J. Gen. Appl. Microbiol., **57**, 19–26 (2011)

Full Paper

Comparative PCR-based fingerprinting of *Vibrio cholerae* isolated in Malaysia

Cindy Shuan Ju Teh,[1,2] Kwai Lin Thong,[1,2,*] Ro Osawa,[3] and Kek Heng Chua[4]

[1] *Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[3] *Department of Bioresources Science, Graduate School Agricultural Science, Kobe University, Nada-ku, Kobe 657–8501, Japan*
[4] *Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

(Received June 10, 2010; Accepted October 26, 2010)

***Vibrio cholerae*, the causative agent of cholera, is endemic in many parts of the world, especially in countries poor in resources. Molecular subtyping of *V. cholerae* is useful to trace the regional spread of a clone or multidrug-resistant strains during outbreaks of cholera. Current available PCR-based fingerprinting methods such as Random Amplified Polymorphic DNA (RAPD)-PCR, Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC)-PCR, and Repetitive Extragenic Palindromic (REP)-PCR were used to subtype *V. cholerae*. However, there are problems for inter-laboratory comparison as these PCR methods have their own limitations especially when different PCR methods have been used for molecular typing. In this study, a *Vibrio cholerae* Repeats-PCR (VCR-PCR) approach which targets the genetic polymorphism of the integron island of *Vibrio*s was used and compared with other PCR-based fingerprinting methods in subtyping. Forty-three *V. cholerae* of different serogroups from various sources were tested. The PCR-fingerprinting approaches were evaluated on typeability, reproducibility, stability and discriminatory power. Overall, Malaysian non-O1/non-O139 *V. cholerae* were more diverse than O1 strains. Four non-O1/non-O139 strains were closely related with O1 strains. The O139 strain in this study shared similarity with strains of both O1 and non-O1/non-O139 serogroups. ERIC-PCR was the most discriminative approach (*D* value = 0.996). VCR-PCR was useful in discriminating non-O1/non-O139 strains. RAPD-PCR and REP-PCR were less suitable for efficient subtyping purposes as they were not reproducible and lacked stability. The combination of the ERIC-PCR and VCR-PCR may overcome the inadequacy of any one approach and hence provide more informative data.**

Key Words——ERIC; RAPD; REP; VCR-PCR; *Vibrio cholerae*

Introduction

Cholera, caused by *Vibrio cholerae*, is endemic in many parts of the world, especially the countries which lack proper sanitary management. This pathogen lives freely in aquatic environments and cholera is always associated with poor sanitation (Singh et al., 2001).

Studies on the genomic variation and molecular

* Address reprint requests to: Dr. Kwai Lin Thong, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: +603-79674437 Fax: +603-79675908
E-mail: thongkl@um.edu.my

epidemiology of O1 and O139 *V. cholerae* are often carried out to track sources and spread of the pathogen (Kumar et al., 2007). PFGE has been considered as the gold standard for subtyping bacterial pathogens since it is highly discriminative and reproducible. It has been used to differentiate strains from outbreaks and from sporadic cases of cholera (Chen et al., 2004). However, PFGE needs strict adherence to standardized protocols for comparable fingerprints. Other sequence-based typing such as Multilocus Variable Number Tandem Repeats Analysis (MLVA) and Multilocus Sequencing Typing (MLST) were employed to provide better definitive subtypes of *V. cholerae* strains. However, these highly discriminatory tools (PFGE, MLVA, and MLST) are time-consuming, expensive and laborious if compared with simpler and easier PCR-based methods.

PCR-based DNA fingerprinting approaches such as Arbitrarily Primed PCR, Box-PCR, Random Amplified Polymorphic DNA (RAPD)-PCR, Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC)-PCR, and Repetitive Extragenic Palindromic (REP)-PCR have been used to study epidemiological relationships among *V. cholerae* isolates (Rivera et al., 2001; Singh et al., 2001; Thong et al., 2002). Although these approaches are occasionally associated with poor reproducibility and low discriminatory ability, there are reports of the usefulness of RAPD-PCR, ERIC-PCR, and REP-PCR to differentiate toxigenic and non-toxigenic (Castañeda et al., 2005) or serogroups of *V. cholerae* isolates (Chakraborty et al., 2000).

A relatively new approach, *V. cholerae* Repeats PCR (VCR-PCR), which was developed by Tokunaga et al. (2010), was reported to be useful for differentiating *V. cholerae* strains from different geographic regions and serogroups. This method targets the interspatial region of the repetitive sequences of the Integron Island in *V. cholerae*. However, this was only tested on a panel of toxigenic O1 El Tor and O139 *V. cholerae* (Tokunaga et al., 2010).

