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Genomic Diversity of Cholera Outbreak Strains in East Malaysia

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ABSTRACT

Thirty one *Vibrio cholera* isolates recovered from cholera outbreak in Bintulu, Sarawak (Malaysia) were detected with the presence of ctx gene by using specific PCR. These isolates were further characterized and differentiated by using the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) and BOX-PCR to determine their genomic fingerprints. The specific PCR result confirmed the identities of 27 isolates out of 31 as pathogenic *V. cholerae*. The ERIC-PCR generated several genetic profiles consisting of 4-6 bands with sizes in the range of 100 to 600 bp, while the BOX-PCR produced profiles numbering 2-7 bands in the sizes between 200 to 1000 bp. Based on the dendrogram generated from the DNA fingerprinting profiles (ERIC-PCR and BOX-PCR), all of the isolates can be divided into 2 main clusters that is further divided into 2 sub-clusters. The low genetic diversity of the isolates indicated the outbreak of *V. cholerae* in the study area was due to the contamination from a single or few sources of *V. cholerae*.

Keywords: Vibrio cholerae, Ctx gene,ERIC-PCR, BOX-PCR, Genetic fingerprinting

INTRODUCTION

Cholera is a severe infectious diarrheal disease caused by toxigenic *Vibrio cholerae.* The disease is characterized by stools of rice water diarrhea that rapidly leads to dehydration. The pathogen lives freely in aquatic environments and cholera is always associated with poor sanitation.[1] In Malaysia, cholera outbreaks caused by the El Tor O1 *V. cholerae* serogroup occur periodically, cases from the 0139 serogroup occur sporadically, and the non–O1/non–O139 *V. cholerae* serogroup has not been implicated in any major outbreak.[2-4] Contaminated drinking water, cooked food, and raw or undercooked seafood served as vehicles of transmission in Malaysia.[5] In Sarawak (East Malaysia), the socio-economic activities practiced by the various ethnic groups, the natural phenomena (the La Nina in 1997), the dry seasons (drought) and other contributing factors such as lack of proper treated water supply and the poor sanitary system encountered by the toxigenic *V. cholerae* in the rural area had facilitated the spread of the diseases in Sarawak.[6]

Studies on genomic variation and molecular epidemiology of O1 and O139 *V. cholerae* are often carried out to track sources and and spread of the pathogen.[7] It has been reported that molecular typing methods such as Enterobacterial Repititive Intergenic Consensus (ERIC) PCR and BOX-PCR were the discriminatory typing methods for Vibrio spp., and were very useful for tracing the temporal and geographic relatedness of epidemic strains of *V. cholerae.*[8] In this study, *V. cholerae* strains were recovered from the cholera outbreaks in Bintulu, Sarawak during August 2012. Preliminary analysis of the isolates was carried out by culturing the isolates on CHROMagar *Vibrio* and followed by serotyping. Molecular characterization was done by screening the isolates for the presence of virulent gene, *ctx* genes. DNA fingerprinting by repetitive sequence based molecular markers like ERIC and BOX PCRs were carried out to study the strain level differences.

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MATERIAL AND METHODS

Bacterial strains

Stool samples or rectal swabs were collected from patients with diarrhea admitted to the district hospital, Bintulu. All strains were reconfirmed using slide agglutination with polyvalent 01, monospecific Ogawa-Inaba antisera and with specific anti-0139 antisera obtained commercially (Denka Seiken, Tokyo). The strains were grown in Luria-Bertani broth overnight at 37°C with shaking at 220 rpm in an orbital shaker.

