram in Malaysian Borneo. Samples of soil (n = 8) and water (n = 7) from the two salt lick sites were collected and plated on Eosine Methylene Blue Agar (EMBA) and MacConkey agar for the isolation of bacteria. Fifteen bacterial colonies were isolated and identified by Matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF-MS), 16S rRNA and API® 20E kit. The bacterial isolates were subjected to $(GTG)_5$-PCR and antimicrobial analyses. The antibiotics used were Ampicillin (AMP), Tobramycin (TOB), Imipenem (IPM), Amikacin (AK), Trimethoprim-sulfamethoxazole (SXT), Tetracycline (TE), Chloramphenicol (C), Ceftazidime (CAZ), Nitrofurantoin (F) and Norfloxacin (NOR). The result of this study shows the presence of two families of bacteria in the salt lick samples which were Enterobacteriaceae and Pseudomonadaceae. The dendrogram plotted based on $(GTG)_5$-PCR shows a close genetic relatedness among the bacteria from water and soil as well as in between and within the two salt lick sites. The resistance index of the bacteria from the salt licks ranged from 0.0 to 0.6. This study suggests the potential risk of antimicrobial-resistant bacteria with diverse transmission pathways associated with the salt licks within the area.

1. Introduction

Salt lick, also known as mineral lick, occurs naturally in many parts of Borneo where much terrestrial wildlife depends on its mineral concentration. The salt deposits in the site usually contain essential mineral elements such as phosphorus, sodium, calcium, iron, zinc, and trace elements which are required for supplementing the diet of the animals (Mahaney *et al*., 1997; Klaus *et al*., 1998; Brightsmith and Munoz-Najar, 2004; Voigt *et al*., 2008; Sim *et al*., 2020). The mineral composition and concentration of salt licks have been reported to be varied depending on sites (Ayotte *et al*., 2006). A study conducted on salt lick composition in several salt lick sites in Malaysian Borneo reported that the mineral range of Na was from 0 to 136 mg/kg, K from 454 to 1834 mg/kg, Ca from 0 to 1017 mg/kg and Mg from 450 to 3627 mg/kg (Sim *et al*., 2020).

Generally, salt lick can be categorized into two types which are the artificial salt lick and the naturally occurring salt lick. Artificial salt lick is normally in the

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form of blocks of salt that farmers place in pastures for livestock to lick for them to get minerals supplements (Chong *et al*., 2005; Magintan *et al*., 2015). Naturally occurring salt licks are common, they provide essential elements such as phosphorus and other biometals including sodium, calcium, zinc, iron and trace elements (Mahaney *et al*., 1997; Klaus *et al*., 1998; Brightsmith and Munoz-Najar, 2004; Matsubayashi *et al*., 2007; Voigt *et al*., 2008; Moe, 2011; Sim *et al*., 2020). These minerals are important for animals for the development and maintenance of their bones, muscles and other growth requirements. Dry weather exposes salty mineral deposits that draw animals from far away to come to the salt lick to get their needed nutrients (Stockstad, 1950; Stockstad *et al*., 1953; Dalke *et al*., 1965; Fraser and Reardon, 1980). Animal footprints have been consistently captured at these natural deposits substantiating their importance to the animals within a particular area. The gathering of animals within the salt lick sites has made the sites become hot spots for hunting by the rural community for wild animal meat for protein and medicine (Blake *et al*., 2011; Kirupaliny and Mohd-Azlan, 2012; Ka-Yi and Mohd-Azlan, 2018; Ka-Yi and Moh-Azlan, 2020).

Enterobacteriaceae is a large family of bacteria that falls under the Gram-negative strains. The bacteria within this family are commonly found in the gastrointestinal tract of humans and animals, water and soil and the bacteria are known to be facultative anaerobes. Janda and Abott (2006) reported that while many of them are harmless, some are pathogenic and can be very harmful to humans and animals. The examples of bacteria under the family Enterobacteriaceae include Escherichia, Enterobacter, Salmonella, Klebsiella, Shigella, Serratia, Proteus and Citrobacter. Pathogenic Enterobacteriaceae have been known to cause diseases like gastrointestinal diseases, meningitis and pneumonia (Guentzel, 1996).