Hence in this study, we further evaluated the usefulness of VCR-PCR on Malaysian non-toxigenic and toxigenic *V. cholerae* strains. The discriminatory ability of VCR-PCR was compared with that of RAPD-PCR, ERIC-PCR, and REP-PCR.

Materials and Methods

Bacterial strains. Twenty-three O1, 1 O139 and 19 non-O1/non-O139 serogroups of *V. cholerae* strains previously isolated from human stools, water, algae and seafood were retrieved from the culture collection of Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya and were used in this study.

DNA preparation for PCR. Crude DNA for each strain was prepared by suspending a loopful of bacteria colonies in 50 μl of deionized water, and boiling at 99°C for 5 min. After a short centrifugation, 5 μl of the supernatant (~50 ng of genomic DNA template) was used for PCR.

Random Amplified Polymorphic DNA (RAPD)-PCR. RAPD-PCR was performed using a single primer, 1281 (5′-AACGCGCAAC) as described in Chakraborty et al. (2000) with minor modifications. The PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 200 μM each dNTP, 1.0 μM primer, 2 U *Taq* DNA polymerase (Promega, USA) in a total volume of 25 μl. The PCR program was set at 94°C, 5 min for initial denaturation, followed by 45 cycles of 94°C for 1 min; 36°C for 1 min; 72°C for 2 min, and one cycle of final extension at 70°C for 10 min.

Enterobacterial Repetitive Intergenic Consensus sequence (ERIC)-PCR. The ERIC-PCR was performed in a volume of 25 μl using 1× PCR buffer, 1.5 mM $MgCl_2$, 125 μM each dNTP, 1.0 μM each primer, 2.5 U *Taq* DNA polymerase (Promega). The primers used were ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic et al., 1991). The PCR program consisted of an initial denaturation of 95°C for 5 min, followed by another 35 amplification cycles of 92°C for 45 s, 52°C for 1 min, 70°C for 10 min and a final extension at 70°C for 20 min.

Repetitive Extragenic Palindromic (REP)-PCR. REP-PCR was performed using REP primer (5′-GCG CCG ICA TGC GGC ATT-3′) as previously described in Navia et al. (1999). PCR was carried out in a total of volume of 25 μl containing 2.5 mM $MgCl_2$, 50 μM of each dNTP, 0.6 μM of primer, and 1.0 U *Taq* DNA polymerase (Promega). The PCR program consisted of an initial denaturation of 4 min at 94°C, followed by 35 cycles of 94°C for 1 min, 42°C for 1 min, 68°C for 8 min and a final extension of 8 min at 72°C.

Vibrio cholerae Repeats (VCR)-PCR. VCR-PCR

was carried out as described by Tokunaga et al. (2010) with a minor modification in PCR conditions. Briefly, the PCR mixture (25 µl) contained 2.5 mM $MgCl_2$, 50 µM of each dNTP, 0.5 µM of primer (VCR-5′ TCCCTCTTG AGGCGTTTGTTAC; VCR-3′ AGCCCCTTAGGCGGG CGTTAA), and 1.5 U *Taq* DNA polymerase (Promega). The PCR program consisted of 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 3 min.

Analysis of PCR amplicons. The amplicons generated by RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR were electrophoresed on a 1.2% agarose gel at 90 V for 6 h. A 1 kb DNA marker (Promega) was used as the molecular size standard. The gels were ethidium bromide stained and then visualized by a Gel Doc XR (Biorad, USA).

Analysis of fingerprinting patterns. Banding patterns generated by RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR were analyzed with BioNumerics 6.0 (Applied Maths, Kortrijk, Belgium). All the PCR fingerprint profiles were assigned arbitrary designations. The quantitative differences among the profiles were defined by the Dice coefficient; F. Cluster analysis was carried out based on the unweighted pair group with arithmetic averages (UPGMA) using a position tolerance of 0.15.

Comparison of PCR-based fingerprinting approaches. The typeability, reproducibility, stability, and discriminatory power of RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR were compared (Castañeda et al., 2005; Hunter and Gaston, 1988). The analysis was repeated 3 times on different days using freshly prepared DNA templates.

Results

Four PCR-based methods were used to subtype the 43 *V. cholerae* strains and the relationships among the strains were deduced from the dendrograms (Figs. 1–4).

