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Original article

Isolation and polymerase chain reaction identification of bacteria from the 2014–2015 flood of Kota Bharu, Kelantan, Malaysia

Pwaveno Huladeino Bamaiyi[a,c], Mohd Sani Nani Izreen[a], Khatijah Mohamad[a], Badrul Hisham Nur Eizzati[a], Che Rostman Siti-Bainum[a], Mat Kamir Norsyamimi Farhana[a], Mohd Nor Mohd Norfaizull[a], Saeid Kadkhodaei[b], Goriman Khan Mohd Azam Khan[a]

[a]*Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Kota Bharu, Kelantan 16100, Malaysia*
[b]*Department of Agriculture Technology, Universiti Putra Malaysia, 43400, Malaysia*
[c]*Department of Public Health, School of Allied Health Sciences, Kampala International University, Uganda*

Background: The transmission of waterborne, foodborne, and airborne infections following flooding is common around the world. There is a need to study and understand the bacterial biodiversity of flood water during massive floods.

Objectives: To determine the 16S rRNA bacterial biodiversity of flood water that affected parts of Kota Bharu, Kelantan, Malaysia from December 2014 to January 2015.

Methods: We collected 31 water samples in 50 mL sterile containers from 6 different locations. Bacteria were cultured by inoculating into blood and nutrient agar using sterile swabs. Various bacteria were identified from the cultures that grew within 24–48 h, using colony morphology, differential/selective media, and biochemical tests. The isolated bacteria were identified using DNA Sanger sequencing and comparing with sequences at NCBI BLAST and SepsiTest BLAST up to species level, and sequences were deposited at GenBank. A 16S rRNA biodiversity chart was obtained. Sequences with low trace score (< 20) were removed, sequences were trimmed, capped (pair-wise assembled) and the 16S biodiversity was analyzed using a 16S biodiversity tool (Geneious version R8.1).

Results: The 16S biodiversity tool results revealed 22 genera of bacteria belonging to 12 families: *Moraxellaceae* (10%), *Aeromonadaceae* (8%), *Comamonadaceae* (13%), *Neisseriaceae* (2%), *Bacillaceae* 1 (16%), *Staphylococcaceae* (8%), *Bacillales Incertae Sedis* XII (3%), *Bacillaceae* 2 (3%), *Streptococcaceae* (2%), *Flavobacteriaceae* (2%), *Enterobacteriaceae* (25%), and *Pseudomonadaceae* (10%). Antibiotic susceptibility tests revealed *Klebsiella pneumoniae* of the family *Enterobacteriaceae* as the most resistant (71.4%) to all 7 antibiotics tested.

Conclusions: The isolation of some relatively new species of bacteria in the floodwater in Malaysia needs to be taken into consideration for epidemiological study of flood pathogens to determine future public health implications. Antibiotic resistance of bacteria should support choice of therapy.

Keywords: Bacteria, biodiversity, flood, isolation, Malaysia, PCR, 16S rRNA

Flood waters can be devastating, especially if proactive measures are not adequately taken ahead of time to mitigate the effects of the flood. In addition to the direct impact of flood water is the transmission of waterborne, foodborne, and airborne infection sequelae. Most of these infections are caused by pathogenic and opportunistic bacteria carried in the water from one location to another and include salmonellosis, leptospirosis, shigellosis, staphylococcus infections, burkholderiosis, vibriosis, and other infections [1, 2]. Different bacteria have been described in water from different sources worldwide, but there is paucity of data on bacteria in flood water during massive flood sessions.

Unexpected massive flood waters, that have defied meteorological forecasts, the worse in the

Correspondence to: Pwaveno Huladeino Bamaiyi, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, 16100, Kota Bharu, Kelantan, Malaysia. Present address: Department of Public Health, School of Allied Health Sciences, Kampala International University, Uganda. E-mail: pwaveno.hb@umk.edu.my; pwaveno.bamaiyi@kiu.ac.ug

history of Malaysia, hit the east coast of Peninsula Malaysia from 15th December 2014 to 3rd January, 2015 with Kelantan being the worst affected state. This great flood was estimated to have destroyed public property worth MYR (Malaysian Ringgit) 2.85 billion (about 814,285,714 USD); caused 25 deaths; affected 541,896 victims; with 2,076 houses destroyed, and a further 6,698 houses damaged; and 168 government healthcare facilities affected with an estimated MYR 380 million (108,571,429 USD) damage, and water levels rose 5–10 m above floodplain [3].

A study in Pahang state of Malaysia identified *Shigella flexneri*, *Escherichia coli*, and *Salmonella typhimurium* in the flood water [4], but generally there has not been any study to determine the bacterial biodiversity of the flood water and describe the antibacterial resistance pattern of flood bacteria in Malaysia. In Thailand, a study of flood water and tap water contaminated by floods in 2011 showed the presence of pathogenic bacteria such as *Shigella* sp., *Leptospira* sp., and *Vibrio cholerae* [5]. Mhuantong et al. [6] reported a predominance of proteobacteria in flood water and sediment samples collected from the great Thailand flood of 2011 with 21 different genera of bacteria found.

This study was conducted to elucidate the bacterial biodiversity of flood water, describe the antibiogram of some bacteria found in flood water, and postulate the possible public health impact of flood water using water samples taken from the Kelantan flood disaster in Malaysia.

Materials and methods

Study area and sampling procedure

Kota Bharu is the capital city of Kelantan state of Malaysia located on the east coast of Peninsula Malaysia at 6°8′N 102°15′E and close to the Thailand border with a population of about 491,237.

During the unexpected massive floods that hit the city, water samples were taken from 6 locations in the city as follows:

1. Taman Bendahara (Universiti Malaysia Kelantan city campus hostel area) with Global Positioning System (GPS) coordinates N06°09.809′E102°17.070′ and elevation of 12 m above sea level.
2. KampungTok Sadang (along Airport Road) with GPS coordinates N06°10.560′E102°17.115′ and elevation of 10 m above sea level.
3. Jalan Gajah Mati (Clock Roundabout) with GPS coordinates N06°07.508′E102°14.222′ and elevation of 23 m above sea level.
4. Kota Bharu mall surroundings with GPS coordinates N06°07.116′E102°14.396′ and elevation of 25 m above sea level.
5. Tesco Bus stop area with GPS coordinates N06°06.789′E102°13.757′ and elevation of 21 m above sea level.
6. Jalan Kuala Krai with GPS coordinates N06°06.285′E102°14.433′ and elevation of 26 m above sea level.