Many animals especially herbivores regularly visit salt lick to consume soil for their nutrient and mineral sources. Some animals require minerals at these sites, not for nutrition, but to eliminate the effects of certain secondary compounds present in the arsenal of plant defences against herbivory (Viogt *et al*., 2008). However, the animals also discard their excretion waste while consuming the soils within the salt lick site (Ayotte *et al*., 2006). Hence, this makes the soil in the area becomes potentially contaminated with pathogenic bacteria, especially those present in the faeces of the animals (Ayotte *et al*., 2006). In addition, large mammals like deer and wild boar have been reported to enter the salt lick area more prevalent than other animals (Matsubayashi *et al*., 2007). Hunted wildlife, especially large mammals are normally dressed at the salt lick sites which could contribute to the contamination of the sites and the meat by faeces of animals from the salt lick environment. This could pose a health hazard to the public and concern for the safety of the animal's meat. $(GTG)_5$ PCR and antibiotic resistance have been successfully utilized for the characterization of Enterobacteriaceae from river water environments (Lihan *et al*., 2017). This study was aimed to investigate the characteristics and also the possible transmission pathways of bacteria in salt lick sites in Upper Baram, Sarawak, Malaysian Borneo.

2. Materials and methods

2.1 Study area and sampling

This study was conducted at two salt lick sites located within the vicinity of Semait river within the Native Customary Right Land (NCR) area belonging to Long Selaan community in Upper Baram, Malaysian Borneo. Fifteen samples of water (n = 7) and soil (n = 8) were collected in April 2016 from the salt lick sites in sterile bottles and stomacher bags, respectively. The locations of the salt licks were Semait Hilir (3° 4'56.15"N 115°5'42.53"E) and Semait Hulu (3° 4'48.51"N 115°5'36.49"E) as described in an earlier report by Sim *et al*. (2020).

The samples were labelled according to salt lick sites and also the type of samples (water or soil) where they were collected. For example, a sample labelled SSL1 S11 corresponds to Semait Salt Lick site 1 (SSL1) which indicate the sample was collected from the first site (Sungai Semait Hilir), whereas S11 stands for soil sample 1 with bacterial colony no. 1.

2.2 Isolation and bacterial growth media

The samples of water and soil from the two salt lick sites were serially diluted and then plated on Eosin Methylene Blue agar (EMBA) (Difco Laboratories, MD, USA) and MacConkey agar (Oxoid Ltd., England, United Kingdom). Bacteria growing on the plates were randomly picked, purified on nutrient agar and then stored in nutrient agar (Scharlab, Barcelona, Spain) slant.

2.3 DNA extraction

DNA extraction was conducted according to the boiled cell method (Samuel *et al*., 2011; Kathleen *et al*., 2014). Briefly, overnight bacterial culture was collected into microcentrifuge tubes and then centrifuged for five min at 10,000 rpm to collect the pellet. The supernatant was discarded and the pellet was collected. A volume of 500 µL of distilled water was added into the microcentrifuge tubes and then the pellet was dissolved.

The tubes containing the bacterial cells were boiled in a heat block at 100°C for 10 mins. The tubes were then quickly placed in ice for 5 mins and then centrifuged at 10,000 rpm for 10 mins. The supernatant was used as a DNA template for the PCR analysis.

2.4 Identification of bacterial isolates

2.4.1 MALDI-TOF-MS

Matrix-assisted laser desorption ionization time-of-flight mass spectrophotometry (MALDI-TOF-MS) was used for the identification of the isolates by using the mass spectrophotometry technique. Prior to the transfer of the bacterial samples on the plate, a purified matrix solution was prepared by using the Bruker Matrix HCCA© method (Bruker Daltonik GmbH, Germany). To prepare the purified matrix solution, 250 µL of Sigma -Aldrich (19182) standard solvent (50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid) was added to a screw cap tube containing Bruker HCCA© (α-Cyano-4 -hydroxycinnamic acid) with a final concentration of 10 mg/ml. The screw cap tube was then closed to minimize solvent evaporation. The screw cap tube was shaken and vortexed to dissolve the Bruker HCCA© at room temperature.

For the sample preparation, the "Direct Transfer" sample preparation method (Bruker Daltonik GmbH, Germany) was used. Firstly, a single fresh bacterial colony from the isolate was transferred as a thin film directly onto a MALDI target plate position. Then, the sample spot was overlaid by 1 µL of Bruker HCCA© solution. The matrix-overlaid sample spots were allowed to dry at room temperature. After homogenous preparation was finally observed on all sample spots on the plate, MALDI-TOF-MS measurement was performed. At the end of the measurement, the results were shown on the computer by using the MALDI Biotyper Realtime Classification™ software by comparing with the standard Bruker Database.