RAPD-PCR subtyped the 43 strains into 38 profiles (F= 0.4 – 1.0) consisting of 14 to 24 bands ranging from 250 bp to 2,000 bp. The banding patterns were reproducible for all except 4 strains which showed the same banding pattern in 2 out of three repeated tests. Four clusters (RAPD 1–4) were observed at a similarity of 75% (Fig. 1). RAPD 1 comprised 2 non-O1/non-O139 and an O1 strains from environmental sources. RAPD 2 was the largest cluster, consisting of 20 O1 strains, 10 non-O1/non-O139 strains and 1 O139 strain. RAPD 3 and RAPD 4 each contained 2 strains. Four non-O1/non-O139 strains and 1 O1 strain were not clustered in any of the groups. Among the 4 strains, VC6 (O1 serogroup) was distinctly different.

ERIC-PCR subtyped 43 *V. cholerae* strains into 40 profiles (F= 0.67 – 1.0) comprising of 12–14 bands. The banding patterns were reproducible. At the similarity of 75%, 4 clusters were observed (ERIC 1 – ERIC 4) (Fig. 2). ERIC 1 was formed by 2 non-O1/non-O139 strains. ERIC 2 comprised 11 O1 and 3 non-O1/non-O139 strains while ERIC 3 comprised 12 O1 and 3 non-O1/non-O139 strains. Cluster ERIC 4 comprised 8 non-O1/non-O139 strains and 1 O139 strain.

REP-PCR subtyped 43 *V. cholerae* into 35 profiles (F = 0.45–1.0) comprising 12–20 bands. The strains were grouped into 5 clusters (REP 1 – REP 5) at a similarity of 75% (Fig. 3). Each of the clusters REP1 and REP 4 was composed of 6 non-O1/non-O139 strains. Cluster REP 2 was formed by 1 non-O1/non-O139 strain and 1 O139 strain. Two non-O1/non-O139 strains were clustered in REP 3. REP 5 comprised 21 O1 *V. cholerae*.

VCR-PCR gave reproducible results and subtyped 43 strains into 31 profiles (F=0.56–1.0). Based on the similarity of 75%, all the 43 strains were grouped according to their serogroups (O1, O139, non-O1/non-O139) (Fig. 4). There were 3 clusters (VCR1–3). VCR 1 and VCR 3 comprised non-O1/non-O139 *V. cholerae* strains. All O1 strains were clustered in VCR 2.

The typeability, reproducibility, stability and discriminatory power of the 4 approaches were determined and are summarized in Table 1. ERIC-PCR performed the best in typeability and discriminatory power. Based on reproducibility, ERIC-PCR and VCR-PCR correctly assigned the 43 strains into same profiles on 3 repeated tests while RAPD-PCR and REP-PCR were not able to show consistency in the banding patterns.

Discussion

Major outbreaks of cholera rarely occur in Malaysia. However, minor outbreaks with low mortality rates and sporadic cases are reported yearly (http://www.dph.gov.my/survelans/Statistik). Hence, prompt identification of geographical or historical origin of the strain is very important to avoid prolonged outbreak. Four PCR-based methods were evaluated and applied to determine the relatedness of the strains. The time required for the PCR analysis including agarose gel electrophoresis was less than 14 h.