The GPS coordinates and elevation above sea level were measured using a Nuvi 255 WT receiver (Garmin, Lenexa, KS, USA). We collected 31 water samples in 50 mL sterile containers from the 6 different locations (5 samples from each of Taman Bendahara, Kampung Tok Sedang, Kota Bharu clock roundabout, Kota Bharu mall area, Tesco mall area, and 6 samples from Jalan Kuala Krai) for bacteriological analysis. Each water sample was inoculated into blood agar and nutrient agar using sterile swabs. The inoculated media were put in an incubator at 37°C for 24–48 hours. The various bacterial colonies were selected based on colony characteristics such as shape, color, hemolysis, and size. The selected colonies were subcultured on nutrient agar to obtain pure colonies. The pure colonies were subjected to the following biochemical tests: catalase, oxidase, triple sugar iron agar (TSI), citrate, urease, motility, indole, methyl red (MR), and Voges–Proskauer (VP) tests. Because biochemical tests were inadequate and not exhaustive, genomic DNA was further isolated from the pure colonies using a commercial genomic DNA extraction and purification kit (Vivantis, Selangor Darul Ehsan, Malaysia and Oceanside, CA, USA) following the manufacturer's instructions. Suitable oligonucleotide universal primers were used in a conventional polymerase chain reactions (PCR) to target 16S rRNA sequences of the unknown bacteria isolates.

Molecular analysis

The sequence of primers used was as follows: forward 5′-GGTGGAGCATGTGGTTTA-3′, reverse 5′-CCATTGTAGCACGTGTGT-3′ [7]. The expected product size was 287 bp. The PCR product was purified and sequenced for identification of isolated bacteria using DNA Sanger sequencing. Decipher software was used to check for any suspected chimeric sequences [8]. These sequences were compared with highly similar sequences at NCBI BLAST and SepsiTest BLAST for identification at

up to species level. The threshold for identification was set at >97% for species identification. Species were not reported for any sequences below the threshold. Sequences were deposited at the GenBank, NCBI, USA, and accession numbers were obtained (isolates and sequences for the rest of this article are referred to by their initial identity without the characters preceding this e.g. SUB882316 UMK1a1, will be referred to as simply 1a1). Two isolates (2d1 and 3d1) of interest to the authors because of their characteristic violet-to-black pigmented colonies were also confirmed using species specific PCR primers (*C. violaceum*) with the sequence recA-Viol-f (5′-AAGACAAGAGCAAGGCGCTGGC-3′) and revA-Viol-r (5′-TCGAAGGCGTCGTCGGCGAAC-3′) and PCR product size of 1047bp [9]. A literature search indicated that little or no report has been made about the following bacterial species at any time in Malaysia: *Acinetobacter ursingii, Curvibacter gracilis, Pseudomonas veronii, Wautersia numazuensis, Bacillus idriensis, Pectobacterium cypripedi, Bacillus luciferensis, Exiguobacterium mexicanum,* and *Pseudomonas vranovensis*.

Metagenomics analysis through 16s rRNA sequencing data

All 16s rRNA sequencing data were subjected to the following preprocessing procedure:

The quality control of the sequences was conducted by analyzing the trace files using SeqScanner version 1.0 (Applied Biosystems, Foster City, CA, USA). The leading vector, tailing and poor-quality (trace score <20) sequences were removed accordingly (file available on request).

The remaining sequences were trimmed at the 3′ or 5′ ends to remove low quality ends of the sequences because of the noise introduced by low quality regions (in Geneious version R8.1 (Biomatters, *http://www.geneious.com*, Kearse et al. [10]) as shown following:

For each sample, the paired reads (forward and reverse) were assembled through assembly in Geneious as shown following:

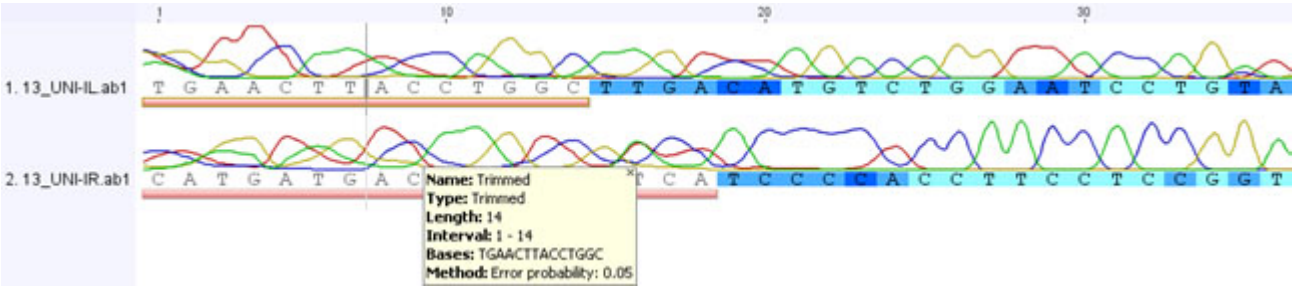

Figure 1. Trimming using Geneious version R8.1 (Biomatters, http://www.geneious.com, Kearse et al. [10])

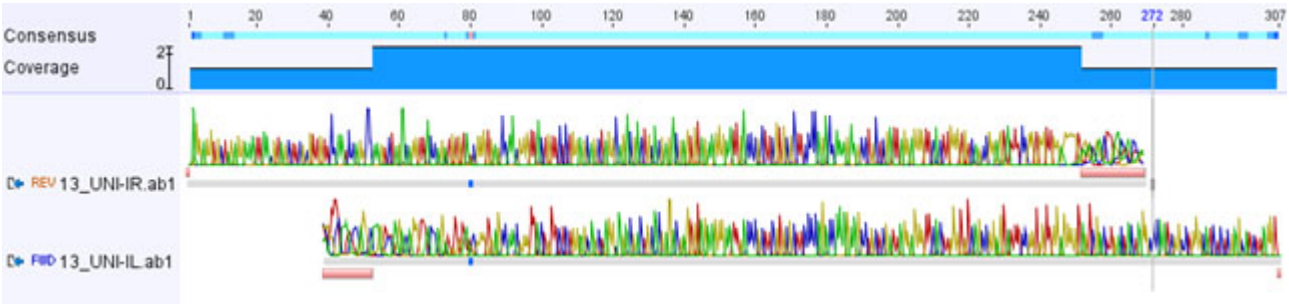

Figure 2. Capping using Geneious version R8.1 (Biomatters, *http://www.geneious.com*, Kearse et al. [10])

