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Random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) PCR of *Vibrio cholerae* from a foodborne outbreak in Limbang, Sarawak

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Abstract

Toxigenic and non-toxigenic *V. cholerae* strains can be monitored for changes in clones or serogroups, linkages between clinical and environmental isolates, genesis and clonal selection of epidemic strains, and population structure. Also, determining genetic relatedness among *V. cholerae* strains is critical for determining population genetic structure and evolutionary trends. In collaboration with the Sarawak Government Hospital, the present work was carried out on a total of 16 *V. cholerae* isolates in order to determine the genetic relatedness or heterogeneity of *V. cholerae* isolates from a foodborne outbreak among guests who attended a wedding ceremony in Limbang, Sarawak, Malaysia. The random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) were conducted to compare and determine strains and trace disease-causing microorganism. The RAPD fingerprinting was conducted using a total of 10-mer oligos 100 nmole random primers (OPAE1 - OPAE20). The primers OPAE7, OPAE10, OPAE14, and OPAE17 were selected because they were the most stable and discriminatory for *V. cholerae*. The PCR fingerprinting of ERIC-PCR was carried out using primer set of ERIC, ERIC1R (5'- ATGTAAGCTCCTGGGGATTCAC-3'), and ERIC2F (5'- AAGTAAGTGACTGGGGTGAGCG-3'). As a result, the 1 confirmed *V. cholerae* O1 samples were successfully fingerprinted. Based on the profiling results, the genetic fingerprint of some of the isolates from the clinical and environmental samples had 100% similarity, as indicated by the dendrogram. This indicated that the strains shared the same genetic profile. The smaller the genetic distance, the more homogeneous the strains are. The clinical and environmental strains shared some genetic characteristics. As indicated by the dendrogram, some strains were found to be closely linked to one another, while others were heterogeneous. Therefore, RAPD-PCR and ERIC-PCR produced the highest discrimination index. By combining these typing methods, evaluation of isolates' genetic diversity may be improved. The findings of the present work demonstrated the need for continued surveillance of *V. cholera* in Sarawak, Malaysia.

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Introduction

The first recorded occurrence of cholera was in 1873 (Yadav and Chee, 1990). In Malaysia, there was a cholera epidemic from 1991 to 2003, as recorded by the Infectious Diseases Division of the Ministry of Health. Around the same time, from 1994 to 2003, Sarawak, a state in East Malaysia, also suffered from the effects of the epidemic (Patrick *et al.*, 2012). In the rural areas of Sarawak, the contributing factors to this were found to be inadequate treatment of sewage and water supply, climate, drought season, and unsanitary living conditions. The epidemic led to 1,888 clusters in Sri Aman, Sarawak, resulting in 80 fatalities. According to the BorneoPost in 2011, the cholera outbreak reported in Limbang on March 6 was under control, and although 111 cases were reported after March 6, two of the cases were a cause for concern, as one of the patients was pregnant while the other was suffering from a medical ailment.

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Bacterial strain typing is important to determine the diagnosis, treatment, and epidemiological surveillance of the infection. Bacterial typing is separated into two categories: traditional epidemiological typing, and DNA-based typing (Abdel-Rhman and Rizk, 2021). By looking into the origins and spread of *Vibrio cholerae*, several biomolecular approaches have been employed in investigating epidemiology and strain relatedness. In order to distinguish between isolates of *V. cholerae* populations belonging to various pulsotypes and ribotypes, researchers have used the following methods: PFGE, AFLP, ERIC, and RAPD (Singh and Mohapatra, 2020). Following the development of DNA-based strain-typing technology, there is a need for an appropriate strain-typing method that is quick, inexpensive, and has good discriminatory power.

The RAPD method uses short random sequence primers to amplify random DNA fragments. Amplification happens when two priming sites are sufficiently close to anneal. Many polymorphisms can be found even among closely related organisms using a large number of possible primers (Abbas and Radhi, 2019). These properties make RAPD useful for species that grow slowly or for which large-scale culture is expensive or risky. However, ERIC uses primers that complement certain regions in the bacterial genome. Intranscriptible ERIC sequences are 126 bp long. ERIC-PCR is less expensive, easier to perform, more reliable, and faster, while RAPD detects genetic variation by randomly amplifying portions of target DNA (Abdel-Rhman and Rizk, 2021). According to Rivera *et al*. (2011), ERIC-PCR is an acceptable approach for typing and epidemiological research of *V. cholerae*.