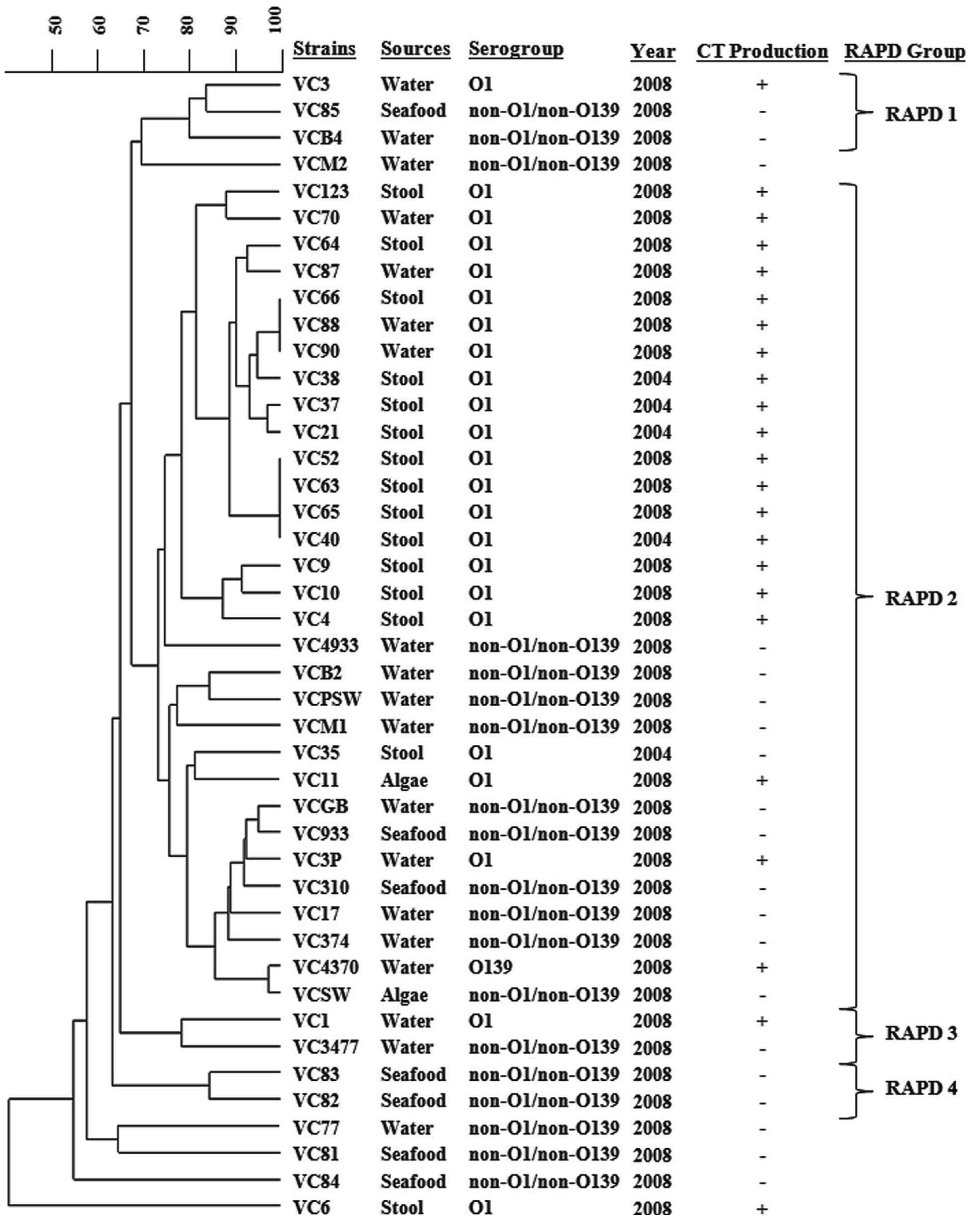

Fig. 1. Dendrogram derived from banding patterns of RAPD-PCR.

In this study, VCR-PCR was useful to differentiate strains of non-O1/non-O139 from the O1 and O139 strains. Therefore, this method is useful to categorize O1, O139 and non-O1/non-O139 *V. cholerae* serogroups. Tokunaga et al. (2010) had reported 8 VCR patterns for O1 and O139 strains from 13 countries. VCR patterns A, B, C, D, F and G were assigned for O1 strains isolated from Asia from 1971−1997, while pattern E was strains isolated from Peru and Bolivia. There was only 1 VCR pattern (Pattern H) for O139 strains isolated from Hong Kong, Denmark, Bangladesh, India, China, Japan, Thailand, Singapore and Nepal from 1993−2006. Based on a visual comparison of VCR patterns for O1 and O139 strains as described by Tokunaga et al. (2010), 12 local O1 strains obtained in this study had similar VCR patterns (A, B, C, D, F and G), except for pattern E. This suggests that these 6 different clones of O1 strains are still widely distributed among Asian countries. DNA fingerprints of strains which differed from the published VCR patterns may be due to mutation at the spacer regions of the integron islands, resulting in altered targets for the primers. In this study, the O139 strain had a VCR pattern similar to pattern H. This is in agreement with Toku-