Species classification and relative abundance measurement using high throughput 16S rRNA amplicon sequencing data from environmental samples were performed using the cloud-based 16S rRNA biodiversity tool (Geneious version R8.1, (Biomatters, *http://www.geneious.com*, Kearse et al. [10])). The final verified sequences were submitted through the Geneious R8.1 bioinformatics platform to a distributed cloud compute resource. The data were then analyzed using the Ribosomal Database Project Database (RDP) Classifier [11]. The RDP Classifier assigns sequences derived from bacterial and archaeal 16S genes and fungal 28S genes to the corresponding taxonomy model using a 'Naïve Bayesian Classifier' for rapid assignment of rRNA sequences. The Geneious 16S biodiversity tool accurately assigned a taxonomy (in the range of domain to genus) along with a confidence-estimate for each sequence by comparing them to the RDP database and can only identify bacteria up to genus level to produce a chart [12]. The output was then displayed in a web browser using Krona [13], which produces an interactive html5 hierarchical graph of the bacterial diversity in the sample. Krona allows hierarchical data to be explored with zoomable pie charts. The difference between the total number of different bacteria identified for each location and sea level was calculated using a χ^2 test at 95% confidence level with SPSS Statistics for Windows version 22 (IBM Corp, Armonk, NY, US). The difference between percentages of bacterial families was elucidated using a χ^2 test at 95% confidence level, and the differences in the antibiotic susceptibility of the 17 bacterial isolates to 7 antibiotics was calculated using one-way ANOVA at 95% confidence level in SPSS version 22. Interpretation of zone of inhibition diameter (**Table 2**) was conducted according to standard procedures of the Clinical and Laboratory Standards Institute, 2007 (http://clsi.org/) and Benedict et al. [14]. Multiple antibiotic resistance was defined as resistance to 2 or more antibiotics tested. The effect size of differences observed in antibiotic sensitivity test was estimated using an η^2 test according to the interpretation of Cohen [15].

Results

Charts, Tables, and Figures can be requested by email from: pwaveno.hb@umk.edu.my; pwaveno.bamaiyi@kiu.ac.ug. PCR results on gel electrophoresis are shown (**Figures 3–7**). The chart (**Figure 8**) classified the samples into 3 bacterial domains, Proteobacteria (67%), Firmicutes (32%), and Bacteroidetes (1%). The chart reveals 12 families of bacteria: *Moraxellaceae* (10%), *Aeromonadaceae* (8%), *Comamonadaceae* (13%), *Neisseriaceae* (2%), *Bacillaceae* 1 (16%), *Staphylococcaceae* (8%), *Bacillales Incertae Sedis* XII (3%), *Bacillaceae* 2 (3%), *Streptococcaceae* (2%), *Flavobacteriaceae* (2%), *Enterobacteriaceae* (25%) and *Pseudomonadaceae* (10%) with $P = 0.03$ for the number of isolates belonging to the families. Please see: *https://16s.geneious.com/16s/results/cdee2e80-b6b7-4a5c-bf33-4e6760294758.html* for a version that can be manupulated. A literature search indicates that 10 of the 12 families of bacteria (83%) contained bacteria pathogenic to man and animals. The isolate sequences that passed the criteria, were successfully submitted to the GenBank, their accession numbers and species identified are listed in **Table 1**. There was no significant difference ($P > 0.05$) between the number of different species of bacteria isolated from the 6 locations studied. The susceptibility of 17 selected bacteria isolates to 7 different antibiotics was found to be significant with $P < 0.0001$ (**Tables 2** and **3**) with the bacteria having the highest susceptibility to gentamycin followed by tetracycline, and the lowest susceptibility was to penicillin G followed by ampicillin (**Figure 9**). The size of this difference was large ($\eta^2 = 0.236$). Some bacterial isolates in **Table 2** (isolates 4d2 and 5d1; 4e2 and 6a1y; 6b2 and 6c) did not show the same antibiotic sensitivity pattern even though they were same bacterial species. *Klebsiella pneumoniae* showed the highest resistance to multiple antibiotics while *Exiguobacterium mexicanum*, *Acinetobacter calcoaceticus*, and *Pseudomonas vranovensis* did not show resistance to any of the antibiotics tested; 82% of bacteria isolated showed resistance to one or more antibiotics while 76% of bacteria isolates tested showed multiple antibiotic resistance. Readers can use the `LINK` and click on the different classes, families and/or species to find out more details about the abundance of each category among the samples. This will provide a comprehensive illustration of the samples.