Specific PCR targeting on *ctx* genes and genotyping by ERIC-PCR and BOX-PCR

DNA was extracted using boiled-cell method as described by Lesley *et al.* (2005). Five hundred microliters of the broth were centrifuged at 12,000 rpm for 3 min in order to pellet the bacterial cells. The supernatant was discarded and the pellet was then resuspended with 400 µl of sterile distilled water and boiled for 10 min followed by freezing in -20°C for 10 min. It was then centrifuged at 10,000 rpm for 5 min to pellet the cell debris. The supernatant was then kept for use in PCR. *V. cholerae* strains were examined for the presence of ctx genes by specific PCR assay as described by previously (Son *et al.,* 2002). PCR DNA fingerprinting by ERIC and BOX primers was carried out as according to Rivera *et al.* (1995) and Rademaker (1997), respectively. Primers used in the PCR reactions; their annealing conditions and the size of amplicons obtained are displayed in the Table 1.

Table 1. Primers used to detect *ctx* genes in *V. cholerae* strains and genotyping of *V. cholerae* strains

Genes	Primer sequences (5' to 3')	Amplicon size (bp)	Annealing conditions	References
ctxA-F	CTCAGACGGGATTTGTTAGGCACG	301	64°C, 1 min	Son *et al.* (2002)
ctxB-R	TCTATCTCTGTAGCCCCTATTACG			
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC		52°C, 1 min	Versalovic *et al.* (1991)
ERIC 2	AAGTAAGTGACTGGGGTGAGCG			
BOXA 1R	CTACGGCAAGGCGACGCTGACG		43°C, 1 min	Rademaker, (1997)

Specific PCR was performed in a reaction volume of 25 µl volume consisting of 1× of PCR buffer, 1.5mM of MgCl, 200µM of each dNTPs and 1 U of *Taq* polymerase using the following program on an i-cycler system (Sensquest Labcycle): an initial denaturation (94°C for 3 min); followed by 35 cycles of amplification with denaturation at 94°C for 1 min, primer annealing for 1 min at 60ºC and extension at 72ºC for 1 min. Lastly, a final extension of the incompletely synthesized DNA at 72°C for 5 min. The following was added to each 25 µl PCR mixture for ERIC-PCR: 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM MgCl2, 0.5 µl of dNTPs, ERIC-1R and ERIC-2 primers (0.2 pmol/µl of each); and 2.5 U of *Taq* polymerase. ERIC-PCR was carried out in the conditions: initial denaturation at 95°C for 5 min; 35 cycles of amplification with denaturation at 92°C for 1 min and primer annealing for 1 min at 52ºC; and extension at 72ºC for 3 min and final extension of the incompletely synthesized DNA at 72°C for 10 min. PCR reaction mixture for BOX-PCR contained 16.2 µl sterile distilled water, 0.2 mM of dNTPs, 2.5 µl 10X PCR buffer, 0.2 pmol of BOXA 1R primer, 0.5 µl *Taq* polymerase, and 1 µl template DNA for a final volume of 25 µl. The amplification protocol was as follows: initial denaturation at 95°C for 2 min; 35 cycles of amplification with denaturation at 94°C for 3 min, 92°C for 30s and primer annealing for 1 min at 43ºC; and extension at 65ºC for 3 min and final extension of the incompletely synthesized DNA at 65°C for 8 min. The amplified products (5 µl) for specific PCR were analyzed by 1% agarose gel electrophoresis in Tris-Borate EDTA (1X TBE), while the amplified products for DNA fingerprinting PCRs were analyzed by 1.2% agarose gel electrophoresis in Tris-Borate EDTA (1X TBE), stained in ethidium bromide and visualized using gel documentation system (Bio-Rad Universal Hood).

Cluster analysis of the ERIC-PCR and BOX-PCR using RAPD Distance software

The fingerprint profiles obtained from ERIC-PCR and BOX-PCR were analyzed using the RAPDistance Package Version 1.04. Scoring process was done. For each band sizes, no. 1 was given to the strains that produced the band and 0 to the strains that failed to produced it. Then, the data was input in the software and a NJT format files was created.

Automatically the dendrogram was generated by this software. The generated dendrogram was then edited by using Corel Draw software.