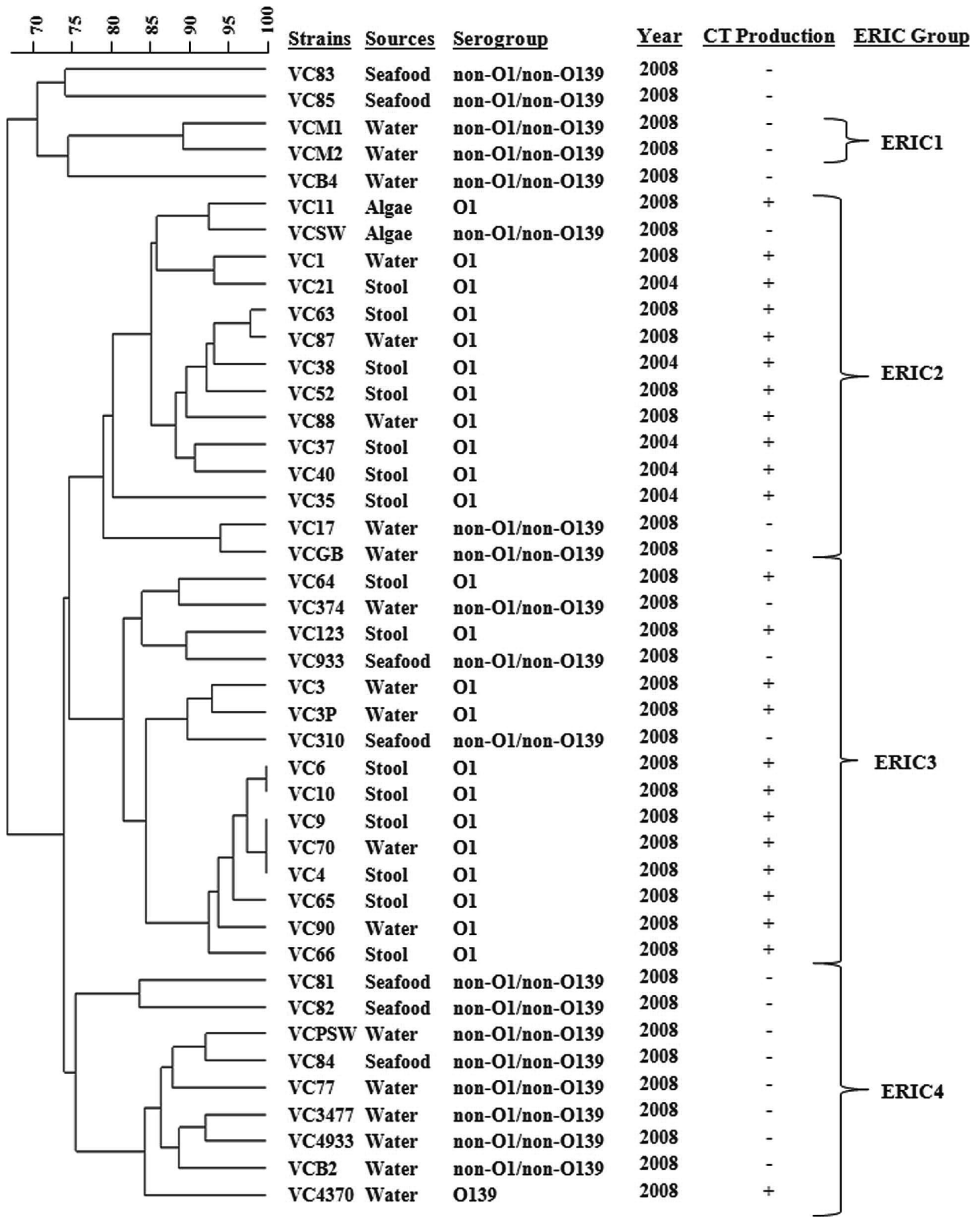

Fig. 2. Dendrogram derived from banding patterns of ERIC-PCR.

naga et al. (2010) that by using the VCR-PCR, all O139 strains formed a homogenous genotype regardless of the geographic origin or time of isolation.

The overall results based on the four PCR-based fingerprinting approaches (RAPD-PCR, ERIC-PCR, REP-PCR, and VCR-PCR) showed that the 43 Malaysian *V. cholerae* were genetically diverse and heterogeneous. The non-O1/non-O139 strains could be divided into 2 major groups: A) Genetically related to O1 strains (>80%), or B) Genetically diverse from O1 strains (<80%). For example, non-O1/non-O139 strains such as VCSW, VC374, VC310, and VC933 were highly related to O1 strains based on RAPD-PCR, ERIC-PCR, REP-PCR and VCR-PCR. Non-O1/non-O139 VCM1 and VCM2 *V. cholerae* were 100% similar when subtyped by REP-PCR and VCR-PCR. However, the similarity of the two strains decreased to 68% and 89% for RAPD-PCR and ERIC-PCR, respectively.

The dendrograms generated based on RAPD-PCR, ERIC-PCR and REP-PCR showed that the O139 strain was closely related to strains of the non-O1/non-O139 serogroup. However, the O139 strain was distinguished from other O1 and non-O1/non-O139 strains by VCR-PCR analysis and was more related to O1 strains.

