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Molecular characterization of clinical isolate of *Vibrio cholerae* isolated from outbreaks cases in Malaysia

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Abstract: A total of 32 clinical strains of *Vibrio cholerae*, including members of the 01 and 0139 serogroup were collected from Klang, Selangor; Penang Island; Samarahan, Sarawak and Miri, Sarawak in Malaysia. In general, all the isolates except the 0139 serotype expressed low resistance to all the antibiotics tested with their Multiple Antibiotic Resistance (MAR) indices ranged from 0.10 to 0.48. The presence of ctx gene that encoded the cholera toxin was confirmed in all these clinical isolates by polymerase chain reaction. The results from the RAPD-PCR were analyzed using the RAPDistance software (Version 1.04). From the dendrogram generated, two main groups were observed which were subdivided into two clusters each. The Selangor's isolates and the 0139 Penang's isolates formed one group whereas the Samarahan, Sarawak isolates and the Miri, Sarawak isolates made up the other group, thus delineating their different sources of origin based on their geographical location.

Keywords: ***Vibrio cholerae*** **O1 and O139, outbreak, antibiotic resistance, PCR**

Introduction

Cholera is an infectious disease of the gastrointestinal tract caused by the Vibrio cholerae serogroup 01 and 0139. The bacteria are typically ingested by drinking water contaminated by improper sanitation or by eating improperly cooked fish, especially shellfish (Kaper *et al.*, 1995; Gunnlaugsson *et al*., 1999). Currently, cholera has caused 7 pandemics globally. The first six pandemics of cholera that originated in Indian subcontinent were caused by the classical biotype of *Vibrio cholerae* 01. But in 1961, the seventh pandemic that originated from the island of Sulawesi in Indonesia and spread to the rest of the world was caused by the El Tor (Carlos and Eduardo, 1996). The seventh pandemic is ongoing, and it continues to cause seasonal outbreaks in many developing countries, especially Bangladesh and India. It is the largest pandemic in terms of geographic area and the number of people affected and it is the longest pandemic ever recorded, lasting more than 42 years (2003) since its beginning. However, in 1992 *Vibrio cholerae* belonging to a 0139 serogroup (also called Bengal strain to indicate its first isolation from the coastal areas of the Bay of Bengal) appeared as large epidemic in India and Bangladesh. The epidemic of *Vibrio cholerae* 0139 has continued to spread and not less than 11 countries in Southern Asia and elsewhere (Carlos and Eduardo, 1996; Faruque *et al.*, 1998). Serogroup 0139 and 01 now coexist and continue to cause large outbreaks of cholera in India and Bangladesh (Sack *et al*., 2004).

In Malaysia, which lies in the cholera endemic South East Asia zone, the period 1991 to 1994 has experienced serious outbreaks of the diseases (Mahalingam *et al.*, 1994). Records from the Infectious Diseases Division of the Ministry of Health showed yearly occurrence of cholera epidemics in the country from 1991 to 2003 (Ministry of Health Malaysia, 2004). Even though most of the outbreak occurred sporadically and of small scale, large outbreak of cholera was also common throughout the country. In 2001 Sabah was badly hits by cholera outbreaks where two third of the total national cases (371/557) were recorded in the state. In the same year, two separate cholera outbreaks occurred in Selangor (Petaling district and Klang district) that resulted in a total of 124 cases. Year 2002 was marked by two

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distinguished cholera outbreak in Malaysia. First, the occurrence of big cholera outbreak among the students of a semi-boarding school in Selangor and secondly the appearance of a small but distinguishable cholera outbreak caused by the newly mutated strain of *Vibrio cholerae* type 0139 or Bengal strain in Penang. The outbreak of cholera due to the serogroup 0139 in Malaysia is not new as in 1988 a cholera outbreak in an army camp in Selangor was caused by this newly mutated strain. The latest outbreaks of cholera were reported from Kota Setar district and Baling district in Kedah in March 2004. At the end of this outbreak, there were 16 confirmed cases and 22 carriers from Kota Setar district whereas 27 confirmed cases and 4 carriers were from Baling district (Ministry of Health Malaysia, 2004).

In Sarawak, one of the two states of Malaysia, which is situated in Borneo Island and separated from the Peninsula Malaysia by the South China Sea, a ten-year trend (from 1994 to 2003) of cholera epidemics had been described recently. The socio-economic activities carried out by the different ethnic group, the natural phenomenon (the La Nina in 1997) and the dry seasons (drought) as well as other contributing factors like the lack of proper treated water supply and the poor sanitary system encountered by the toxigenic *Vibrio cholerae* in the rural area had facilitated the spread of the diseases in Sarawak (Patrick *et al*., 2005). Hence, this endemic trend of cholera indicates the importance of more studies to be conducted on the epidemic strains of Vibrio cholerae circulating in Malaysia. It is equally important to monitor their possible development of antibiotic resistance, particularly to tetracycline, as mass chemophrophylaxis using doxycycline (an analogue to tetracycline) were commonly instituted by the health personnel to the entire community in the cholera-affected area in times of outbreak in Malaysia particularly in the state of Sarawak (Patrick *et al*., 2005).