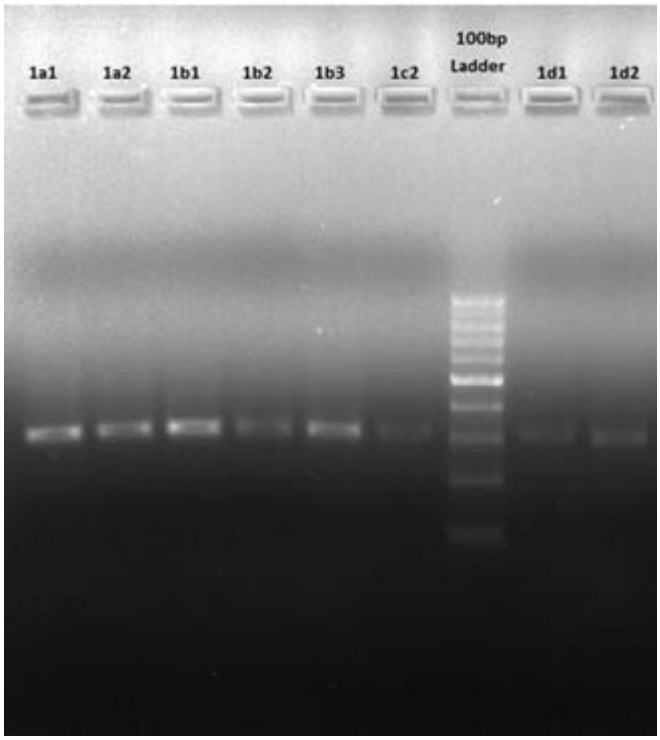

Figure 3. PCR results on gel electrophoresis are shown isolates 1a1 to 1d2

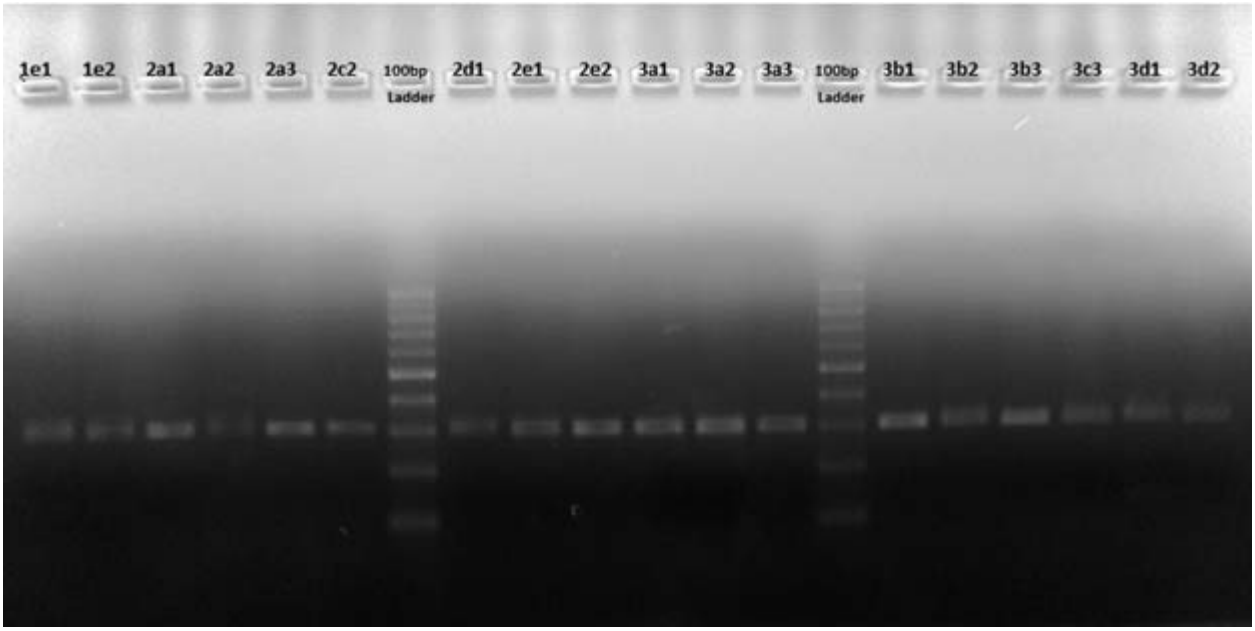

Figure 4. PCR results on gel electrophoresis are shown isolates 1e1 to 3d2

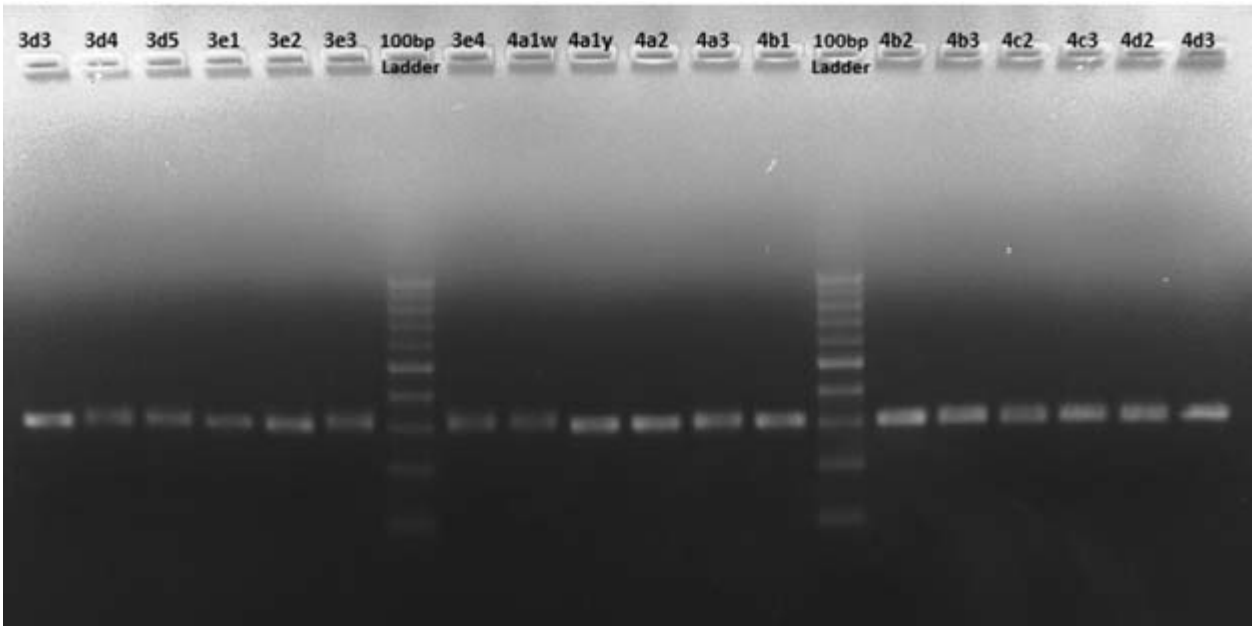

Figure 5. PCR results on gel electrophoresis are shown isolates 3d3 to 4d3

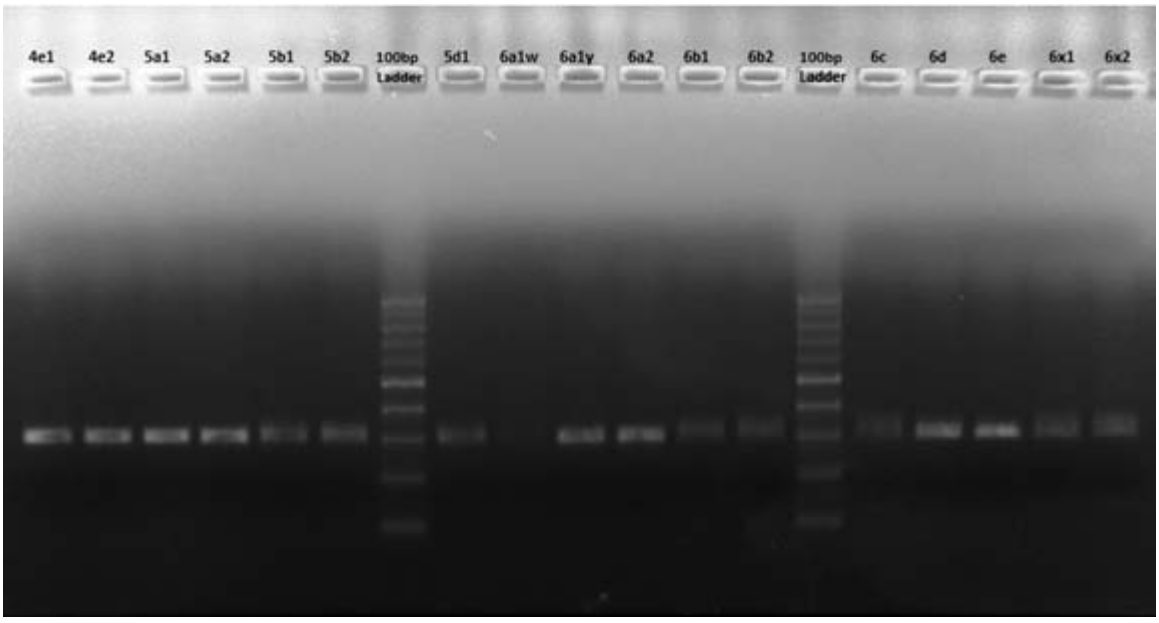

Figure 6. PCR results on gel electrophoresis are shown isolates 4e1 to 6x2