2.4.2 Identification with 16S rRNA sequencing

The 16S rRNA sequencing was used to identify some of the isolates of bacteria that were unable to be identified by the MALDI-TOF-MS method earlier. Prior to the sequencing step, the polymerase chain reaction (PCR) was conducted. The PCR mastermix was prepared by adding the following components: 10 µL of 5× Green GoTaq® Flexi buffer (Promega, USA), 6 µL of 25 mM magnesium chloride ($MgCl_2$), 3 µL of 10 mM deoxyribonucleotide phosphates (dNTPs), 1 µL of 10 µM forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG-3'), 1 µL of 10 µM reverse primer 519R (5'- GWATTACCGCGGCKGCTG -3'), 8 µL of ultra-pure water (ddH_2O) and 1 µL of GoTaq® Flexi DNA Polymerase (Promega, USA). The mastermix was swirled then distributed into 20 µL volume before a 5 µL DNA sample was added. The PCR mixture was run with the following cycles: 10 mins of initial denaturation at 95°C, denaturation for 30 s at 94° C, annealing for 1 min at 55°C, extension at 72°C for 90 sec and final extension at 72°C for 10 mins. The PCR was run for a total of 26 cycles. The purified DNA products were sent to Apical Scientific Laboratories Sdn. Bhd. (Malaysia) for sequence analysis. The search of database similarity for the nucleotide sequences was carried out with Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI), website (https:// blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4.3 API® 20 E

The Analytical Profile Index® or API® 20E kit is an identification system for Enterobacteriaceae and other non-fastidious Gram-negative rods (bioMérieux, France). This kit was used to identify two bacteria strains that were unable to be identified by MALDI-TOF-MS and 16S rDNA Sequencing methods. The strip was prepared for each of the isolates. An incubation box (with tray and lid) was prepared and about 5 mL of water was distributed into the honeycomb wells of the tray to create a humid atmosphere. The tray was labelled with the name of the sample at the elongated flap of the tray. The strip was then removed from its packaging and was placed in the incubation box. After that, the inoculum was prepared for each of the bacterial isolates. Two ampules of API® Suspension Medium (5 mL) were opened and a single fresh (18 hr old) well-isolated colony of each bacteria was emulsified into the respective ampules of the API® Suspension Medium to achieve a homogenous bacterial suspension. The suspensions were then used in the inoculation of the strip. Using a pipette, both tube and cupule of the tests CIT, VP and GEL were filled with the bacterial suspension while for the other tests, only the tubes were filled with the bacterial suspension. The incubation boxes were then closed and incubated at 37°C for 24 hrs. The API20E online database which can identify bacteria to species level was utilised for the identification of the bacteria isolated in this study.

2.5 $(GTG)_5$-PCR and gel electrophoresis

$(GTG)_5$-PCR was conducted to amplify $(GTG)_5$ repetitive elements in the whole genome of the bacterial isolates. The PCR was conducted according to Matsheka *et al.* (2006). The PCR mastermix was prepared by adding the following reagents: 5 µL of 5× Green GoTaq® Flexi buffer (Promega, USA), 3 µL of 25 mM magnesium chloride ($MgCl_2$), 0.5 µL of 10 mM

deoxyribonucleotide phosphates (dNTPs), 0.5 µL of 10 µM $(GTG)_5$ primer (5'-GTGGTGGTGGTGGTG-3'), 10.7 µL of ultra-pure water (ddH_2O) and 0.3 µL of GoTaq® Flexi DNA Polymerase (Promega, USA). The master mix was distributed and a volume of 5 µL of DNA sample was added. A negative control reaction tube was included where 5 µL of sterile distilled water was added to the mixture. The samples were then placed in the PCR machine with the following cycles: 2 mins of initial denaturation at 95°C, 1 min of denaturation at 95° C, 1 min of annealing at 50°C, 1 min of extension at 72° C, then the addition of another 5 mins of final extension at 72°C. Overall, 30 cycles were included in the PCR amplification. After PCR was completed, agarose gel electrophoresis (AGE) was conducted to separate DNA for visualization and purification (Addgene, MA, USA). Briefly, the solidified agarose gel was placed into the gel box of the electrophoresis system. The gel box was filled with 1x TBE buffer until the gel was covered. A molecular weight (1 kb) ladder was loaded as a reference marker into the first lane of the gel. Then the samples were loaded in the subsequent wells of the gel. The gel (1.2% w/v) was run at 80 V for about 105 mins. Once it was done, the gel was carefully removed from the gel box and stained with EtBr and then later observed under UV light (Addgene, MA, USA). The DNA profiles obtained from $(GTG)_5$ PCR were at first analysed manually by looking at the presence and absence of bands at a particular size on the gel photograph. The absence of a band was keyed as zero "0" and the presence of the band was keyed as one "1" using the RAPDistance V. 1.04 software.