In this study, we characterize strains of *Vibrio cholerae* 01 biotype El Tor and serotype 0139 isolated from infected patients in four different areas in Malaysia from 1999 to 2003. RAPD-PCR was used for the genotypic characterization whereas the NCCLS method was applied in the antibiotic sensitivity test. We also try to determine whether all these epidemic strains carry the enterotoxin producing gene (the ctx gene), the toxin responsible for the development of acute-profuse diarrhea in the infected person.

Materials and Methods

Source of clinical Vibrio cholerae strains

A total of 30 *Vibrio cholerae* 01 and two strains of *Vibrio cholerae* 0139 isolates were collected from different laboratories in different location across the country (Figure 1) were used in this study. Thirteen of the Vibrio cholerae 01 strains were obtained from Hospital Miri Laboratory, labeled VC1 to VC13, and were isolated from the stool of patients during the cholera outbreak in Miri Division (VC1-VC13) in the year 2003. Seven of the *Vibrio cholerae* 01 strains were obtained from Central Laboratory, Sarawak General Hospital Kuching labeled VC14 to VC20 were isolated from the stools of patients during the cholera outbreak in Samarahan Division in the year 1999. Twelve of the Vibrio cholerae strains were obtained from National Public Health Laboratory, Selangor labeled VC21 to VC32 were isolated from the stools of patients during the cholera outbreak in Selangor (VC21-VC30; 01 strain) and Penang (VC31-VC32; 0139 strain) in the year 2001 and 2002, respectively. All the strains were reconfirmed using slide agglutination with polyvalent 01, monospecific Ogawa-Inaba antisera and with specific anti-0139 antisera obtained commercially (Denka Seiken, Tokyo).

Antibiotic sensitivity testing

The antimicrobial susceptibility tests were performed using National Committee for Clinical Laboratory Standards (NCCLS, 2004) method, which adopted the disc diffusion method as described by Bauer *et al*. (1966). Pure colony of Vibrio cholerae 01 grown on TSA overnight at 37oC were picked using sterile inoculating loop and suspended in a test-tube containing 1.5 ml sterile saline solution (0.9%) to obtain a turbidity of 0.5% Mac Farland standard measured using Densimat, Bio Murieux. A sterile cotton swab was dip into the bacterial suspension and pressed against the side of the testtube before it is over the entire surface of Mueller-Hinton agar. The antibiotic discs used in this study were of Oxoid brand (Oxoid Ltd., England). Antibiotics tested were: penicillin 10 µg (P10), ampicillin 10 µg (AM10), cephalothin 30 µg (KF 30), piperacillin 100 µg (PIP 100), cefuroxime 30 µg (CXM 30), cefotaxime 30 µg (CTX 30), cefoperazone 75 µg (CFP 75), ceftazidime 30 µg (CAZ 30), ceftriazone 30 µg (CRO 30), amp/sulbactam 20 µg (SAM 20), amoxy/clavu acid (augmentin) 30 µg (AMC 30), trimeth./sulfamethoxazole (cotrimoxazole) 25 µg (SXT 25), gentamycin 30 µg (GN30), netilmicin 30 µg (NET 30), ciprofloxacin 5 µg(CIP 5), rifampicin 5 µg (RD

5), chloramphenicol 30 µg (C30), tetracycline 30 µg (TE30), nalidixic acid 30 µg (NA 30), metronidazole 5 µg (MTZ 5), bacitracin 10 µg (B10), trimethoprim 1.25µg (W1.25), sulphamethoxazole 25µg (RL25), streptomycin 10µg (S10). The antibiotic discs were placed on the agar surfaces using mechanical dispensing apparatusand the plates were inverted and incubated at 37°C for 18-24 h. After incubation, the diameters of the zone of inhibition were measured in millimeter and the susceptibility of each strain was interpreted based on the National Committee for Clinical Laboratory Standards (2004). *E. coli* ATCC25922 was used as control with each test.

Genomic DNA preparation

The *Vibrio cholerae* 01 strains were grown in Luria Bertani (LB) broth at 37°C for 24 h in an orbital shaker at 120 rpm. 1.5 ml of the liquid culture was transferred to microfuge tube of 1.5 ml volume. Bacteria cells were harvested by centrifugation at 10, 000 rpm for 2 min. The supernatant was discarded and the pellet washed twice with sterile distilled water and was resuspended in 1.5 ml sterile distilled water. The bacteria suspension was boiled for 10 min to lyses the cells and releases the DNA followed by a 'cold shock' treatment in ice for 10 min, centrifuged at 12,000 rpm for 5 min and the clear supernatant was transferred to a new microfuge tube. The supernatant containing bacterial template DNA was used directly in specific PCR for the detection of ctx genes and RAPD-PCR fingerprinting.