Therefore, the objectives of the present work were to determine the genetic relatedness or heterogeneity of *V. cholerae* isolates from a foodborne outbreak in Limbang, Sarawak, and to study the efficacy of two fingerprinting techniques namely random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC).

Materials and methods

Bacterial strains

In collaboration with the Sarawak Government Hospital (SGH), a total of 28 clinical and environmental samples from Limbang, Sarawak were collected, and analysed in the laboratory of Faculty of Resource Science and Technology (FRST), UNIMAS. According to SGH, all samples were identified through passive case detection (PCD) and active case detection (ACD) as shown in Table 1.

Next, the strains were subjected to enrichment and streaked on selective agar for *V. cholerae* species. Thiosulfate-citrate-bile-sucrose (TCBS) agar and CHROMagar Vibrio were used to select colony for culturing. After streaking and incubated overnight at 37°C, the specific colony (TCBS = yellow; CHROMagar Vibrio = turquoise blue) were selected for sub-culturing in alkaline peptone water (APW) broth. The broth was then incubated at 37°C for 24 h, and thereafter stored at -20°C.

DNA extraction for PCR amplification

The boiling-cell method was used to extract DNA following Radu *et al.* (1999) with some modifications. Briefly, 300 µL of the culture was centrifuged at 10,000 rpm for 5 min. The supernatant was then discarded, and the pellet was re-suspended in 200 µL of sterile double-distilled water and boiled at 95°C for 10 min. After boiling the DNA, the boiled suspension were immediately cooled in ice at 4°C for 5 min. Next, the DNA was centrifuged at 10,000 rpm for 10 min, and the supernatant was used for PCR analysis or stored at -20°C.

Fingerprinting of RAPD-PCR

The RAPD fingerprinting was conducted using a total of 20 10-mer oligos 100 nmole random primers (OPAE1 - OPAE20). The samples were collected from rectal swabs, three samples each from stools, water samples, and Moore swabs from villagers as shown in Table 1. The primers OPAE7 (GTGTCAGTGG), OPAE10 (GTGAACCCCA), OPAE14 (TCTGTCCTGG), and OPAE17 (GACCGCTTGT) were selected because they were the most stable and discriminatory for *V. cholerae* strains (Maiti *et al.,* 2009). The experiment was carried out with modification from Sahilah *et al.* (2010): 5 min at 94°C (initial denaturation); 45 cycles at 94°C for 1 min (denaturation), 36°C for 1 min (annealing), 72°C for 2 min (extension); and 7 min at 72°C (final extension). A total of 30 µL of exTEN 2× PCR Master Mix (Base Asia), 1.2 µL of 10 µM primer, 10.2 µL of sterile double-distilled water, and 3.6 µL of DNA template were used in the PCR reaction for RAPD-PCR. Each reaction mixture was

Table 1. Bacterial samples collected from Limbang, Sarawak in collaboration with Sarawak Government Hospital (SGH).