2.6 Antibiotic sensitivity test

An antibiotic sensitivity test was carried out to identify the susceptibility of the isolates towards certain antibiotics. Briefly, bacterial was swabbed on the entire surface of the Mueller-Hinton agar. Then, five disks of antibiotics were placed on each agar at an equal distance from each other. A total of 10 types of antibiotics were tested for each isolate according to the Performance Standards for Antimicrobial Disk Susceptibility Tests determined by The Clinical Laboratory Standards Institute (CLSI, 2012) for the antibiotic sensitivity test for Enterobacteriaceae. The antibiotics used were Ampicillin (AMP), Tobramycin (TOB), Imipenem (IPM), Amikacin (AK), Trimethoprim-sulfamethoxazole (SXT), Tetracycline (TE), Chloramphenicol (C), Ceftazidime (CAZ), Nitrofurantoin (F) and Norfloxacin (NOR). After the addition of the antibiotics, the agar plates were incubated at 37°C for 24 hrs. The diameter of each inhibition zone was measured in accordance with the standards provided by Performance Standards for Antimicrobial Disk Susceptibility Tests (CLSI, 2012).

3. Results

3.1 Growth on media culture

The growth of the bacterial isolate on EMBA after 24 hrs of incubation at 37°C is shown in Figure 1. The growth of *E. coli* is seen as green-coloured colonies on the agar plate in Figure 1 (A). The growth of other lactose fermenter bacteria is shown on plate agar in Figure 1 (B). Meanwhile, Figure 2 shows the growth of the bacteria on MacConkey agar after 24 hrs of incubation at 37°C. The colour has changed from pink to yellow indicating the presence of lactose fermenter bacteria. This colour change is due to the decreasing pH of the agar due to lactic acid production resulting from the fermentation of lactose by the bacteria.

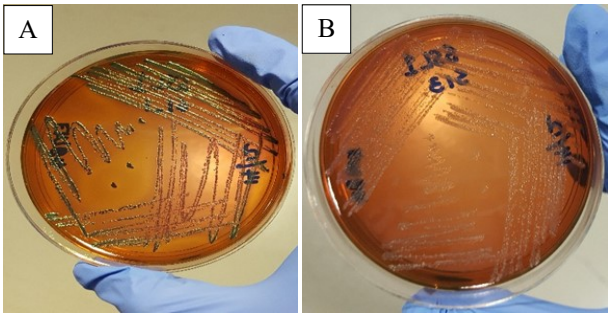

Figure 1. Growth of bacteria on EMBA. Green colonies were reflected on the agar plates indicate the growth of *E. coli* (A). Purple colonies indicated the presence of other lactose fermenters Enterobacteriaceae (B).

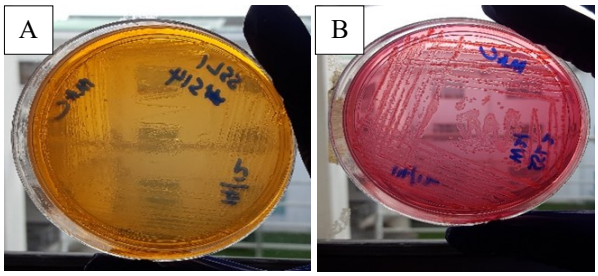

Figure 2. The growth of bacteria on MacConkey agar after 24 hrs of incubation at 37°C. The colour changed from pink to yellow (A) indicating the presence of lactose fermenter bacteria. This colour change is due to the decreasing pH of the agar resulting from the fermentation of lactose by the bacteria. Certain bacterial growth did not change the colour of the agar (B).