PCR Amplifications and cycling conditions

All amplifications were performed on a thermocycler (Thermo Hybaid, Thermo Electron Corp., USA). The primer pairs used for ctx toxin genes amplifications were C2F (5'-AGGTGTAAAATTCCTTGACGA-3') and C2R (5'-TCCTCAGGGTATCCTTCATC-3') as described by Shangkuan et al. (1995). For RAPD-PCR, primer GEN 1-50-03 (5'-CTTGAGTGGA-3' and GEN 1-50-08 (5'-GAGATGACGA-3') with 50% GC content (Genosys, Biotechnologies, USA) were used. The reaction mixture for the ctx gene amplification was 20 µl and consisted of 2 µl 10X PCR buffer, 1.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTP's mixture, 0.1 µl of 5 U/µl of Taq DNA polymerase (Fermentas, USA), 0.8 µl each of 10 pmol primer (C2F and C2R), 2.0 µl of extracted bacterial DNA and 11.1 µl of sterile distilled water. Amplifications were performed as follows: an initial pre-denaturation at 94°C for 4 min followed by 35 cycles at 94°C for 1 min (denaturation), 60°C for 1min (primer annealing), 72°C for 1 min (DNA extension) and a final elongation was performed at 72°C for 5 min. The RAPD-PCR reactions were performed in a 25 µl volume in 0.2 ml microfuge tube containing reaction mixture of 17.0 µl sterile distilled water, 2.5 µl 10x PCR buffer, 0.5 µl mM dNTP's mix (Fermentas, USA), 1.0 µl of 0.5 µM of either primer GEN 1-50-3 or GEN 1-50-8, 0.5 µl of 5 U/µl Taq DNA polymerase and 2.0 µl of template DNA. Amplifications were carried out under the following conditions: 35 cycles of 1 min at 95°C, 1min at 36°C and 1min at 72°C. A final elongation step at 72°C for 5 min was included.

The PCR amplification products were fractionated by electrophoresis through 1.2% agarose gel (Promega, USA), visualized by staining the gel with ethidium bromide and the gel pictures were taken using a Gel Documentation System (Gene Genius, Syngene, Cambridge, UK).

Cluster analysis of RAPD-PCR

RAPD banding patterns obtained were analyzed by using the RAPD Distance Package Software (ver 1.04) programme. The information obtained from the band patterns on all the gels was scored in the binary data format for each of the primer used. The score '1' indicates the presence of bands, while the score '0' indicates the absence of the band patterns subjected to the software-assisted RAPD Distance Software Package for further analysis in the form of a dendrogram.

Results

The results obtained showed that all the *V. cholerae* strains were found to be resistant to two or more of the 21 antibiotics tested. All the isolates (100%) were sensitive to ampicillin, piperacin, cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, nadilic acid, ciprofloxacin, gentamycin, and netilmycin; 94% were sensitive to amoxicillin/clavulanic acid (augmentin), ampicillin/sulbactam (septrin), trimethoprim, trimethoprim/sulfamethoxazole (co-trimoxazole), chloramphenicol and rifampicin; and 38% sensitive to streptomycin and sulfamethoxazole. However, all the strains were resistant to methronidazole and teicoplanin. Table 1 illustrated the antibiotic resistance profiles and multiple antibiotic resistances (MAR) indices of all the isolates. Three types of antibiotic resistance patterns, with MAR indices of 0.10, 0.19 and 0.48 were exhibited. The *Vibrio cholerae* 01 Selangor strains and the 01 Sarawak strains each produced one type of resistance pattern with MAR indices of 0.1 and 0.19 (Pattern I and II) respectively. Meanwhile, Vibrio cholerae 0139 produced a resistance pattern (Pattern III) with the highest MAR index of 0.48. Thus,

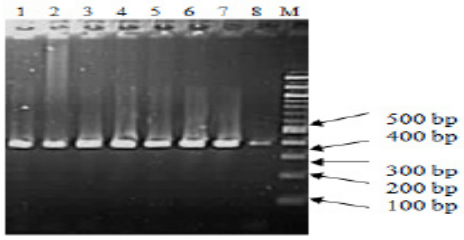

Figure 2. Agarose gel (1.2%) electrophoresis of Ctx gene detected in representative clinical strains of *Vibrio cholerae.*
Lane M: Standard markers of 100 bp ladder. Lane 1-2: VC1-VC2 (strains from Miri), Lane 3-4: VC14-VC15 (strains from Samarahan), Lane 5-6: VC21-VC22 (strains from Selangor), Lane VC7-8: VC31-VC32 (strains 0139 serotype).