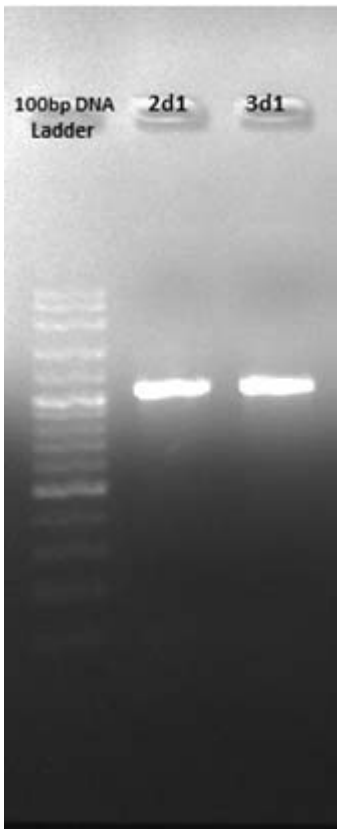

Figure 7. *Chromobacterium violaceum* confirmed by species specific PCR for isolates 2d1 and 3d1

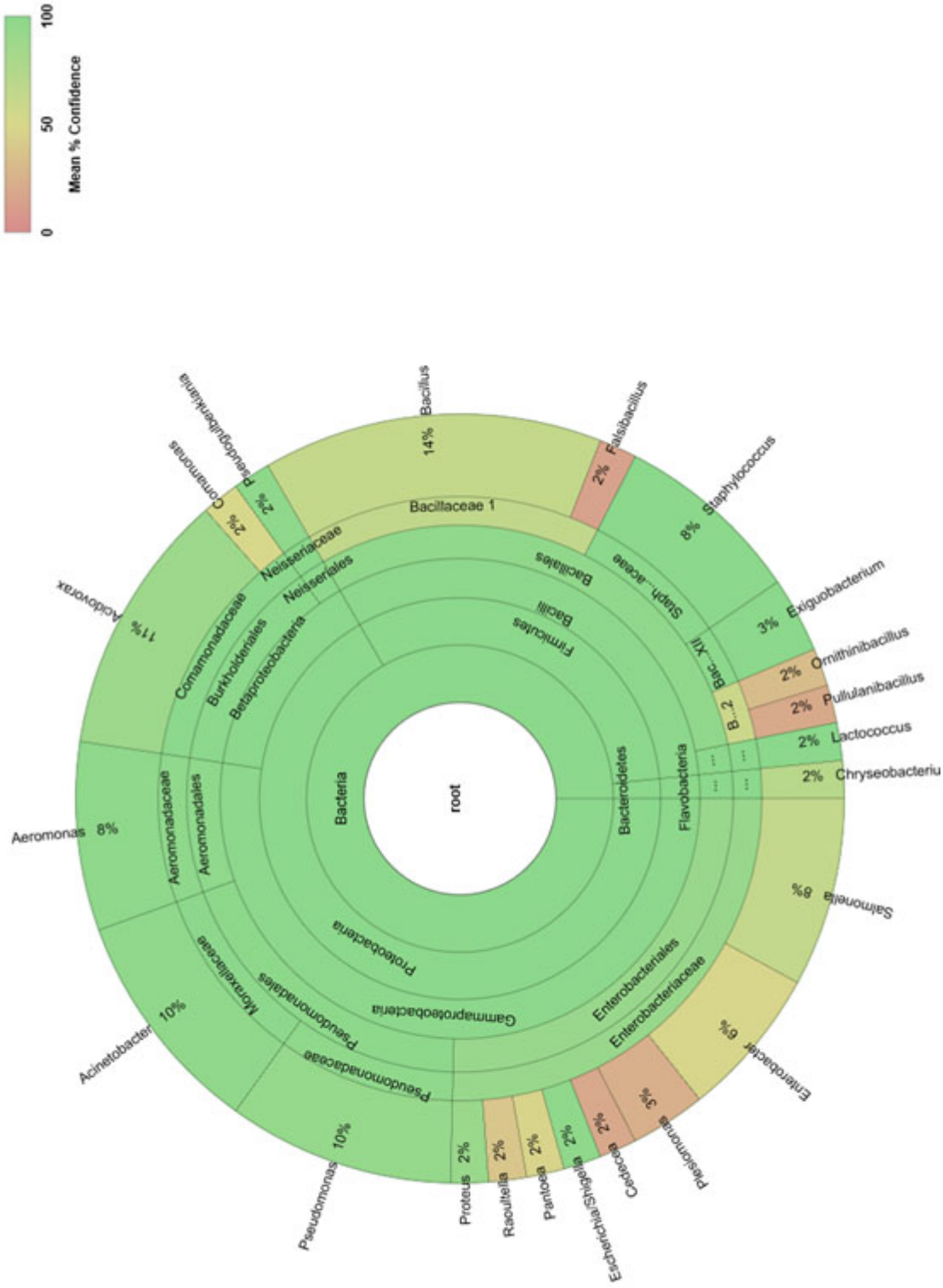

Figure 8. Preprocessed 16S rRNA amplicon data submitted to the Ribosomal Database Project Database Classifier (Wang et al. [11]) and visualized using the Krona Interactive Hierarchical Browser (Ondov et al. [13]).