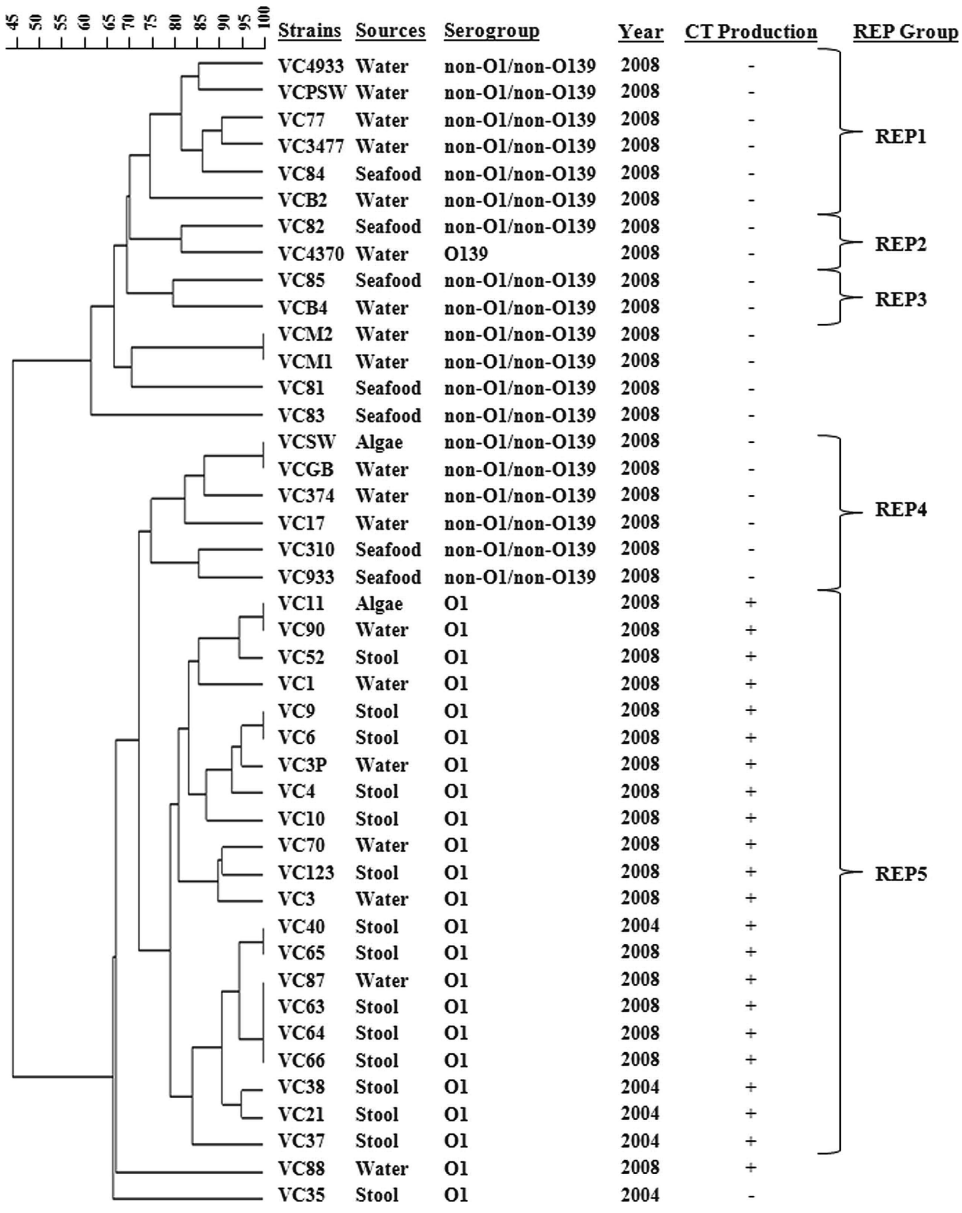

Fig. 3. Dendrogram derived from banding patterns of REP-PCR.

Hence, this particular O139 strain which was isolated from sea water could be the result of horizontal gene transfer of O1 antigen biosynthetic and housekeeping genes of O1 El Tor strains into the non-O1/non-O139 environmental strain. This observation was in agreement with Faruque et al. (2004) and Singh et al. (2001), who described the derivation of O139 strain from O1 and environmental non-O1/non-O139 strains.