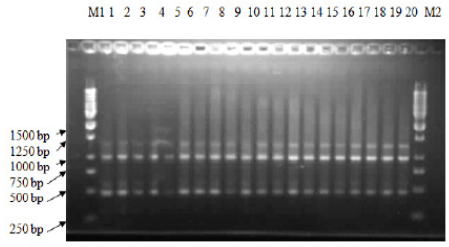

Figure 3a. RAPD fingerprints of type able clinical isolates of *Vibrio cholerae* 01 and 0139 with primer Gen 1-50-03.
Lane M1 and M2: Standard markers of 1kb ladder. Lane 1-20: VC1-VC20.

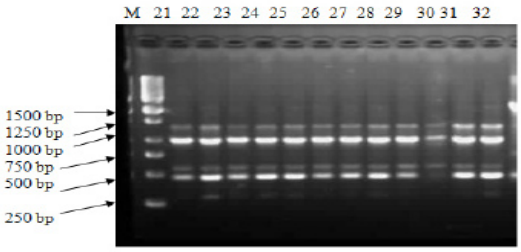

Figure 3b. RAPD fingerprints of type able clinical isolates of *Vibrio cholerae* 01 and 0139 with primer Gen 1-50-03.
Lane M; Standard markers of 1kb ladder; Lane 21-32: VC21-32

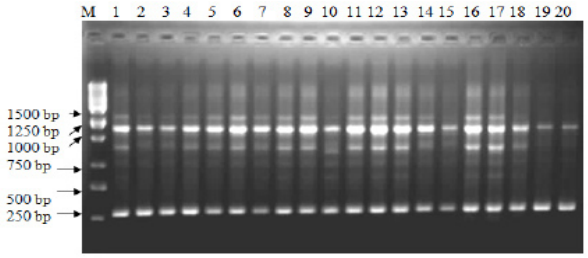

Figure 3c. RAPD fingerprints of type able clinical isolates of *Vibrio cholerae* 01 and 0139 with primer Gen 1-50-08.
Lane M: Standard markers of 1kb ladder. Lane 1-20: VC1-VC20.

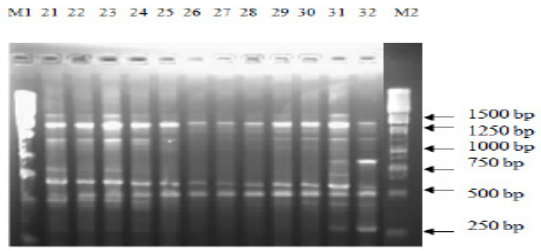

Figure 3d. RAPD fingerprints of type able clinical isolates of *Vibrio cholerae* 01 and 0139 with primer Gen1-5-08.
Lane M1 and M2: Standard markers of 1kb ladder. Lane 21-32: V21-V32.

Table 1. The antibiotic resistance profiles and multiple antibiotic resistances (MAR) index of the Vibrio cholerae

Pattern	Strain No.	Antibiotic Profiles[a]	MAR[b]	No. of isolates/ Total isolates (% of Occurrence)
I	VC21-VC30	TEC, MTZ	0.10	10/32 (31.25)
II	VC1-VC20	S, RL, TEC, MTZ	0.19	20/ 32 (62.5)
III	VC31-VC32	SAM, AMC, S, SXT, W, RL, RD, C, TEC, MTZ	0.48	2/ 32 (6.25)

[a]Tested for AM, ampicillin ; PIP, piperacin; KF, cephalothin; CXM, cefuroxime; CTX, cefotaxime;CAZ, ceftazidime; SAM, ampicillin/ sulbactam; AMC, amoxycillin/ clavulanic acid; GM, gentamicin; NET, netilmicin; S, streptomycin; SXT, trimethoprim/sulfamethoxazole; W, trimethoprim; RL, sulfamethoxazole; RD, rifampin; C, chloramphenicol; TEC, teicoplanin; NA, nalidixic acid; MTZ, metronidazole; TE, tetracycline; CIP, ciprofloxacin.
[b]Note: Calculation of MAR index = $\frac{\text{No. of antibiotics resisted}}{\text{Total no. of antibiotics tested}}$.

the *Vibrio cholerae* 0139 strains possessed the most resistance to the antibiotics tested followed by the *Vibrio cholerae* 01 Sarawak strains while the Vibrio cholerae Selangor strains exhibited the least resistant. The antibiogram obtained clearly demonstrates that all the isolates were grouped according to their geographical location.

Toxigenic strains of *Vibrio cholerae* possessed essential genetic element called the CTX genetic element (Juliana *et al*., 2000; Chen *et al*., 2004). The ctx genes are located in the CTX element (Waldor *et al*., 1996) and encode the cholera toxin CT. This toxin is primary responsible for the severe secretory diarrhea in infected person. Thus we screened all the isolates for the presence of ctx gene. Our results showed the presence of ctx gene of approximately 400 bp in all the tested strains. Figure 2 showed the positive electrophoresis detection of the ctx gene in the representative strains.