Results and Discussions

In the current study, all of the 31 isolates of clinical sample collected were tested positive on the CHROMagar *Vibrio* (appear in turquoise). Further genetic identification on the presumptive *V. cholerae* by using specific PCR targeting the ctx gene confirmed that 27out of 31 isolates were pathogenic *V. cholera,* whereas four isolates (JM-VC2, 1-VC2, 13-VC2 and 20-VC2) were not. Figure 1 shows the electrophoresis of PCR product of the ctx gene in the positive strains. Toxigenic strains of *V. cholerae* possessed essential genetic element called the CTX genetic element (Chen *et al.,* 2004). The ctx genes are located in the CTX element and encode the cholera toxin CT. This toxin is primary responsible for the severe secretory diarrhea in infected person (Waldor *et al.,* 1996). All of the isolates were selected for further molecular characterization by using ERIC-PCR and BOX- PCR.

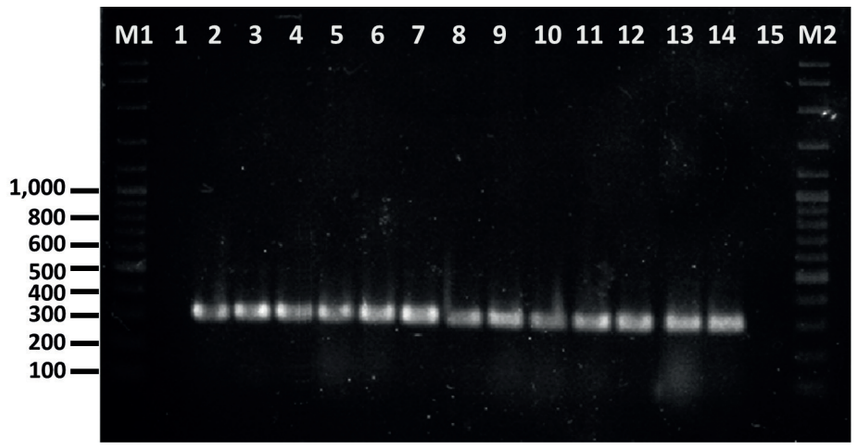

Figure 1: Representative image of agarose (1%) gel electrophoresis of specific PCR products (ctx A) of *V. cholerae* isolates. Lanes: M1: 100 bp ladder; 1: no template; 2: *V. cholerae* strain serotype Ogawa; 3: 39-VC2; 4: 4-VC2; 5: 11-VC2; 6: 16-VC2; 7: 3-VC2; 8: 2-VC2; 9: J-VC2; 10: MSS-VC2; 11: BE-VC2; 12: JK-VC2; 13: NA-VC2; 14: AB-VC2; 15: no template; M2: 100 bp ladder.

ERIC-PCR and BOX-PCR was performed to illustrate the relationship between the 31 isolates. DNA fingerprinting with ERIC-PCR revealed that the size of the bands ranged from 100 to 600 bp, with the number of bands observed ranged 4 to 6 as shown in Figure 2. On the other hand, the BOX fingerprinting profiles obtained is displayed in Figure 3. The findings showed that the size of the bands ranged from 200 to 1000 bp, with the number of bands observed in the range of 2 to 7.