Sample	Source	Case	Location	Coordinate (approximate)
VC001	Water sample 1	NA	Kampung Limpaku Pinang	4.879674, 115.019880
VC002	Water sample 2	NA	Kampung Limpaku Pinang	4.879674, 115.019880
VC003	Water sample 4	NA	Kampung Limpaku Pinang	4.879674, 115.019880
VC004	Moore swab 1	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC005	Moore swab 2	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC006	Moore swab 3	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC007	Moore swab 4	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC008	Moore swab 5	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC009	Moore swab 6	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC010	Moore swab 7	NA	Kampung Limpaku Pinang	4.880785, 115.019258
VC011	Rectal swab	PCD	Kampung Pahlawan	4.687784, 115.025586
VC012	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC013	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC014	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC015	Rectal swab	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC016	Rectal swab	PCD	RH John Kelati	4.569667, 114.835611
VC017	Rectal swab	PCD	Kampung Bukit Luba	4.664801, 114.943439
VC018	Rectal swab	PCD	Kampung Lubok Tuan	4.603333, 114.888611
VC019	Rectal swab	PCD	Kampung Bukit Luba	4.664801, 114.943439
VC020	Rectal swab	PCD	RPR Rangau	4.756868, 115.007478
VC021	Rectal swab	PCD	Kampung Limpaong	4.767524, 115.001710
VC022	Rectal swab	PCD	Kampung Batu Danau	4.652792, 114.826880
VC023	Rectal swab	PCD	Kampung Pemukat	4.627540, 114.951687
VC024	Rectal swab	PCD	RH Imau	4.653031, 115.004280
VC025	Rectal swab	ACD	Kampung Merasam Parit	4.731611, 114.832736
VC026	Stool	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC027	Stool	PCD	Kampung Limpaku Pinang	4.880785, 115.019258
VC028	Stool	ACD	Kampung Limpaku Pinang	4.880785, 115.019258

PCD: passive case detection, ACD: active case detection, NA: not applicable.

viewed under UV light. Thermo Scientific GeneRuler 1 kb and 100 bp DNA ladders were utilised as DNA markers.

Fingerprinting of ERIC-PCR

The PCR fingerprinting of ERIC-PCR was carried out using primer set of ERIC, ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2F (5'- AAGTAAGTGACTGGGGTGAGCG-3'). Briefly, 30 µL of reaction mixture that contained 15 µL of exTEN 2× PCR Mastermix (Base Asia), 1.2 µL of 10 mM primers, 9 µL of sterile double-distilled water, and 3.6 µL of DNA template were added to form reaction master mix. The following cycling conditions were used: 95°C for 5 min (initial denaturation); 35 cycles of 94°C for 30 s (denaturation), 47°C for 30 s and 52°C for 1 min (annealing); 72°C for 4 min (extension); and 72°C for 16 min (final extension) (Bilung *et al*., 2018). The PCR product was then viewed under UV light.

Agarose gel electrophoresis (AGE)

Next, 5 µL of PCR products were loaded on 2% agarose gel in 1X TE buffer, and ran at 100 V for 55 min. The agarose gel was stained with ethidium bromide (EtBr) solution for 40 min before visualising

the gel under a UV transilluminator (MaestroGen, Australia). A 100 bp ladder (Promega, USA) was used to show the sizes of DNA fragments.

Cluster analysis

PyElph version 1.4 was used to analyse the banding patterns. Dendrograms were created using UPGMA (Pavel and Vasile, 2012).

Discrimination Index

The discrimination indices (D) for RAPD-PCR and ERIC-PCR were derived using the Hunter and Gaston formula (1988) as shown in Eq. 1:

$$D = 1 - \frac{1}{N\,(N-1)} \sum_{j=1}^{S} \quad n_j(n_j - 1) \qquad \text{(Eq. 1)}$$

where, N = total number of strains in the sample population, S = total number of types described, and n_j = number of strains belonging to the j^{th} type. A value of 1 indicated discrimination, while a value of 0 indicated no discrimination.

Results and discussions

Molecular techniques would benefit in learning more about the spread of cholera, and the relationship between different strains, as well as how many copies and types of CTX proteins there are in each strain. In order to investigate the geographical distribution of *V. cholerae*, DNA-based typing approaches are being used to explore the relatedness among bacterial strains. In the present work, a total of 16 confirmed *V. cholerae* O1 isolates with the *ctx* gene from the foodborne outbreak patients from Limbang, Sarawak were examined using two genotypic molecular typing methods to detect their genetic relatedness.

Random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) fingerprinting are two of the approaches employed. The number of types and how often each type was found can show how good a typing method is at distinguishing between different types. DI stands for discrimination index, and it can be defined as the chances that two random isolates from the test population are separated into different types of DNA (Abdel-Rhman and Rizk, 2021).