The two primers (Gen 1-50-03 and Gen 1-50-08) generated polymorphism in all the 32 strains, producing the amplification patterns for strains differentiation. The RAPD fingerprinting profiles obtained were displayed in Figure 3. The dendrogram generated by the RAPDistance package using the combined data of the two primers for the 32 Vibrio cholerae strains is shown in Figure 4. The dendrogram displayed two main groups (group I and group II) with two main clusters for each group (cluster A, B, C, D) demonstrating the heterogeneity of the isolates. All the 10 Selangor 01 serotype and the 0139 serotype form group I while all the 20 Sarawak 01 serotype made up the group II, thus defining their different sources of clonal origin. Seven and five types of RAPD profiles were produced by the 10 Selangor's strains and the 20 Sarawak's strains, respectively. For the Sarawak's isolates, three of the profiles (Profile S1, S4 and S5) were specific to Miri outbreak while the other two profiles (Profile S2 and S3) were both common to Miri and Samarahan outbreaks. The dendrogram obtained clearly showed that all the isolates were grouped according to their geographical location.

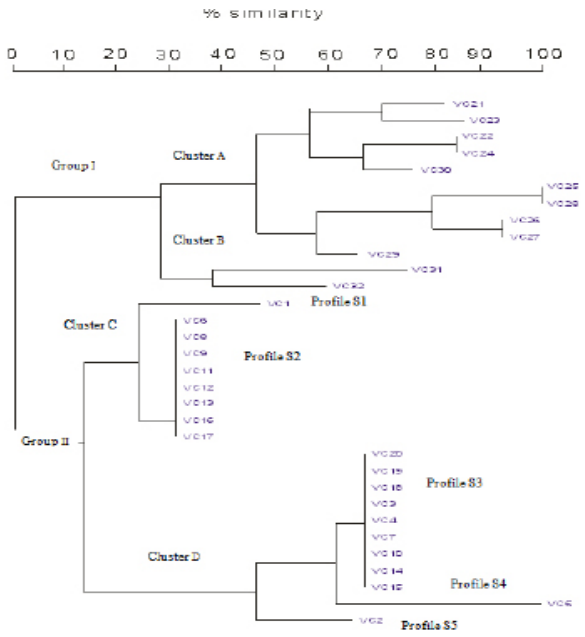

Figure 4. Dendrogram showing relationship between the 30 strains of Vibrio cholerae 01 isolated from Sarawak and Selangor and two strains of *Vibrio cholerae* 0139 isolated from Penang.

Discussion

Resistance to a number of antimicrobial agents has been reported with increasing frequency in *Vibrio cholerae* isolated from around the world (Carlos and Eduardo, 1996; Gunnlaugsson *et al.*, 1999; Son *et al.*, 1999; Garg *et al.*, 2000; Dalsgaard *et al.*, 2000; Folgosa *et al.*, 2001; Kondo *et al.*, 2001; Ramamurthy *et al.*, 2003). On the contrary, in this study all the Malaysian isolates except the 0139 strains showed limited resistance to antibiotics tested. These isolates can be grouped on the basis of their antibiotic resistance pattern into the following categories: - (1) TecMtz, isolates resistance to teicoplanin and methronidazole ; (2) TecMtzSRl, isolates with additional resistance to streptomycin and sulfamethoxazole and (3) TecMtzSRlamAmcSxtWRdC, 0139 isolates resistance to Tec, Mtz, S, Rl, septrin, augmentin, co-trimoxazole, rifampin and chloramphenicol (Table 1). All the Selangor isolates generated the first profile, all the Sarawak isolates generated the second profile and the 0139 strains produced the third profile. These resistance patterns correspond to the geographical location and serotypes of the isolates. The antibiotyping allowed a clear distinction between *Vibrio cholerae* strains and therefore found to be acceptable clonal marker for this set of isolates. As antibiotyping is a first line of method for detecting strains relatedness, it may allow quick and early recognition of a previously defined epidemic strain in a particular environmental setting (Son *et al.*, 1998; Pourshafie *et al.*, 2001).

Kondo *et al.* (2001) demonstrated in their study a strain of tetracycline-resistant El Tor 01 Ogawa unique to the Southern Thailand. Chen *et al.* (2004) then reported the close relationship between tetracycline-resistant El Tor 01 Ogawa from Peninsular Malaysia seafood and that of El Tor 01 Ogawa isolated from patient in Southern Thailand. On the other hand, in this study we reported two different groups of 01 Ogawa from two different locations (Peninsular Malaysia and Sarawak) both sensitive to tetracycline but showed different antibiogram (Table 1). The 01 Ogawa Selangor strains were resistant to streptomycin and metronidazole and the 01 Ogawa Sarawak strains were resistant to the same antibiotics in addition to teicoplanin and metronidazole. Based on their antibiogram these strains could not be linked to the tetracycline-resistant 01 Ogawa strain of the Southern Thailand. The recommended treatment of cholera patients is replacement of lost fluid mainly by oral rehydration solution administered orally or intravenously and often followed by antibiotic therapy (Sack *et al.*, 2004). The antibiotic therapies for cholera patient, commonly with tetracycline or doxycycline, had shown to reduced the fecal elimination of *Vibrio cholerae* and decrease the lethargy of cholera (Kumate *et al.*, 1998). Since doxycycline (an analog of tetracycline) is the drug of choice in the treatment of cholera in Malaysia, the present study indicates that antibiotic treatment of cholera patient in Malaysia is not of worrying concern as all the 32 clinical isolates including the 0139 strains were sensitive to tetracycline and ciprofloxacin. Tetracycline is the drug of choice for the treatment of cholera patient while ciprofloxacin is a common drug for the treatment of diarrhea in recent years (Ramamurthy *et al.*, 2003).