3.2 Identification of bacterial isolates

The identity of the 15 bacterial isolates after the identification with MALDI-TOF-MS, 16S rRNA sequencing and API® 20 E techniques is shown in Table 1. Using MALDI-TOF-MS, 8 of the isolates were successfully identified as *E. coli* (2 isolates), *Enterobacter cloacae* (3 isolates), *Klebsiella oxytoca* (1 isolate), *Enterobacter asburiae* (1 isolate) and *Pseudomonas putida* (1 isolate). Seven isolates were not detected by the MALDI-TOF-MS thus indicated as "not reliable identification" or "no peaks found". These bacterial isolates were then subjected to 16S rRNA

Table 1. Species identification for all fifteen isolates

No	Isolates	Species identified	Identification method
1	SSL1 S11	*Escherichia coli*	MALDI-TOF
2	SSL1 S12	*Raoultella ornithinolytica*	16S rRNA
3	SSL1 S13	*Klebsiella oxytoca*	MALDI-TOF
4	SSL1 S14	*Enterobacter asburiae*	MALDI-TOF
5	SSL1 W11	*Pseudomonas monteilii*	16S rRNA
6	SSL1 W12	*Enterobacter cloacae*	MALDI-TOF
7	SSL1 W13	*Pseudomonas azotoformans*	16S rRNA
8	SSL1 W14	*Enterobacter cloacae*	MALDI-TOF
9	SSL1 W22	*Enterobacter cloacae*	MALDI-TOF
10	SSL1 W23	*Pseudomonas putida*	API20E
11	SSL2 S21	*Pseudomonas monteilii*	16S rRNA
12	SSL2 S22	*Enterobacter cloacae*	MALDI-TOF
13	SSL2 S23	*Escherichia coli*	MALDI-TOF
14	SSL2 S24	*Enterobacter cloacae*	API20E
15	SSL2 W21	*Escherichia coli*	16S rRNA

SSL1: Salt Lick Site 1, SSL2: Salt Lick site 2, S: soil sample, W: water sample.

sequencing. The 16S rRNA sequencing identified five isolates as *Raoultella ornithinolytica* (1 isolate) and *Pseudomonas monteilii* (2 isolates), *Pseudomonas azotoformans* (1 isolate) and *Escherichia coli* (1 isolate). The remaining two isolates were further analysed using API® 20E kit and both of the isolates were identified as *Enterobacter cloacae*. Based on the identification tests, all of the bacteria identified falls into two families of Gram-negative which were Enterobacteriaceae and Pseudomonadoceae.

3.3 $(GTG)_5$-PCR

$(GTG)_5$-PCR was conducted to characterise all of the isolates (Matsheka *et al.*, 2006). Figure 3 shows the DNA bands of the PCR products of the fifteen isolates. From the bands, a dendrogram was generated. Figure 4 shows the dendrogram generated by using RAPDistance V. 1.04 software (Armstrong *et al.*, 1994). In general, based on the dendrogram, the isolates were grouped according to their genus. For example, Enterobacter, Escherichia, Pseudomonas were grouped into their own clusters. The bacteria species, despite being isolated from different sources (soil and water) and different locations of salt licks, these isolates were grouped into the same sub-clusters. For instance, some of the isolates from soil and water samples were grouped in the same

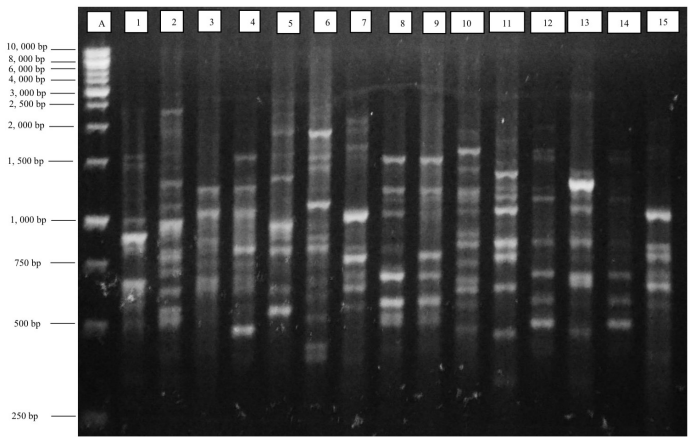

Figure 3. PCR bands of all 15 bacterial isolates. Lane A was the 1kb DNA ladder (Promega, USA) while Lane 1 until Lane 15 contained the PCR products of the isolates of SSL1 S11, SSL1 S12, SSL1 S13, SSL1 S14, SSL1 W11, SSL1 W12, SSL1 W13, SSL1 W14, SSL1 W22, SSL1 W23, SSL2 S21, SSL2 S22, SSL2 S23, SSL2 S24, SSL2 W21. The bands were used to generate dendrogram for the characterisation of the isolates.