Among the four approaches, both RAPD-PCR and ERIC-PCR were highly discriminative but failed to categorize the strains according to serogroups. REP-PCR and VCR-PCR were better in categorizing strains according to serogroups. Overall, all the four approaches gave comparable discriminatory power for distinguishing *V. cholerae* strains ($>$0.90). Although RAPD-PCR and REP-PCR showed higher discriminatory power than VCR-PCR, they were not useful for typing *V. cholerae* strains as neither was reproducible in certain cases as shown in this study (Table 1). Based on the criteria evaluated (typeability, reproducibility, stability and discriminatory power), ERIC-PCR was the better molecular typing tool for discriminating purpose. However, VCR-PCR may be more useful for epidemiological study of *V. cholerae* on a serogroup basis and was

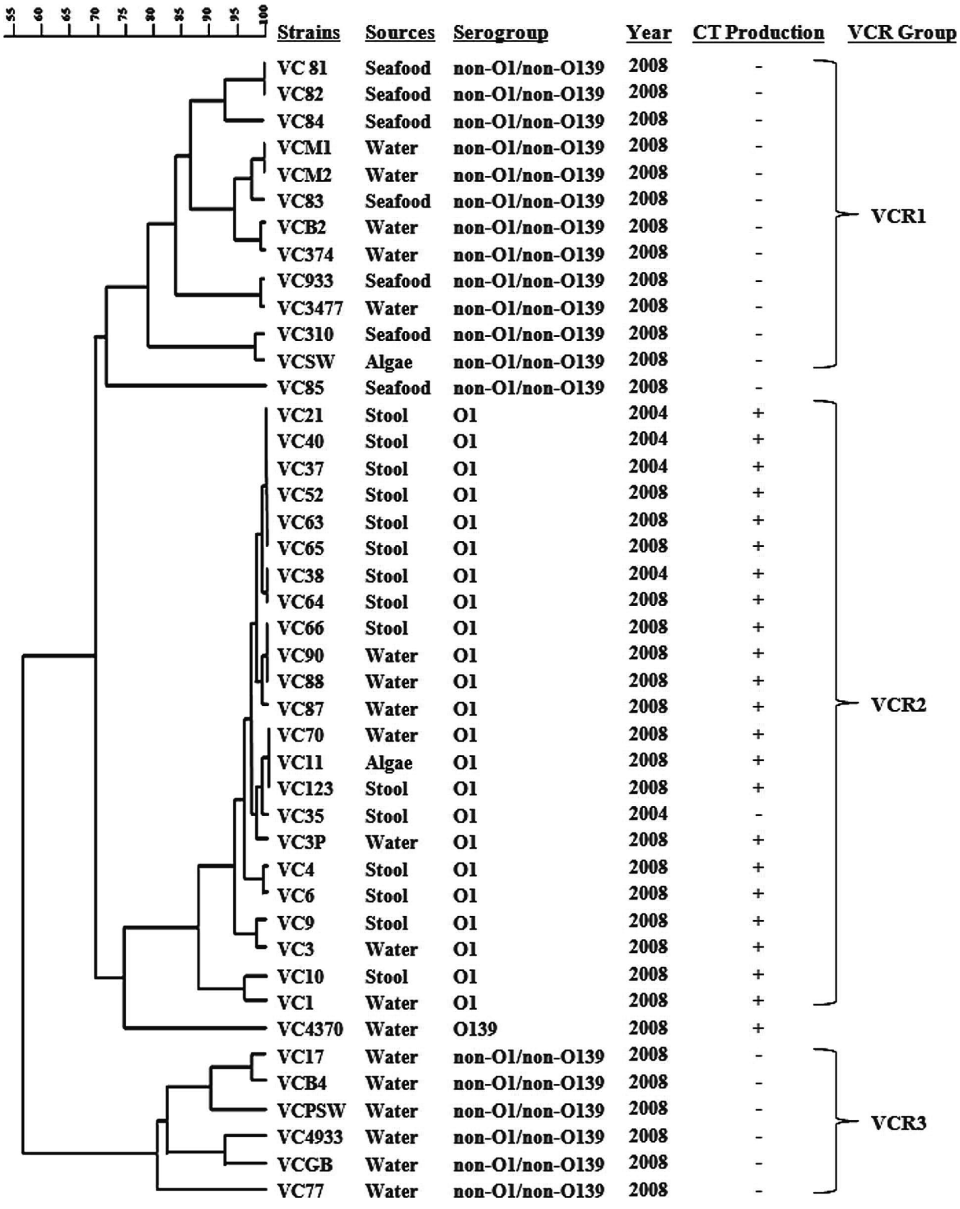

Fig. 4. Dendrogram derived from banding patterns of VCR-PCR.

the only approach which can enable interlaboratory comparison. Therefore, to overcome the inadequacy of a single approach, the combination of ERIC-PCR and VCR-PCR is recommended.

Acknowledgments

This study was funded by a University Malaya Research Grant (PS250/2008C) and MTSF Science and Technology Grant (MTSF1015-2009A). CSJT was supported by University of Malaya Fellowship Scheme. Parts of this work were presented in the 3rd Ditan International Conference on Infectious Disease (2009).