Twenty random RAPD primers were utilised in the present work, and only seven of the 20 primers generated bands. However, only four of the seven primers created reproducible patterns with a sufficient number of amplified products for proper analysis. Therefore, OPAE7, OPAE10, OPAE14, and OPAE17 primers were used to analyse all 16 isolates. The remaining primers either did not amplify well for the strains, or produced a minimal number of PCR products, since several strains had no bands for RAPD. These findings could be interpreted as the absence of specific primer binding sites in these isolates' chromosomal DNA as explained by Radu *et al.* (1999). A pattern or profile was a collection of replicable bands generated for a given primer.

The repeatability of the RAPD-PCR and ERIC-PCR procedures was determined by performing the experiments thrice with each primer, at the very least. The representative profiles of the RAPD-PCR patterns produced with primers OPAE7, OPAE10, OPAE14, and OPAE17 are depicted in Figure 1. Meanwhile, Figure 2 depicts the ERIC-PCR patterns.

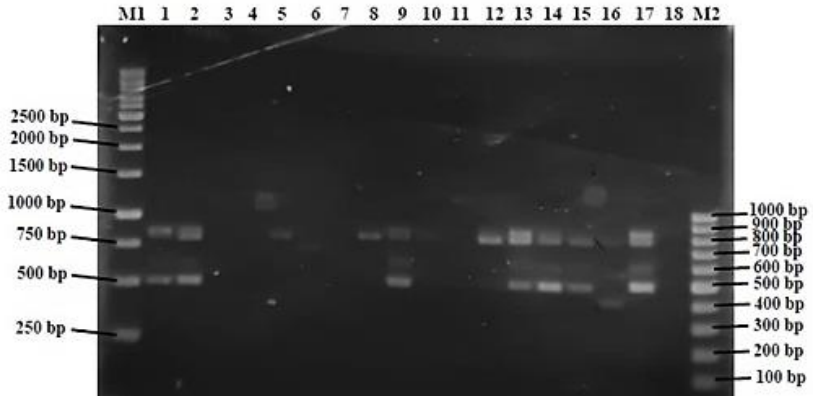

Figure 1. RAPD-PCR profile of 16 strains of *V. cholerae* isolates obtained using OPAE14 primer. Lane M1 = 1 kb DNA ladder; lane M2 = 100 bp DNA ladder; lane 1 = positive control (ATCC 14035); lanes 2 – 17 = VC001, VC002, VC003, VC005, VC006, VC012, VC015, VC016, VC017, VC018, VC019, VC020, VC021, VC023, VC026, VC028; and lane 18 = negative control (sterile double-distilled water).

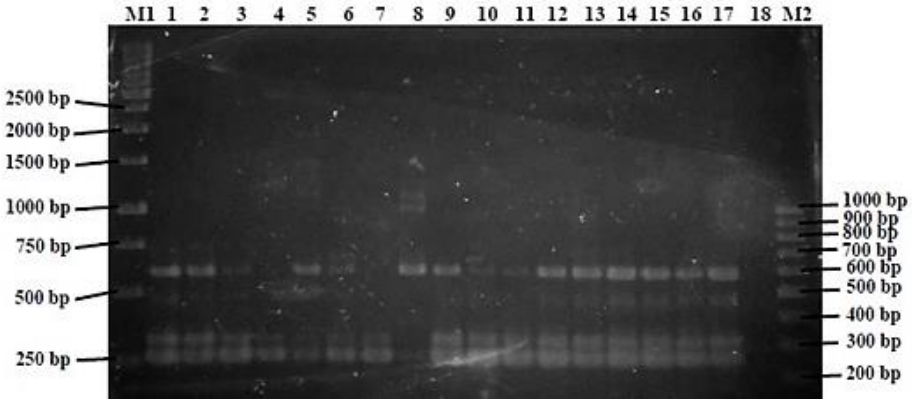

Figure 2. ERIC-PCR profile of 16 strains of *V. cholerae* isolates obtained using ERIC1R and ERIC 2 primer. Lane M1 = 1 kb DNA ladder; lane M2 = 100 bp DNA ladder; lane 1 = positive control (ATCC 14035); lanes 2 – 17 = VC001, VC002, VC003, VC005, VC006, VC012, VC015, VC016, VC017, VC018, VC019, VC020, VC021, VC023, VC026, VC028; and lane 18 = negative control (sterile double-distilled water).