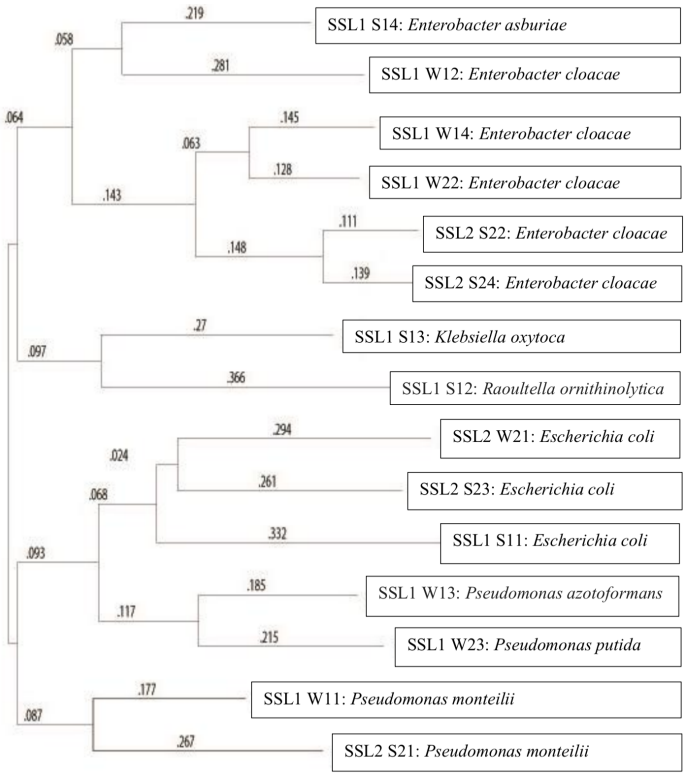

Figure 4. Dendrogram generated from the PCR profiles. From the dendrogram, the isolates from the same genus were grouped in the same cluster. Some isolates of the same species from the water and soil samples were also shown to be in the same cluster such as *E. coli* from SSL2 W21 and SSL2 S23.

cluster such as isolates SSL2 S23 and SSL2 W21.

3.4 Antibiotic sensitivity test

A total of 15 isolates were examined for their susceptibility to antimicrobial agents. The isolates were tested against ampicillin (AMP), tobramycin (TOB), imipenem (IPM), amikacin (AK), trimethoprim-sulfamethoxazole (SXT), tetracycline (TE), chloramphenicol (C), ceftazidime (CAZ), nitrofurantoin (F) and norfloxacin (NOR). *E. coli* strain ATCC 25922 was used as quality control for the standardization of the test. Figure 5 shows the agar plate of inhibition zone for each antibiotic after 24 hr incubation at 37°C. Table 2 summarizes the susceptibility, intermediate and resistance of the bacteria towards the antibiotics. Ten out of fifteen isolates were found to be resistant to more than three antibiotics tested. The antibiotic that most isolates (93.33%) were resistant to was ampicillin (AMP), while 100% of the isolates were susceptible to amikacin (AK).

MAR index was calculated on each isolate by using a formula described by Krumperman (1983) which is *y/x* where *y* represents the total number of resistance displayed by the isolates and *x* represents the total number of antibiotics that were tested against the isolates. The value 0.2 of MAR index differentiate between the low and high risk of contamination (Christopher *et al.*, 2013) where a value below 0.2 indicates a low risk while a value higher than 0.2 indicates a high risk of contamination (Krumperman, 1983). From Table 3, only isolates SSL1 S11 and SSL1 S12 show values below 0.2 whereas the rest show values higher than 0.2.

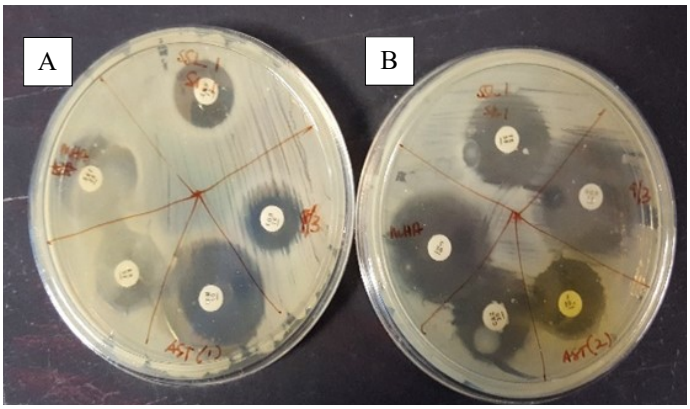

Figure 5. The isolates were cultured on Mueller Hinton agar for antibiotic susceptibility testing. There were 10 antimicrobial agents used and five disks were placed on each agar therefore two agar plates were utilised for each isolate. The first agar plate (A) shows the isolate SSL1 S11 tested with AMP, TOB, IPM, AK and SXT. The second agar (B) show the isolate SSL1 S11 tested with TE, C, CAZ, F and NOR. The inhibition zone is seen as the clear zone surrounding the antibiotics.