References

Castañeda, N. C., Pichel, M., Orman, B., Binsztein, N., Roy, P. H., and Centron, D. (2005) Genetic characterization of *Vibrio cholerae* isolates from Argentina by *V. cholerae* repeated sequences-polymerase chain reaction. *Diag. Microbiol. Infect. Dis.*, **53**, 175–183.

Chakraborty, S., Mukhopadhyay, A. K., Bhadra, R. K., Ghosh, A. N., Mitra, R., Shimada, T., Yamasaki, S., Faruque, S. M., Takeda, Y., Colwell, R. R., and Nair, G. B. (2000) Virulence genes in environmental strains of *Vibrio cholerae*. *Appl. En-*

Table 1. Comparison of RAPD-PCR, ERIC-PCR, REP-PCR, and VCR-PCR based on typeability, reproducibility, stability and discriminatory power.

	Typeability[a] $T = N_t / N$	Reproducibility[a] $R = N_r / N$	Stability[a] $S = N_s / N$	Discriminatory power[b] $D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j (n_j - 1)$
RAPD-PCR	0.88	0.90 (n=39)	0.6 (2/3)	0.990
ERIC-PCR	0.93	1.00 (n=43)	1.00 (3/3)	0.996
REP-PCR	0.81	0.95 (n=41)	0.6 (2/3)	0.988
VCR-PCR	0.72	1.00 (n=43)	1.00 (3/3)	0.973

N_t, number of strains assigned to a profile; N, number of strains; N_r, number of strains assigned to the same profile on repeated testing; N_s, number of tests in which the same strains were correctly assigned to the same profile on repeated testing; S, number of different profiles; n_j, number of strains belong to the jth type. [a]Formula obtained from Castañeda et al. (2005). [b]Formula obtained from Hunter and Gaston (1988).

viron. Microbiol., **66**, 4022–4028.

Chen, C. H., Shimada, T., Elhadi, N., Radu, S., and Nishibuchi, M. (2004) Phenotypic and genotypic characteristic and epidemiological significance of ctx^+ strains of *Vibrio cholerae* isolated from seafood in Malaysia. *Appl. Environ. Microbiol.*, **70**, 1964–1972.

Faruque, S. M., Chowdhury, N., Kamruzzaman, M., Dziejman, M., Rahman, M. H., Nair, G. B., and Mekalanos, J. J. (2004) Genetic diversity and virulence potential of environmental *Vibrio cholerae* population in a cholera-endemic area. *Proc. Natl. Acad. Sci. USA*, **101**, 2123–2128.

Hunter, P. R. and Gaston, M. A. (1988) Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *J. Clin. Microbiol.*, **26**, 2465–2466.

Kumar, A. R., Sathish, V., Nair, G. B., and Nagaraju, J. (2007) Genetic characterization of *Vibrio cholerae* strains by inter simple sequence repeat PCR. *FEMS Microbiol. Lett.*, **272**, 251–258.

Navia, M. M., Capitang, L., Ruiz, J., Vargas, M., Urassa, H., and Schellemberg, D. (1999) Typing and characterization of mechanisms of resistance of *Shigella* spp. isolated from feces of children under 5 years of agent from Ifakara, Tanzania. *J. Clin. Microbiol.*, **37**, 3113–3117.

Rivera, I. N. G., Chun, J. S., Huq, A., Sack, R. B., and Colwell, R. R. (2001) Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. *Appl. Environ. Microbiol.*, **67**, 2424–2429.

Singh, D. V., Matte, M. H., Matte, G. R., Jiang, S., Sabeena, F., Shukla, B. N., Sanyal, S. C., Huq, A., and Cowell, R. R. (2001) Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: Clonal relationships between clinical and environmental isolates. *Appl. Environ. Microbiol.*, **67**, 910–921.

Thong, K. L., Goh, Y. L., and Cam, P. C. (2002) Molecular subtyping of clinical isolates *Vibrio cholerae* from Malaysia and Vietnam by Pulsed-field Gel Electrophoresis. *Asia-Pac. J. Mol. Biol. Biotechnol.*, **10**, 103–108.

Tokunaga, A., Yamaguchi, H., Morita, M., Arakawa, E., Izumiya, H., Watanabe, H., and Osawa, R. (2010) Novel PCR-based genotyping, using genomic variability between repetitive sequences of toxigenic *Vibrio cholerae* O1 El Tor and O139. *Mol. Cell. Probes*, **24**, 99–103.

Versalovic, J., Koeuth, T., and Lupski, J. R. (1991) Distribution of repetitive DNA sequences in eubacteria and applications to fingerprinting of bacterial genomes. *Nucleic Acids Res.*, **19**, 6823–6831.