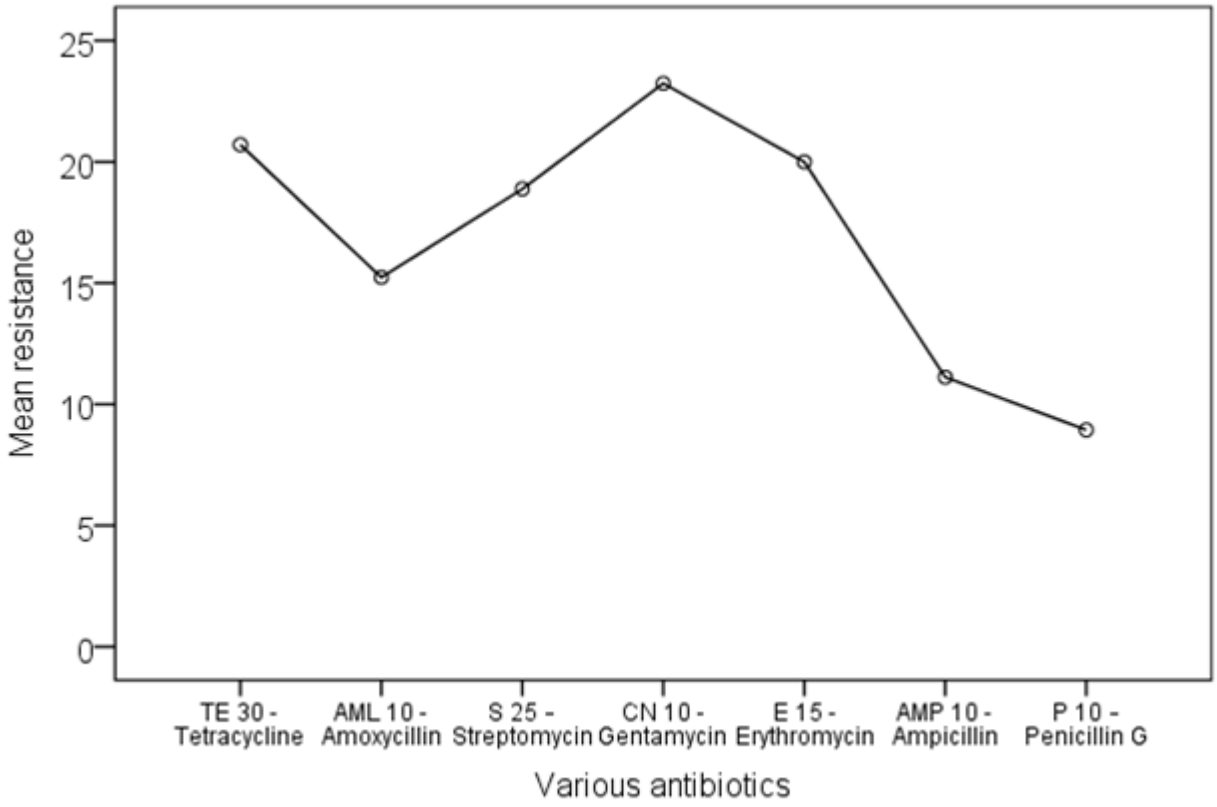

Figure 9. Antibiotic resistance plot showing the mean resistance of various antibiotics tested

Table 1. Bacteria Identified to species level from the flood and deposited at the GenBank

S/No.	Submission ID	Accession Number	Bacteria Identified
1	SUB882316 UMK1a1	KR027927	*Staphylococcus xylosus*
2	SUB882316 UMK1a2	KR027928	*Acinetobacter ursingii*
3	SUB882316 UMK1b1	KR027929	*Aeromonas aquariorum (A. dhakensis)*
4	SUB882316 UMK1b2	KR027930	*Bacillus pseudofirmus*
5	SUB882316 UMK1b3	KR027931	*Bacillus altitudinis*
6	SUB882316 UMK1c2	KR027932	*Acinetobacter radioresistens*
7	SUB882316 UMK1d1	KR027933	*Acinetobacter radioresistens*
8	SUB882316 UMK1d2	KR027934	*Lactococcus lactis subsp. Lactis*
9	SUB1092331 UMK1e1	KT731961	*Acidovorax caeni*
10	SUB1092331 UMK1e2	KT731962	*Acidovorax caeni*
11	SUB882316 UMK2a1	KR027935	*Staphylococcus xylosus*
12	SUB1092331 UMK2a2	KT731963	*Acidovorax caeni*
13	SUB882316 UMK2a3	KR027936	*Aeromonas veronii*
14	SUB1092331 UMK2d1	KT731964	*Chromobacterium violaceum*
15	SUB882316 UMK2e1	KR027937	*Staphylococcus xylosus*
16	SUB882316 UMK2e2	KR027938	*Aeromonas veronii*
17	SUB882316 UMK3a1	KR027939	*Acinetobacter junii*
18	SUB882316 UMK3a2	KR027940	*Klebsiella pneumoniae subsp. Rhinoscleromatis*
19	SUB882316 UMK3a3	KR027941	*Raoultella terrigena*
20	SUB882316 UMK3b1	KR027942	*Pseudomonas trivialis*
21	SUB1092331 UMK3b2	KT731965	*Curvibacter gracilis*

Table 1. (Con) Bacteria Identified to species level from the flood and deposited at the GenBank

S/No.	Submission ID	Accession Number	Bacteria Identified
22	SUB882316 UMK3b3	KR027943	*Pseudomonas veronii*
23	SUB1092331 UMK3c3	KT731966	*Rhodococcus equi*
24	SUB1092331 UMK3d2	KT731967	*Chromobacterium violaceum*
25	SUB882316 UMK3d3	KR027944	*Bacillus megaterium*
26	SUB1092331 UMK3d4	KT731968	*Aquitalea magnusonii*
27	SUB1092331 UMK3d5	KT731969	*Wautersia numazuensis (Cupriavidus numazuensis)*
28	SUB882316 UMK3e1	KR027945	*Exiguobacterium acetylicum*
29	SUB882316 UMK3e2	KR027946	*Chryseobacterium gambrini*
30	SUB883111 UMK4a1w	KR048048	*Salmonella enterica subsp. Diarizonae*
31	SUB883111 UMK4a1y	KR048049	*Bacillus idriensis*
32	SUB883111 UMK4a2	KR048050	*Staphylococcus xylosus*
33	SUB882316 UMK4b1	KR027947	*Aeromonas aquariorum (A dhakensis)*
34	SUB882316 UMK4b2	KR027948	*Pectobacterium cypripedii (Pantoea cypripedii)*
35	SUB882316 UMK4b3	KR027949	*Pseudomonas trivialis*
36	SUB882316 UMK4c2	KR027950	*Bacillus luciferensis*
37	SUB882316 UMK4c3	KR027951	*Enterobacter asburiae*
38	SUB882316 UMK4d2	KR027952	*Bacillus luciferensis*
39	SUB882316 UMK4d3	KR027953	*Aeromonas aquariorum (A dhakensis)*
40	SUB882316 UMK4e1	KR027954	*Acinetobacter calcoaceticus*
41	SUB882316 UMK4e2	KR027955	*Bacillus pseudofirmus*
42	SUB882316 UMK5a1	KR027956	*Proteus mirabilis*
43	SUB882316 UMK5a2	KR027957	*Escherichia coli*
44	SUB882316 UMK5b1	KR027958	*Exiguobacterium mexicanum*
45	SUB882316 UMK5d1	KR027959	*Bacillus luciferensis*
46	SUB883111 UMK6a1w	KR048051	*Staphylococcus xylosus*
47	SUB883111 UMK6a1y	KR048052	*Bacillus pseudofirmus*
48	SUB1092331 UMK6a2	KT731970	*Acinetobacter calcoaceticus*
49	SUB1092331 UMK6b1	KT731971	*Rubrivivax gelatinosus*
50	SUB1092331 UMK6b2	KT731972	*Acidovorax caeni*
51	SUB1092331 UMK6c	KT731973	*Acidovorax caeni*
52	SUB882316 UMK6d	KR027960	*Raoultella terrigena*
53	SUB882316 UMK6e	KR027961	*Pseudomonas vranovensis*
54	SUB1092331 UMK6x1	KT731974	*Acidovorax caeni*
55	SUB1092331 UMK6x2	KT731975	*Acidovorax caeni*