4. Discussion

This study shed light on the transmission of bacteria between water and soil within and between the salt lick sites as shown by the dendrogram plotted from the $(GTG)_5$-PCR profile (Figure 4). The contamination can be from various sources but one of the ways to find out is to look at the genetic relatedness of the bacteria obtained from the water and soil of that area. It is reflected in the dendrogram that there is a close genetic relation between the isolates from samples where they were collected and in different sample types such as water and soil. For example, the dendrogram in Figure 4 shows that *E. coli* isolate SSL2 W21 is closely related to the *E. coli* isolate SSL2 S23. Since isolate SSL2 W21 was isolated from the water sample and SSL2 S23 from the soil sample, there is a possibility that the *E. coli* strains were transmitted from the soil to the water or vice versa.

The free-roaming of animals within their home range and within the salt lick area may pose a high risk of transmission of pathogens, which exist in soil, to another environment such as the water. Gram-negative enteric pathogens, particularly from the Enterobacteriaceae family are more often acquired from the soil rather than from the water. According to Baumgardner (2012), any soil organism may potentially enter the water, thus the soil is often the origin of water-borne infections. In addition, the soil may be easily contaminated by animal waste through their faeces. Soil moisture and adsorption

Table 2. Number of isolates susceptible, intermediate and resistant to the selected antimicrobial agents

No.	Antimicrobial agent (µg per disk)	Number of isolates susceptible (%)	Number of isolates intermediate (%)	Number of isolates resistant (%)
1	Ampicillin [AMP] (10)	0 (0)	1 (6.67)	14 (93.33)
2	Tobramycin [TOB] (10)	9 (60)	2 (13.33)	4 (26.67)
3	Imipenem [IPM] (10)	8 (53.33)	3 (20)	4 (26.67)
4	Amikacin [AK] (30)	15 (100)	0 (0)	0 (0)
5	Trimethoprim-sulfamethoxazole [SXT] (1.25/23.75)	4 (26.67)	0 (0)	11 (73)
6	Tetracycline [TE] (30)	8 (53.33)	3 (20)	4 (26.67)
7	Chloramphenicol [C] (30)	10 (66.67)	1 (6.67)	4 (26.67)
8	Ceftazidime [CAZ] (30)	5 (33.33)	3 (20)	7 (46.67)
9	Nitrofurantoin [F] (300)	3 (20)	1 (6.67)	11 (73.33)
10	Norfloxacin [NOR] (10)	13 (86.67)	2 (13.33)	0 (0)

Table 3. Multiple antibiotic resistance (MAR) index, antibiotic resistance profile and the number of resistances of each isolate

No.	Isolates	Source	Antibiotic resistance profile	Number of resistances	MAR Index
1	SSL1 S11	Soil	None	0	0
2	SSL1 S12	Soil	AMP	1	0.1
3	SSL1 S13	Soil	AMP-SXT-C-CAZ- F	5	0.5
4	SSL1 S14	Soil	AMP-SXT-CAZ-F	4	0.4
5	SSL1 W11	Water	AMP-SXT-C-F	4	0.4
6	SSL1 W12	Water	AMP-SXT-CAZ-F	4	0.4
7	SSL1 W13	Water	AMP-TOB-IPM-SXT-TE-F	6	0.6
8	SSL1 W14	Water	AMP-TOB-IPM-TE-F	5	0.5
9	SSL1 W22	Water	AMP-TOB-IPM-SXT-F	5	0.5
10	SSL1 W23	Water	AMP-SXT-F	3	0.3
11	SSL2 S21	Soil	AMP-SXT-TE-C-CAZ-F	6	0.6
12	SSL2 S22	Soil	AMP-SXT-TE-C-CAZ-F	6	0.6
13	SSL2 S23	Soil	AMP-SXT-CAZ	3	0.3
14	SSL2 S24	Soil	AMP-SXT-CAZ	3	0.3
15	SSL2 W21	Water	AMP-TOB-IPM-F	4	0.4

AMP: Ampicillin, SXT: Trimethoprim-sulfamethoxazole, C: Chloramphenicol, CAZ: Ceftazidime, F: Nitrofurantoin, TOB: Tobramycin, IPM: Imipenem, MAR: Multiple Antibiotic Resistance

to clay particles also promote the survival of enteric pathogens (Santamaria and Toranzos, 2003). Besides that, the soil is also capable to harbour *E. coli* O157:H7 due to its ability to replicate within *Acanthamoeba polyphaga*, a common soil protozoan (Barker *et al.*, 1999).