The pathogenicity of cholera is mainly associated with their ability to produce a cholera enterotoxin (CT) encoded in the ctx gene (Spangler, 1992). In this study, specific PCR method was used to detect the presence of ctx gene in all the clinical isolates (VC1-VC32) tested. The ctx genes are hardly detected in Vibrio cholerae strains isolated from environmental samples, including seafood, that are not implicated in outbreaks. *Vibrio cholerae* strains belonging to the 01 and 0139 serogroups almost exclusively carry the ctx and the O serotype is often used as a marker for the toxigenic strains (Chen *et al.*, 2004). However, some researchers had reported that some strains of *Vibrio cholerae* 01 isolated from seafood's and also human samples did not carry the ctx gene. For examples, a DNA probe study carried out in Japan revealed that 26.6% of the *Vibrio cholerae* 01 strains isolated from imported sea foods did not carry the ctx genes

(Minami *et al*., 1991) while a PCR test carried out in Malaysia revealed that a strain of *Vibrio cholera* 01 isolated from human stool during cholera epidemic were negative for the ctx genes (Son *et al*., 1999). Moreover there are many other types of organism causing diarrhea and not all *Vibrio cholerae* are toxigenic and regular examination of Vibrio cholerae isolated from clinical specimen for the presence of ctx gene are needed to ensure the toxigenic *Vibrio cholerae* are the cause of diarrhea. This support the idea that the detection of ctx genes by PCR and the O serotyping would provide the best set of test to confirm toxigenic or epidemic strain of *Vibrio cholerae* 01 and O139 from human sample.

The application of RAPD-PCR in molecular typing of *Vibrio cholerae* has been successful as the method possessed strong discriminatory power, cost effective, rapid and simple to use (Son *et al*., 1998, 1999; Kondo *et al*., 2001; Juliana *et al.*, 2002; Leal *et al*., 2004; Chen *et al*., 2004). In this study, the dendrogram constructed from RAPD fingerprinting grouped the 32 toxigenic *Vibrio cholerae* isolates into two groups with two main clusters for each group (illustrated in Figure 4). Group I consisted of the Selangor isolates and the 0139 strains, which form two main clusters, cluster A and cluster B, respectively. On the other hand, all the Sarawak isolates formed group II, although they were subdivided into two main clusters that is cluster C and D. Seven and five types of profiles were produced by the Selangor isolates and the Sarawak isolates, respectively. This demonstrated higher genetic polymorphism in the Selangor isolates. The profile of the 0139 serotype isolated from Penang was slightly closer to the Selangor 01 serotypes than to these of Sarawak 01 serotypes. The RAPD analysis indicates that the Selangor 01 serotypes and the 0139 serotypes can form different clusters, although 0139 serotypes were proposed to have diverged from an El Tor strains (Mekalanos *et al*., 1997). For the Sarawak isolates, three of the profiles (Profile S1, S4 and S5) were specific to Miri outbreak while the other two profiles (Profile S2 and S3) were both common to Miri and Samarahan outbreaks (Figure 4). Six Miri strains (VC6, VC8, VC9, VC12, and VC13) and two Samarahan strains (VC16-VC17) were having profile S2 and were indistinguishable from each other, and four of the Miri strains (VC3, VC4, VC7 and VC10) and five of Samarahan strains (VC14, VC15, VC18, VC19 and VC20) were also having the same profile (Profile S3). However, three isolates (VC1, VC2, and VC5) each with different profile were shown to be specific to Miri division. Molecular epidemiological surveillance of cholera in areas of endemic infection has also revealed temporal changes in the properties of toxigenic *Vibrio cholerae* and a continual emergence of new epidemic clones, which often replace existing clones (Mitra *et al.*, 1996; Sharma *et al*., 1997; Son *et al*., 1998; Matsumoto *et al*., 2000; Kondo *et al.*, 2001; Juliana *et al*., 2002; De *et al*., 2004).