Table 2. Antibiotic sensitivity test of some bacteria isolates from the flood

No.	Isolate (bacteria)	TE 30 – tetracycline	AML 10 – amoxycillin	S 25 – streptomycin	CN 10 – gentamycin	E 15 – erythromycin	AMP 10 – ampicillin	P 10 – penicillin G	% Total resistance to all antibiotics
1	2d1 (*Chromobacterium violaceum*)	31 (S)	R	15 (I)	19 (S)	20 (I)	R	**R**	**43%**
2	3a2 (*Klebsiella pneumoniae subsp. Rhinoscleromatis)*	25 (S)	R	11 (R)	19 (S)	12 (R)	R	**R**	**71%**
3	3a3 (*Raoultella terrigena)*	22 (S)	12 (R)	15 (I)	19 (S)	11 (R)	**R**	**R**	**57%**
4	4b3 (*Pseudomonas trivialis)*	16 (I)	R	20 (I)	20 (S)	24 (S)	R	**R**	**43%**
5	4d2 (*Bacillus luciferensis)*	28 (S)	13 (R)	23 (S)	25 (S)	30 (S)	12 (I)	**R**	**29%**
6	4d3 (*Aeromonas aquariorum)*	21 (S)	27 (S)	18 (I)	22 (S)	15 (I)	**R**	**R**	**29%**
7	4e2 (*Bacillus pseudofirmus)*	12 (R)	20 (S)	21 (S)	25 (S)	23 (S)	**R**	22 (S)	**29%**
8	5a2 (*Escherichia coli)*	14 (R)	15 (I)	12 (R)	18 (S)	12 (R)	17 (S)	**R**	**57%**
9	5b1 (*Exiguobacterium mexicanum)*	26 (S)	40 (S)	22 (S)	25 (S)	28 (S)	44 (S)	30 (S)	0%
10	5d1 (*Bacillus luciferensis)*	21 (S)	22 (S)	20 (I)	25 (S)	19 (I)	20 (S)	12 (R)	14%
11	6a1w (*Staphylococcus xylosus*)	20 (S)	13 (R)	22 (S)	24 (S)	15 (I)	12 (R)	**R**	**43%**
12	6a1y (*Bacillus pseudofirmus)*	22 (S)	**R**	17 (I)	18 (S)	23 (S)	**R**	**R**	**43%**
13	6a2 (*Acinetobacter calcoaceticus)*	25 (S)	31 (S)	25 (S)	31 (S)	28 (S)	31 (S)	28 (S)	0%
14	6b1 (*Rubrivivax gelatinosus)*	23 (S)	**R**	20 (I)	33 (S)	13 (R)	**R**	**R**	**57%**
15	6b2 (*Acidovorax caeni)*	11 (R)	17 (I)	18 (I)	22 (S)	25 (S)	**R**	18 (S)	29%
16	6c (*Acidovorax caeni)*	12 (R)	12 (R)	18 (I)	25 (S)	26 (S)	12 (I)	7 (R)	43%
17	6e (*Pseudomonas vranovensis)*	23 (S)	32 (S)	24 (S)	25 (S)	16 (I)	32 (S)	25 (S)	0%

Key: R = Resistant; I = Intermediate; S = Susceptible

Table 3. Analysis of variance of antibiotics sensitivity (multiple comparisons) with Tukey honest significant difference post hoc test

(I) Antibiotic	(J) Antibiotics	Mean Difference (I–J)	Std. Error	*P*	95% Confidence Interval	
					Lower	Upper
TE 30 – tetracycline	AML 10 – amoxycillin	5.47	3.09	0.57	–3.79	14.73
	S 25 – streptomycin	1.82	3.09	>0.99	–7.44	11.09
	CN 10 – gentamycin	–2.53	3.09	0.98	–11.79	6.73
	E 15 – erythromycin	0.71	3.09	>0.99	–8.56	9.97
	AMP 10 – ampicillin	9.59*	3.09	0.04	0.33	18.85
	P 10 – penicilling	11.77**	3.09	0.004	2.50	21.03
AML 10 – amoxycillin	TE 30 – tetracycline	–5.47	3.09	0.57	–14.73	3.79
	S 25 – streptomycin	–3.65	3.09	0.90	–12.91	5.62
	CN 10 – gentamycin	–8.00	3.09	0.14	–17.26	1.26
	E 15 – erythromycin	–4.77	3.09	0.72	–14.03	4.50
	AMP 10 – ampicillin	4.12	3.09	0.83	–5.14	13.38
	P 10 – penicilling	6.29	3.09	0.40	–2.97	15.56
S 25 – streptomycin	TE 30 – tetracycline	–1.82	3.09	>0.99	–11.09	7.44
	AML 10 – amoxycillin	3.65	3.09	0.90	—5.62	12.91
	CN 10 – gentamycin	–4.35	3.09	0.80	–13.62	4.91
	E 15 – erythromycin	–1.12	3.09	>0.99	–10.38	8.14
	AMP 10 – ampicillin	7.77	3.09	0.16	–1.50	17.03
	P 10 – penicilling	9.94*	3.09	0.03	0.68	19.20
CN 10 – gentamycin	TE 30 – tetracycline	2.53	3.09	0.98	–6.73	11.79
	AML 10 - amoxycillin	8.00	3.09	0.14	–1.26	17.26
	S 25 – streptomycin	4.35	3.09	0.80	–4.91	13.62
	E 15 – erythromycin	3.24	3.09	0.94	–6.03	12.50
	AMP 10 – ampicillin	12.12**	3.09	0.003	2.86	21.38
	P 10 – penicilling	14.29***	3.09	<0.001	5.03	23.56
E 15 – erythromycin	TE 30 – tetracycline	–0.71	3.09	>0.99	–9.97	8.56
	AML 10 – amoxycillin	4.76	3.09	0.72	–4.50	14.03
	S 25 – streptomycin	1.12	3.09	>0.99	–8.14	10.38
	CN 10 – gentamycin	–3.24	3.09	0.94	–12.50	6.03
	AMP 10 – ampicillin	8.88	3.09	0.07	–0.38	18.14
	P 10 – penicilling	11.06*	3.09	0.009	1.80	20.32

Table 3. (Con) Analysis of variance of antibiotics sensitivity (multiple comparisons) with Tukey honest significant difference post hoc test