In the parameters of the water quality studies, there is certainly one or more indicators to determine whether the water is polluted or not. From the microbiological aspect, one of the indicators that the water is contaminated is the presence of coliform bacteria such as *E. coli* in the water sample (World Health Organization, 2017). Therefore, when *E. coli* is detected in the water samples, the water is deemed as contaminated with faecal and it also indicates the possible presence of pathogenic bacteria. From this study, water samples collected show the presence of *E. coli* beside other Enterobacteriaceae and Pseudomonas. When the water quality is known to be poor, it is important to know the source of contamination.

Some members of Enterobacteriaceae are normal flora of the gastrointestinal tract of humans and animals. While the bacteria may also be found in soil and water, some may impose a parasitic character and become pathogenic to humans. *E. coli* has become one of the most important model organisms and its characteristics from various sources have been closely studied (Radu *et al*., 1999; Cabello, 2006; Apun *et al*., 2011; Samuel *et al*., 2011; Ng *et al*., 2014). The majority of Enterobacteriaceae possess peritrichous fimbriae which assist in their adhesion to their hosts. The pathogenic groups produce endotoxins where these endotoxins will stay in the cell cytoplasm. They will then be released extracellularly upon the cell lysis in the host cellular system. One of the major conditions resulting from the release of endotoxins is endotoxic shock, which can lead to death in a short period of time (Ramachandran, 2014). In this study, other potentially pathogenic bacteria were also isolated from the salt lick samples such *Enterobacter cloacae*, *Klebsiella oxytoca*, and *Raoultella ornithinolytica*, which are known to impose harmful risk to humans (Krzymińska *et al*., 2009; Herzog *et al*., 2014; Hajjar *et al*., 2020).

An antibiotic sensitivity test was carried out to determine the susceptibility of bacterial strains isolated from the salt licks towards several antibiotics. The majority (93.33%) of isolates were resistant towards ampicillin (Amp) while about 86.67% of the bacteria were susceptible to norfloxacin (Nor). Since wild animals are not supposed to be in contact with antibiotics, one of the possible explanation for the high ampicillin resistance is probably due to the possession of mobile elements in the bacterial genome which has been reported everywhere including pristine ecosystems (Martinez *et al*., 2009). From the MAR index (Table 3), 86.67% of the isolates showed a MAR index value higher than 0.2. About 75% of isolates that originated from soil showed a high risk of contamination whereas 100% isolates from water showed a high risk of contamination. Krumperman (1983) described that bacteria which show the high-risk source of faecal contamination from the MAR index are potentially hazardous to humans. Hence, MAR index is often used in the identification of high-risk bacterial sources. From the MAR index data obtained from this study, it is shown that most of the antibiotic-resistant isolates were from the water samples. These data show that the bacteria

from the salt lick water are potential threats and may potentially be transmitted to wild animals and later to humans, especially to the community who are involved in hunting activities within the salt lick sites. According to Solomon *et al.* (2013), antibiotic-resistant bacteria in animal-based foods are a potential threat to health not only because the bacteria themselves can be transmitted but their resistance can be transferred to other bacteria as well. Thus, when these bacteria infect humans, it will be difficult to provide treatment unless the specific antibiotic that the bacteria are susceptible to is determined.

5. Conclusion

Based on this study, there is evidence of bacterial transmission from one salt lick to another and from the soil to the water or vice versa. Some potential pathogenic bacteria with multiple resistance characteristics which are known to impose harmful risks to the animals and humans were present in the salt licks. The findings of this study can be used to suggest a preventive measure, especially on the microbiological risk associated with activities in salt licks and also to improve the quality of the salt licks for the well-being of the animals and village communities who are depending on the salt licks. This study showed empirical evidence on the occurrence of potential pathogens which is of health concern in remote areas of Sarawak Borneo. This study also indicated the importance of managing hunting near salt lick as local communities often hunt wildlife in the area. Hunting is not allowed at the salt lick areas in Peninsular Malaysia and Sabah but such regulation does not apply to Sarawak. As such, future management prescription in Sarawak should consider the findings from this study.

Conflict of interest

The authors declare no conflict of interest.

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