Based on the dendrograms, each primer generated at least one cluster, thus indicating that these strains were somehow related despite their clinical and environmental origins as shown in Figure 3. These primary clusters were subsequently separated into many sub-clusters, thereby establishing the genetic heterogeneity of the isolates. The discrimination index and cluster size for each primer are listed in Table 2.

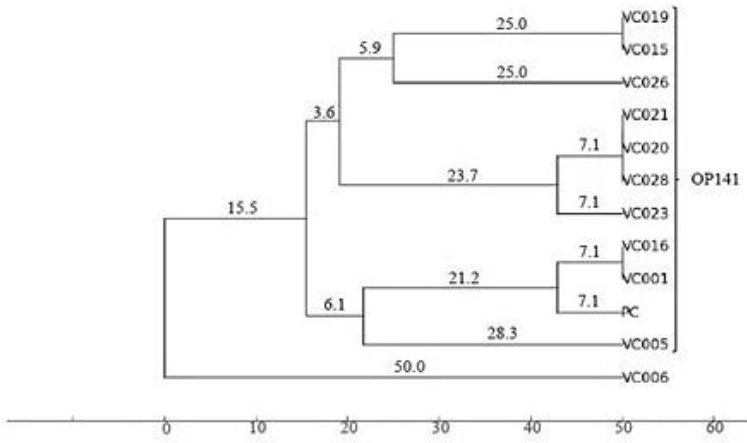

Figure 3. Dendrogram generated from RAPD-PCR (OPAE14) molecular typing by PyElph1.4 Software. Genetic distances are shown on top of each branch. One cluster and one isolate were formed.

Table 2. Discrimination indices of RAPD-PCR and ERIC-PCR in genotyping of *Vibrio cholerae* isolates (n = 16).

Primer	Number of cluster	Cluster size	Number of a single isolate	Discriminatory index
Opae7	1	12	1	0.154
Opae10	1	15	1	0.125
Opae14	1	11	1	0.167
Opae17	2	8, 8	0	0.533
ERIC	2	11, 6	0	0.485

Bands ranging from 300 to 1,000 bp appeared on the agarose gel image (Figure 1) for 12 out of 16 samples; however, there were no bands expressed at OPAE7 in VC002 strain, VC003 (water samples), VC012 (rectal swab), and VC015 strains (rectal swab). There was only one significant cluster developed at a genetic distance of 4.7%, which consisted of 12 strains, including the positive control *V. cholerae* O1 El Tor ATCC 14033. Six strains showed 100% similarity based on the dendrogram, which showed genetic distance of 21.4% for VC019 (rectal swab) and VC017 (rectal swab) strains; 10.0% for VC028 (stool), VC023 (rectal swab), and VC020 (rectal swab, as well as genetic distance of under 4.7%, respectively, for one VC006 strain (Moore swab). At 4.7 and 50.0% genetic distance, two major clusters developed. The overall similarity of the strains was 84.6% because the discrimination index for OPAE7 was 0.154.

However, at genetic distance of 4.7%, two sub-clusters were created with 0.400 and 0.286 discrimination index, respectively, at genetic distance of 7.2 and 25.8%. Only strains VC019 (rectal swab) and VC017 (rectal swab) demonstrated 100% similarity with VC005 strain (Moore swab) at genetic distance of 21.4% under 7.2% sub-clusters. This was similar for isolates VC028 (stool), VC023 (rectal swab), and VC020 (rectal swab) which had a genetic distance of 10.0% from VC016 (rectal swab). Except for stool samples, all strains from rectal swabs showed 100% similarity. Moore swab sample yielded a single isolate. The clinical and environmental strains exhibited heterogeneity across samples on the dendrogram.