(I) Antibiotic	(J) Antibiotics	Mean Difference (I–J)	Std. Error	*P*	95% Confidence Interval	
					Lower	Upper
AMP 10 – ampicillin	TE 30 – tetracycline	–9.59*	3.09	0.04	–18.85	–0.33
	AML 10 – amoxycillin	–4.12	3.09	0.83	–13.38	5.14
	S 25 – streptomycin	–7.76	3.09	0.16	–17.03	1.50
	CN 10 – gentamycin	–12.12**	3.09	0.003	–21.38	–2.86
	E 15 – erythromycin	—8.88	3.09	0.07	–18.14	0.38
	P 10 – penicillin G	2.18	3.09	>0.99	–7.09	11.44
P 10 – penicillin G	TE 30 – tetracycline	–11.76**	3.09	0.004	–21.03	–2.50
	AML 10 – amoxycillin	–6.29	3.09	0.40	–15.56	2.97
	S 25 – streptomycin	–9.94*	3.09	0.03	–19.20	–0.68
	CN 10 – gentamycin	–14.29***	3.09	<0.001	–23.56	–5.03
	E 15 – erythromycin	–11.06**	3.09	0.009	–20.32	–1.80
	AMP 10 – ampicillin	–2.18	3.09	>0.99	–11.44	7.09

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ $F = 5.875$; $\eta^2 = 0.239$

Discussion

The unexpected nature of this study, which was conducted during the course of a devastating flood, may have affected some parameters that could have improved this study. The use of only culturing of water samples and looking for only bacterial colonies that grew within 48 hours may have excluded other bacteria such as *Leptospira* spp, *Vibrio* spp, and *Burkholderia pseudomallei* from being documented. Nevertheless, the isolation of several species of bacteria and genera of bacteria indicates a rich bacterial biodiversity of pathogenic or potentially pathogenic bacteria in the flood water during the great flood of December 2014 to January 2015 in Kelantan, Malaysia.

After the flooding, the prevalence of bacterial infections usually increases triggering episodes of intestinal symptoms such as diarrhea, vomiting, stomach aches, and other gastrointestinal disease symptoms as contaminated water moves from one geographical location to another, carrying a "cocktail" of bacteria along with it [16-18]. The distribution of the bacteria isolated did not show any remarkable difference between the different locations from which water samples were taken. This is probably because there was no large difference in the elevation between the locations, and during flood there is a massive movement of water from one location to the other, which can carry and distribute bacteria almost uniformly from one flood water location to the other. Different water depths would be expected to produce a variety of bacteria species [19]. This has potential public health implications because it implies waterborne infections can easily be carried from one flood location to another.

The predominance of proteobacteria making up 67% of bacteria in this study is similar to a study conducted in Thailand, which reported that majority of bacteria from the 2011 Thailand flood were from the phylum proteobacteria, which made up 56.5% to 91.4% of bacteria in different water samples in Thailand [6]. However, the majority of families and genera of bacteria reported from Thailand differ from the ones in this study, demonstrating the heterogeneity of bacterial communities across different flooded environments. *Enterobacteriaceae* from the phylum proteobacteria were the most common bacteria encountered in water samples from this study. *Enterobacteriaceae* also constitute the majority of waterborne and foodborne infections known in man and animals. Their ubiquitous nature and ability to thrive of the environment, helps them to be widespread in nature. They have been reported in various kinds of water, including flood water, by other investigators worldwide [16, 17, 20]. They are usually opportunistic bacteria, but can enter animals and human hosts, where some cause illnesses such as salmonellosis, shigellosis, and other intestinal infections. The high prevalence of this family in our study is a cause for concern because several members are known to be resistant to many antibiotics including those considered the current last line of antibiotic defense [21]. The present study has revealed *Klebsiella pneumoniae subsp. Rhinoscleromatis* as most resistant to the antibiotics tested. This is consistent with findings worldwide [22-24]. Three bacteria, *Exiguobacterium mexicanum*, *Acinetobacter calcoaceticus*, and *Pseudomonas vranovensis* did not show resistance to any of the antibiotics tested. This is consistent with other findings, but little is known about the antibiotic sensitivities of the relatively new bacterial species *Pseudomonas vranovensis* [25-27]. These bacteria species seem not to pose threats to health as do *Klebsiella pneumoniae*.

Flood water is a conglomeration from different sources including overflowing seas, rivers, streams, springs, wells, and other water. Humankind's activities during floods including bathing, swimming, washing, and excretion of waste into flood water affects its bacterial composition [28]. Some of the pollution may also have come from industrial waste, sewage water contamination, and admixtures of water from all manner of unhealthy sources during the course of the flooding [29, 30]. The study from Thailand showed pathogenic bacteria and high cross-contamination between flood water and other water sources [5]. The degree of pollution of soil surface and the metallic components of the soil also determine the richness and diversity of the bacteria present with presence of zinc decreasing both diversity and levels of species richness [31]. The rich bacterial diversity of our flood water showed bacteria from human and animal sources, and bacteria from the environment. A study in Brazil found that usual environmental water is a rich source of many species of bacteria with varying degrees of antibiotic resistance, showing some bacterial communities tolerating up to 600 times the clinical treatment levels of common antibiotics [32].

There appeared to be some strain variation in pathogenicity of some of the isolates as indicated by the same species showing different antibiotic sensitivities. Further studies that go beyond species

identification to identification of different strains and genes coding for resistance are required to establish if indeed these are different strains of the same bacteria with varying pathogenicity.

There were 9 bacteria in the present study not previously reported from any source in Malaysia based on our literature search. All of them were bacteria recently reported and classified within the last 20 years. *Wautersia numazuensis (Cupriavidus numazuensis)* was first reported in 2011 from Mexico in soils and agricultural plants [25, 33-37]. The epidemiological and public health impact of these water microbes and their ecological roles require future study.

Conclusion

During this massive flood session, there was a rich bacterial biodiversity including some species of potentially pathogenic bacteria that could endanger public health. This altered bacterial composition of normal water outside of flooding, and may explain why there are outbreaks of various infectious diseases during and after flood disasters. During the flood disaster period the only functional tertiary hospital (Hospital Universiti Sains Malaysia) in Kelantan handled 180 cases/day in the emergency department [38]. In adjacent Pahang state, 1,220 flood-related cases were handled within the first 6 days of the Kelantan flood disaster [39].

Most of the bacteria isolated from this study were resistant to one or more commonly used antibiotics. This is of interest to health practitioners and health policy makers because the presence of multidrug resistant bacteria should guide clinicians in the choice of antibiotics during flood disasters for effective treatment and control of waterborne infections. Gentamycin and tetracycline antibiotic classes appeared to be the best antibiotics to consider, but this may be an ever-changing picture. During flooding human and animal contact with flood water should be minimized, if not avoided completely, and adequate provisions should be made for provision of clean water to avoid outbreaks of waterborne diseases.

Conflict of interest

The authors declare that they have no conflict of interest.

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