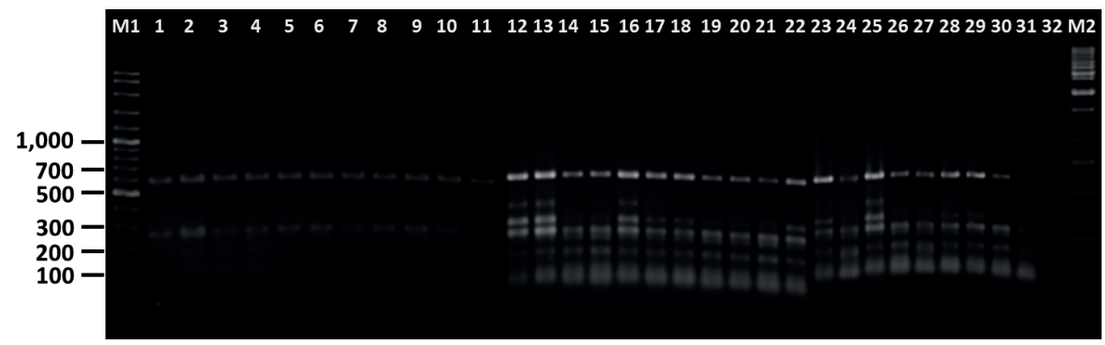

Figure 2: Agarose (1.2%) gel electrophoresis of ERIC-PCR products of *Vibrio cholera* isolates. M1: 100bp ladder; 1: SA-VC2; 2: NSA-VC2; 3: FA-VC2; 4: 3051-VC2; 5: 20-VC2; 6: 15-VC2; 7: 7-VC2; 8: AB-VC2; 9: JK-VC2; 10: MC-VC2; 11: M-VC2; 12: 16-VC2; 13: 11-VC2; 14: 39-VC2; 15: RN-VC2; 16: J-VC2; 17: BS-VC2; 18: MSS-VC2; 19: 33-VC2; 20: 17-VC2; 21: 13-VC2; 22: 9-VC2; 23: HC-VC2; 24: JM-VC2; 25: NA-VC2; 26: BE-VC2; 27: 34-VC2; 28: 18-VC2; 29: 14-VC2; 30: 1-VC2; 31: VP; 32: negative control; M2: 1 kb ladder.

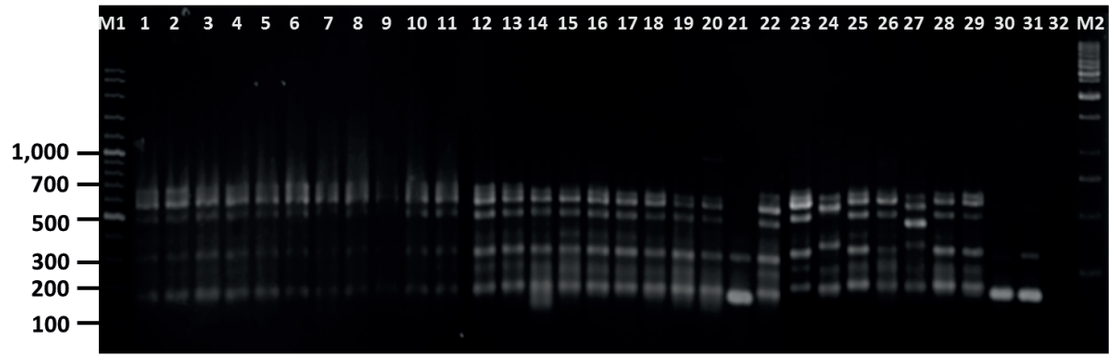

Figure 3: Agarose (1.2%) gel electrophoresis of BOX-PCR products of *Vibrio cholera* isolates. M1: 100 bp ladder; 1: SA-VC2; 2: NSA-VC2; 3: FA-VC2; 4: 3051-VC2; 5: 20-VC2; 6: 15-VC2; 7: 7-VC2; 8: AB-VC2; 9: JK-VC2; 10: MC-VC2; 11: M-VC2; 12: 16-VC2; 13: 11-VC2; 14: 39-VC2; 15: RN-VC2; 16: J-VC2; 17: BS-VC2; 18: MSS-VC2; 19: 33-VC2; 20: 17-VC2; 21: 13-VC2; 22: 9-VC2; 23: HC-VC2; 24: JM-VC2; 25: NA-VC2; 26: BE-VC2; 27: 34-VC2; 28: 18-VC2; 29: 14-VC2; 30: 1-VC2; 31: VP; 32: negative control; M2: 1 kb ladder.