Primers OPAE10 revealed that bands ranging from 380 to 2,400 bp were formed, but only VC002 strain (water sample) did not form any bands out of the 16 isolates tested. One major cluster of 15 strains was generated at a genetic distance of 12.2%, and this primer formed one single isolate, VC003 (water sample). VC021 (rectal swab), VC012 (rectal swab), VC006 (Moore swab), VC026 (stool sample), VC023 strains (rectal swab); VC018 (rectal swab); and VC016 strains (rectal swab) were the three groups of strains that exhibited 100% similarity at a genetic distance of 16.7%. At genetic distance of 12.2 and 44.7%, a discrimination index of 0.125 (overall similarity: 87.5%) was determined. With a discrimination index of 0.327 and 0.500, two sub-clusters were created, with genetic distance of 12.2, 1.7, and 1.8%, respectively. Isolate groups consisted of VC021 (rectal swab), VC012 (rectal swab), VC006 (Moore swab), VC026 (stool) and VC023 (rectal swab), and VC018 (rectal swab) with VC016 strains (rectal swab) demonstrating 100% similarity between isolates at 16.7% genetic distance under 7.5% sub-cluster, but not with VC017 (rectal swab), VC015 (rectal swab), and VC005 (Moore swab).

On the other hand, the OPAE14 primer identified bands ranging in size from 400 to 880 bp. As seen in Figure 3, 11 of 16 samples formed bands, but VC002 (water samples), VC003 (water samples), VC012 (rectal swab), VC017 (rectal swab), and VC018 (rectal swab) did not. One significant cluster was formed at a genetic distance of 15.5%. The cluster consisted of 11 strains. Three sets of strains had 100% genetic similarity namely VC019 (rectal swab) and VC015 (rectal swab); VC021 (rectal swab), VC020 (rectal swab), and VC028(stool swab); and VC016 (rectal swab) and VC001(water sample) as indicated by the dendrogram at genetic distance of 25.0, 7.1, and 7.1%, respectively. At the same genetic distance, isolates VC026 (stool), VC023 (rectal swab), and the positive control seemed to be monoclonal. As a result, the isolates showed distinct genetic profiles.

Two large clusters emerged at genetic distance of 15.5 and 50.0%, respectively, with an overall similarity of 83.3% and a discrimination value of 0.167. At genetic distance of 15.5%, two sub-clusters with discrimination indices of 0.571 and 0.500 were formed at genetic distance of 3.6 and 6.1%, respectively. As demonstrated in OPAE10, there were some similarities in *V. cholerae* strains isolated from clinical and environmental samples with this OPAE14. One of the 100% comparable sets demonstrated the different sources of sample (rectal swab and water sample). VC006 (Moore swab) originated as a single isolate.

The agarose gel image of OPAE17 contained bands ranging in size from 270 to 880 bp. Excluding VC012 (rectal swab) which lacked bands, all the other isolates exhibited bands. There were two primary clusters created by this primer: one at a genetic distance of 11.2%, which contained eight strains; and another at a genetic distance of 17.9%, which also contained eight strains. There were numerous strains that shared 100% of their DNA at 16.7, 16.7, 10.0, 11.7, and 20.8%, respectively, namely the strain VC001 (water samples) and VC016 (rectal swab);

VC003 (water samples), VC002 (water samples), and VC006 (Moore swab); VC023 (rectal swab), VC018 (rectal swab), and VC028 (stool sample); VC021 (rectal swab); and VC019 (rectal swab), VC026 (stool sample), and VC017 (rectal swab). VC015 (rectal swab), VC005 (Moore swab), and VC020 (rectal swab) on the other hand, developed as single isolates. Two large clusters emerged at genetic distance of 11.2 and 17.9%, respectively, with a discrimination score of 0.533 (overall similarity of 46.7%). At genetic distance of 11.2 and 17.9%, a discrimination index of 0.429 was determined. As with OPAE10 and OPAE14, there were correlations between clinical and environmental samples (water sample and rectal swab). Apart from that, one similarity came from water and Moore swab samples, which demonstrated a strong correlation between environmental samples.

RAPD fingerprinting is easy, sensitive, and inexpensive for molecular genetic typing. This technique has been applied in the investigation of genetic diversity in a variety of bacterial species, clinical samples, and environmental samples including *V. cholerae* non-O1/non-O139 (Chomvarin *et al.*, 2014). The RAPD profile was quite diverse, with similarities as low as 25%. Each strain was subjected to RAPD-PCR thrice, and the banding patterns remained consistent. RAPD-PCR has been proved to be useful for categorising a variety of bacterial species in recent years. The findings were able to be obtained in less than 24 hours after collection. Therefore, RAPD-PCR can be further utilised as a diagnostic tool to investigate the source of infection linked to seafood consumption (Høi *et al.*, 1997).