The immigrants coming into Sarawak to work as laborer in the construction industry and as domestic helper were highly suspected to be in part the sources of *Vibrio cholerae* that caused epidemic in Miri, Kuching and Samarahan division in 1997 and 1998 (Patrick *et al*., 2005) and this might explain the two profiles (profile S2 and S3) common to both Miri and Samarahan division. The immigrants who came from the same geographical location may have shed the S2 and S3 strains in the two divisions. For the three profiles (profile S1, S2 and S3) specific to Miri, although may originated from the same clone as the other two profiles, it is possible that these strains may transmitted earlier to Miri division and may persisted in the coastal seawater of Miri for a longer period and may be the main cause of repeated cholera outbreaks in Miri in the last decades (Patrick *et al*., 2005). These strains, although had retain their antibiogram, could have undergone genetic re-assortment indicating the importance of genetic monitoring of emerging strains of the organisms in Sarawak. However, join study should be done with Indonesian counterpart to compare the molecular phenotype of the S1-S5 strains with the Indonesia isolates in order to confirm the origin of the Sarawak isolates.

In conclusion, it was demonstrated in this study that antibiotyping and random amplification of polymorphic DNA analysis had proved to be useful in discriminating the isolates and useful information for treatment of cholera patient, monitoring and control of cholera disease by the health authorities. More specifically, the unique fingerprints will help map sources of Vibrio cholerae in future outbreaks as well as to investigate the relatedness of isolates from different sources.

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References

Bauer, A. W., Kirby, W. M M., and Sherris, J. C. 1966. Antibiotic susceptibility testing by a standard single discs method. American Journal of Clinical Microbiology 45: 493-496

Carlos S. and Eduardo G., 1996. Cholera: Overview of Epidemic, Therapeutic, and Preventive Issues Learned from Recent Epidemics. International Journal of Infection 1:37-46.

Chen, C.H., Shimada, T., Elhadi, N., Radu, S., and Nishibushi, M. 2004. Phenotypic and Genotypic characteristic and epidemiological significance of ctx $^{+}$ strains of *Vibrio cholerae* isolated from seafood in Malaysia. Applied and Environmental Microbiology 70: 1964-1972.

Dalsgaard, A., Forshund, A., Petersen, A., Brown, D. J., Dias, F., Monteiro, S., Molbak, K., Aaby, P., Rodrigues, A., Sandstorm, A. 2000. Class 1 Integron-Borne, Multiple-Antibiotic Resistance Encoded by 150 kilobase Conjugative Plasmid in Epidemic *Vibrio cholerae* 01 strains Isolated in Guinea-Bissau. Journal of Clinical Microbiology 38:. 3774-3779.

De, K., Ramamurthy, T., Faruque, S.M., Yamasaki, S., Takeda, Y., Nair, G.B., and Nandy, R.K. 2004. Molecular characterization of rough strains of *Vibrio cholerae* isolated from diarrheal cases in India and their comparison to smooth strains. FEMS Microbiology Letter 232: 23-30.

Faruque, S. M., Albert, M.J., and Mekalanos, J. 1998. Epidemiology, Genetics, and Ecology of Toxigenic *Vibrio cholerae*. Microbiology and Molecular Biology Review 62: 1301-1314.

Folgosa, E., Mastrandrea, S., Cappucinelli, P., Uzzau, S., Rappelli, P. Brian M.J., and Colombo, M.M. 2001. Molecular identification of pathogenicity gene and ERIC types in *Vibrio cholerae* 01 epidemic strains from Mozambique. Epidemiology and Infection 127: 17-25.

Garg, S., Chakraborthy, P., Basu, I., Datta, S., Rajendran, K., Bhattacharya, T., Yamasaki, S., Bhattacharya, S.K., Takeda, Y., Nair, G.B. and Ramamurthy T. 2000. Expanding multiple antibiotic resistances among clinical strains of *Vibrio cholerae* isolated from 1992-1997 in Calcutta, India. Epidemiology and Infection 124: 393-399.

Gunnlaugsson G., Angulo F. J., Einarsdottir J., Passa A., and Tauxe R. V. 1999. Epidemic Cholera in Guinea-Bissau: The challenge of Preventing Deaths in Rural West Africa. International Journal of Infection 4: 8-13

Juliana, M.V.B., Ronaldo M.A., Irma N.G.R., Dalia P. R., David K.R.K. and Campos C, 2002. Prevalence of virulence-associated genes in clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 1999. FEMS Microbiology Letters 215: 15-21.

Kaper, J.B., Morris, J.G., and Levine, M.M. 1998. Cholera. Clinical Microbiology Review 8: 48-86.

Kondo, S., Kongmuang, U., Kalnauwakul, S., Matsumoto, C., Chen, C.H., and Nichibuchi, M. 2001. Molecular epidemiologic analysis of *Vibrio cholerae* 01 isolated during the 1997-8 cholera epidemic in Southern Thailand. Epidemiology and Infection 127: 7-16.

Kumate, J., Sepulveda, J. and Gutierrez G. 1998. Cholera epidemiology in Latin America and perspectives for eradications. Bulletin Institute Pasteur 96: 217-226.