Based on the dendrogram of ERIC-PCR shown in Figure 4, the 31 isolates were grouped into 2 main and minor clusters (Cluster 1 and Cluster 2). The main cluster 1 was divided into 2 sub-clusters which are Sub-cluster 1.1 and Sub-cluster 1.2. In Sub-cluster 1.1, the bacterial isolates were 16-VC2, 11-VC2, J-VC2, BS-VC2, MSS-VC2, 9-VC2, HC-VC2, NA-VC2, 18-VC2 and 14-VC2. The highest number of bands was found in this cluster with 6 bands and showed the high DNA banding complexity. In Sub-cluster 1.2, isolates 39-VC2, RN-VC2, 33-VC2, 17-VC2, 13-VC2, BE-VC2, 34-VC2 were found. This had showed high genetic diversity was observed in Cluster 1. The bacterial isolates that were categorized under minor Cluster 2 were SA-VC2, VP, 1-VC2, JM-VC2, JK-VC2, MC-VC2, M-VC2, AB-VC2, FA-VC2, 3051-VC2, 20-VC2, 15-VC2, 7-VC2 and NSA-VC2. This cluster showed the lowest number of band, which is 4 bands. The isolates under each main and minor clusters showed no observable genetic differences or identical among one another in each cluster. Generally, isolates with identical banding patterns or high similarity were commonly originated from the same source (Gevers *et al.,* 2001). This indicated that 3 clusters were representing 3 sources of *V. cholerae*. Moreover, isolates from these 3 clusters were found to have similarity in individual fingerprinting. This showed that the genetic diversity was low and isolates were closely related to each other. The similarity of fingerprinting in the 3 clusters reveal mutation and evolution of the *V. cholerae* strains from a single source.

The dendrogram of BOX-PCR was shown in Figure 5, all of the isolates were grouped into 2 clusters (Cluster A and Cluster B). The bacterial isolates that were categorized under minor Cluster A were 13-VC2, 1-VC2 and VP. This cluster showed the lowest number of bands, which are 2 bands. The main cluster B was divided into 2 sub-clusters that are Sub-cluster B1 and Sub-cluster B2. Sub-cluster B1 comprised of 39-VC2, RN-VC2, 33-VC2, 17-VC2, JM-VC2 and BE-VC2. The highest number of bands was found in this cluster with 7 bands and showed the high DNA banding complexity. The list of isolates in Sub-cluster B2 were isolates SA-VC2, 14-VC2, 18-VC2, 34-VC2, AB-VC2, JK-VC2, MC-VC2, M-VC2, 16-VC2, 11-VC2, J-VC2, BS-VC2, MSS-VC2, 9-VC2, HC-VC2, NA-VC2, 7-VC2, 15-VC2, 20-VC2, 3051-VC2, FA-VC2 and NSA-VC2. Hence, high genetic diversity of isolates was observed in Cluster B. Similarly (in ERIC-PCR), the isolates under each main and minor clusters showed no observable genetic differences among one another in each cluster. These indicated that 3 clusters were representing 3 sources of *V. cholerae*. Furthermore, isolates from these 3 clusters were also found to have similarity in individual fingerprinting, showing that the isolates were closely related to each other.

Comparison of the results between ERIC-PCR and BOX-PCR had proven that BOX-PCR banding patterns have showed great heterogeneity among the isolates although the origin of the isolates were the same and showed a high diversity within a population. The bands produce by BOX-PCR ranged from 2 to 7 which was more than 4 to 6 bands produced by ERIC-PCR. In addition, both of the molecular typing methods gave the relative same or similar result which was 3 clusters with identical fingerprinting isolates. Previous studies have shown that these methods are sufficient to differentiate outbreak strains (Radu *et al.,* 2002; Singh *et al.,* 2001). Both fingerprinting analyses revealed identical patterns among the Bintulu outbreak isolates, suggesting that the outbreak was probably caused by a single or a few clone of *V. cholerae* strains.