In the present work, RAPD analysis was proven to be an extremely useful technique for this investigation of a cholera outbreak from Limbang, Sarawak. Results shown in Figures 1 and 3 can conclude that RAPD-PCR fingerprinting is a technique to determine the heterogeneous profile. This is because RAPD uses random sequences; the strains' sequences may vary, with some strains sharing the same genetic profile while others may not. The smaller the genetic distance, the more homogeneous the strains are. In fact, clinical and environmental strains shared several genetic characteristics as indicated by the dendrogram, and some strains were found to be closely linked to one another, while others had some heterogeneity.

Based on PyElph1.4, details on data analysis based on banding patterns produced using ERIC-PCR ranged in size from 260 to 1,800 bp as shown in Figure 2. Two major clusters emerged at genetic distance of 1.1 and 24.2%, respectively, with a discrimination score of 0.485 (overall similarity is 51.5%). There were 11 strains with a genetic distance of 1.1%. The other cluster contains six strains. At a genetic distance of 1.1%, two sub-clusters emerged at genetic distance of 3.1 and 9.3%, respectively. The discrimination value was 0.5 at genetic distance of 9.3%, and two sub-clusters formed at genetic distance of 3.8 and 8.2%. Through ERIC-PCR, it was found that three single isolates with genetic distance of 20.0, 20.0, and 5.6%, respectively, were identified as VC015 (rectal swab), VC005 (Moore sample), and VC002 (water sample).

Single isolates established that VC015 (rectal swab) and VC005 strains (Moore swab) did not share 100% genetic similarity, and had distinct genetic chromosomal or cloning structures. The remaining strains were identical to one another. At 10.0% genetic distance, 100% similarity was observed between VC018 (rectal swab), VC017 (rectal swab), VC016 (rectal swab), and VC006 strains (Moore swab); at 10.0% genetic distance, 100% similarity was observed between VC012 (rectal swab) and VC003 strains (water sample). Apart from that, VC001 (water sample) was identical to the positive control in this fingerprinting but not identical to VC002 (water sample). Based on the dendrogram in Figure 4, the second cluster comprised of strains VC023 (rectal swab), VC021 (rectal swab), VC020 (rectal swab), VC019 (rectal swab), VC028 (stool), and VC026 (stool). Clinical and environmental isolates exhibited similarity in some strains and also heterogeneity.

The ERIC sequence was around 124 - 127 bp in length, and contained approximately 44 bp highly conservative core sequences in its centre. It is most frequently found in numerous copies in the genomes of Enterobacteriaceae and *Vibrio*. ERIC-PCR was used to investigate the diversity of phylogenetic classifications among several *V. cholerae* bacterial isolates (Shuan *et al.*, 2021). While the nucleotide sequences of the ERIC sequences are highly conserved, their chromosomal positions vary between species (Hulton *et al.*, 1991). In *V. cholerae*, the ERIC sequence is found near the haemolysin gene (Rao and Surendran, 2010). The ERIC-PCR investigation revealed that this technique had the potential to be used in place of other molecular characterisation techniques such as RFLP (Merza *et al.*, 2021).