Leal, N.C.; Sobreira, M., Leal Balbino, T. C., de Ameida, A.M., de Silva M. J., Mello, D.M., Seki, L.M. and Hofer, E. 2004. Evaluation of RAPD-based typing scheme in a molecular epidemiology study of *Vibrio cholerae* 01, Brazil. Journal of Applied Microbiology 96: 447-454.

Mahalingam, S., Cheong, Y.M., Kan, S., Rohani, M. Y., Jamuna, V. and Pang, T. 1994. Molecular Epidemiologic Analysis of *Vibrio cholerae* 01 Isolates by Pulsed-Field Gel Electrophoresis. Journal of Clinical Microbiology 32: 2975-2979.

Matsumoto, C., Okuda, J., Inhabit, M., Wang, M., Gag, P., Rammamurthy, T., Wong, H.C., De Polar, A., Kim V. B., Albert, M. T. and Nishibuchi, M. 2000. Pandemic spread of an 03: K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrary primed PCR and tox RS sequence analysis. Journal of Clinical Microbiology 14: 129-134.

Mekalanos, J.J., Rubin E.J., and Waldor M.K. 1997. Cholera:Molecular basis for emergence and pathogenesis. FEMS Immunology and Medical Microbiology 18: 241-248.

Minami, A., Hashimoto, S. and Abe, H. 1991. Cholera enterotoxin production in *Vibrio cholerae* 01 strains isolated from environment and from humans in Japan. Applied and Environmental Microbiology 1991: 57: 2152-7.

Ministry of Health Malaysia. 2004. Internal Report. Infectious Diseases Division, Ministry of Health, Malaysia.

Mitra, R., Basu, A., Dutta, D., Nair, G.B., and Takeda, Y. 1996. Resurgence of *Vibrio cholerae* 0139 Bengal with altered antibiogram in Calcutta, India. Lancet 348: 1181.

National Committee for Clinical Laboratory Standards, 2004.Performance Standards for Antimicrobial Susceptibility Testing. Fourteen Informational Supplement Document Number: NCCLS/CLSI M100-514. Clinical and Laboratory Standards Institute, Villanova, P.A., USA.

Patrick, G.B., Jurin, W.G., Son, R., Suhaimi, N., Fatimah, A.B., Meting, B., Adom, B., Clement, W. D., Selvanesan, S., Faizul, M., Rody, j., Nakaguchi, Y., and Nishibuushi, M. 2005. Journal of Southeast Asian Studies 43: 109-140.

Pourshafie, M., Grimont, F., Kohestani, S., and Grimont, P.A.D. 2002. A molecular and phenotypic study of *Vibrio cholerae* in Iran. Journal of Medical Microbiology 5: 392-398.

Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S.K., Shimada, T., Takeda, T. and Nair, G.B. 1993. Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in southern India. Lancet 341: 703-704.

Ramamurthy, T., Yamasaki, S., Takeda, Y., and Nair, G.B. 2003. *Vibrio cholera*e 0139 Bengal; odyssey of a furtutious variant. Microbe and Infections 5: 329-344.

Sack, D. A., Sack, R.B., Nair, G. B. and Siddique, A.K., 2004. Cholera. Lancet 363: 223-33.

Shangkuan, Y. H. and Lin, H. C. 1998. Application of Randomly Amplified Polymorphic DNA analysis to differentiate strains of *Salmonella Typhi* and other *Salmonella* spp. Journal of Applied Microbiology 85: 693-702.

Sharma, C., Nair, G.B., Mukhopadhyay, A.K., Bhallacharya, S.K., Ghosh, R.K., and Ghosh, A. 1997. Molecular characterization of *Vibrio cholerae* 01 biotype El Tor strains isolated between 1992 and 1995 in Calcutta, India evidence for the emergence of a new clone of the El Tor biotype. Journal of Infectious Diseases 175: 1134-1141.

Son, R., Ho, Y.K., Lihan, S., Yuherman, Rusul, G.,Yasin, R.M., Khair, J., and Elhadi, N. 1999. Molecular characterization of *Vibrio cholerae* 01 and non-01 from human and environmental sources in Malaysia. Epidemiology and Infection 123: 225-232.

Son, R., Rusul, G., Samuel, L., Yuherman., Senthil, S., Rasip. A., Naseldin, E.H., and Nichibuchi, M. 1998. Characterisation of *Vibrio cholerae* 0139 Bengal isolated from water in Malaysia. 1998. Journal of Applied Microbiology 85: 1073-1077.

Spangler, B.D. 1992. Structure and function of cholera toxin and related *Escherichia coli* heat-stable enterotoxin. Microbiology Review 56: 622-47.

Waldor, M. K., Tschape, H. and Mekalanos J. J. 1996. A new type of conjugative transposon encoded resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* 0139. Journal of Bacteriology 178: 4157-65