CONCLUSION

ERIC-PCR and BOX-PCR can be applied successfully in the study of genetic diversity of *V. cholerae.* In this study of determination of the relatedness of *V. cholerae* isolates from clinical sample from hospital, Bintulu, Sarawak, the low diversity obtained from both molecular typing methods indicated that the outbreak is due to contamination from a single or few sources of *V. cholerae*. It was believed that the patients infected by *V. cholerae* sharing or using the same source of water contaminated by *V. cholerae*. Thus, health officials should be able to monitor environments and thereby predict the emergence of virulent strains, which allow the institution of preventive measures such as water sanitation, public education on proper food handling, and personal cleanliness in cholera-endemic regions.

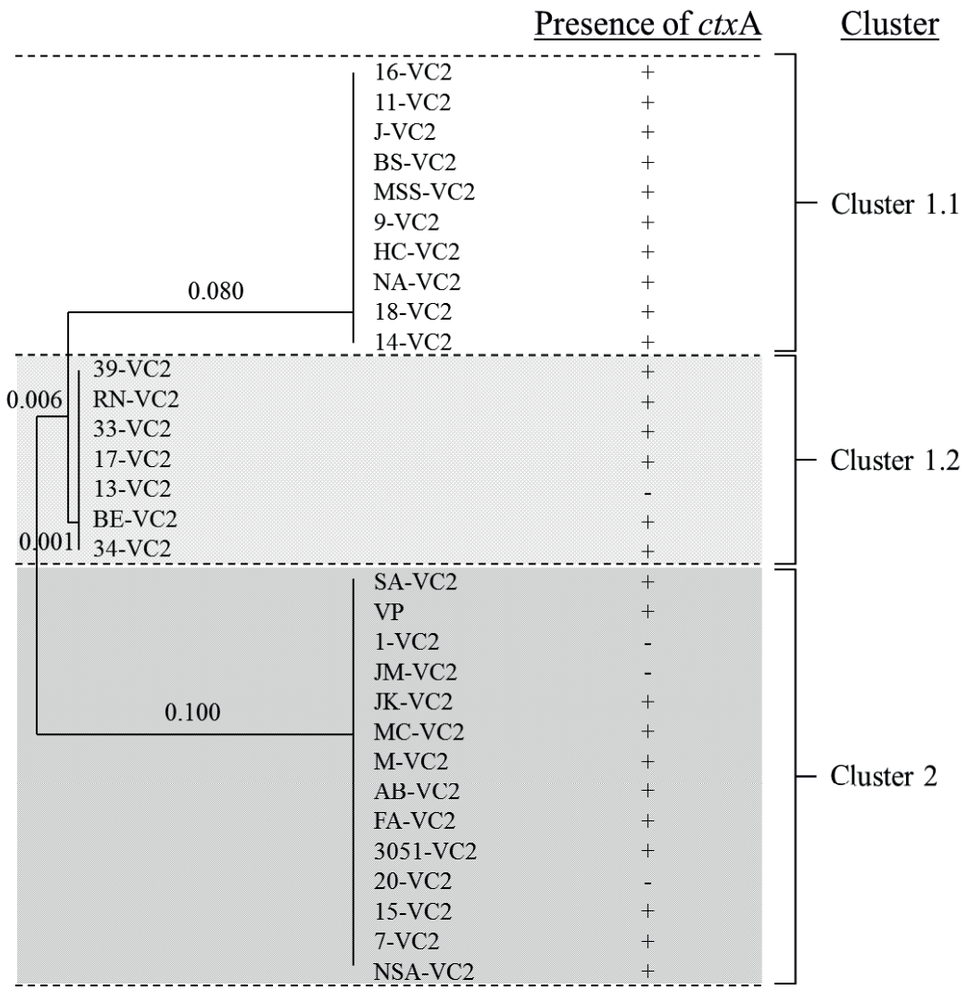

Figure 4: A dendrogram illustrating the relationship between the bacterial isolates analysed by repetitive sequence-based PCR using ERIC primer.