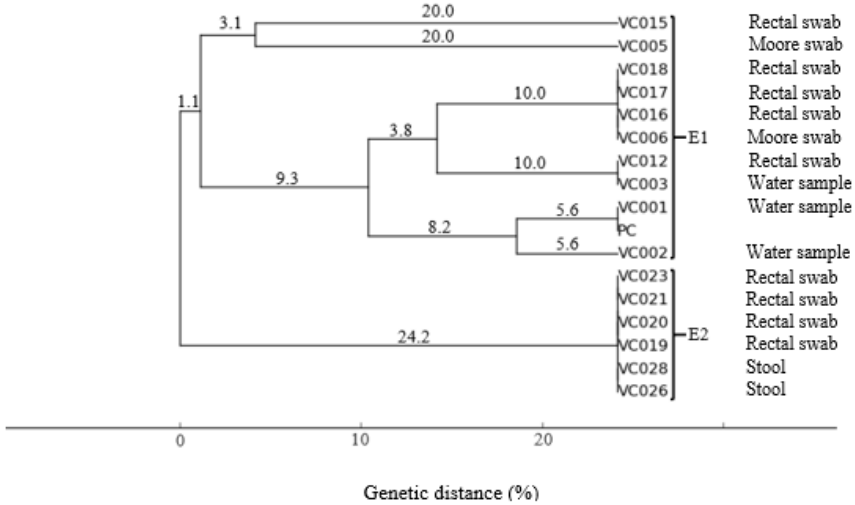

Figure 4. Dendrogram generated from ERIC-PCR molecular typing by PyElph1.4 Software. Genetic distances are shown on top of each branch. Two clusters were formed.

In the present work, ERIC-PCR was successfully employed to investigate the clonal diversity of *V. cholerae*. Individual strains isolated from clinical and environmental sources had a high degree of similarity in their fingerprints (Singh and Mohapatra, 2020). The ERIC-PCR method demonstrated the greatest discrimination power. Additionally, ERIC-PCR was demonstrated to be a highly effective technique for molecular investigation of other *Vibrio* species, including *V. alginolyticus* and *V. parahaemolyticus*. This assay utilises a mixture of primers designed to target conserved ERIC areas to generate amplified products based on the frequency and orientation of ERIC sequences (Bandyopadhyay *et al.*, 2020)

ERIC-PCR was capable of differentiating even isolates with an identical RAPD profile. The discrepancy in results between RAPD and ERIC typing could have been due to the fact that ERIC searches for intergenic sections of untranslated areas, whereas RAPD searches only randomly selected regions of the chromosome. As a result, the grouping of isolates for RAPD and ERIC may differ significantly. Therefore, both methods yielded nearly identical results in the present work, with some strains were found to be closely linked, and others being determined to be distinct from one another. Only a few isolates exhibited identical genetic fingerprints, even when they came from the same source and sampling period. Although the present work was unable to detect any distinct pattern or clusters between clinical and environmental strains, the results, however, indicated a high degree of variability among *V. cholerae* isolates.

Additionally, the discrimination index was used to determine the clonal diversity of the *V. cholerae* isolates in the present work. The discrimination index for each respective typing method indicated the frequency with two strains randomly picked from a population that fell into distinct criteria. Based on the results, RAPD-PCR and ERIC-PCR both demonstrated a discrimination index of 0.245 for RAPD-PCR and 0.485 for ERIC-PCR. As a result, ERIC-PCR exhibited the greatest discrimination index.

The comparison of typing approaches enables the user to retain a convenient method while discarding an ineffective one (Abdel-Rhman and Rizk, 2021). As proved in the previous study, majority of these methods have limitations in terms of resolving evolutionary relationships, particularly when employed in isolation—no single method is sufficient to analyse the genetic relatedness of the endemic environmental *V. cholerae* population in an unambiguous manner (Lamon *et al*., 2019). Occasionally, single analyses may fail to give sufficient resolution or may produce inconsistent results. As a result, a combination of approaches may be necessary to completely type all *V. cholerae* strains, hence increasing phylogenetic accuracy and resolution (Lamon *et al*., 2019).

Therefore, both RAPD-PCR and ERIC-PCR can be used to determine the genetic relatedness of strains, as both fingerprinting techniques can generate

a highly selective profile between strains. Increased discriminatory power does not always imply more accurate epidemiologic association. The efficiency of a molecular typing method is shown by its capacity to cluster and significantly distinguish unrelated strains. Continuous diarrheal surveillance remains critical to monitor the shifting pattern of *V. cholerae* infection that causes cholera epidemic.

Conclusion

Both approaches were simple, rapid, and economical for epidemiological typing of *V. cholerae* isolates; however, as compared to RAPD-PCR, ERIC-PCR produced the highest discriminatory index. Combining two or more typing approaches could improve the evaluation of the isolates' genetic diversity toward outbreak of cholera in the near future. Using several typing methods could also allow more precise characterisation of isolate genetic profiles and identification of clones that are not indicated by conventional approaches.

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