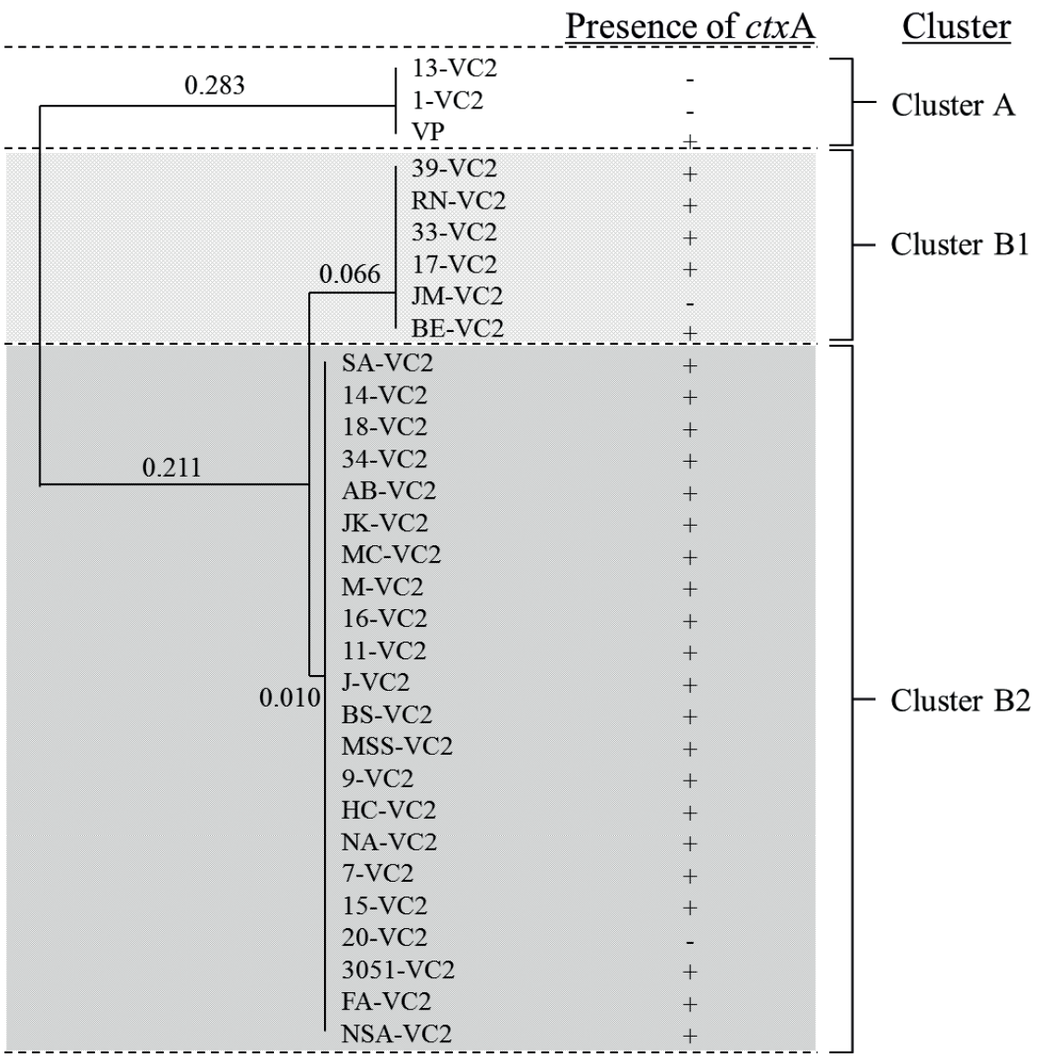

Figure 5: A dendrogram illustrating the relationship between the bacterial isolates analysed by repetitive sequence-based PCR using BOX primer.

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