ORIGINAL ARTICLE

Detection of *Vibrio Cholerae* 01 from Aquatic Environment in Sarawak

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Summary

The detection of *Vibrio cholerae* 01 from the aquatic environment of Daro and Bintulu in Sarawak was carried out following an outbreak of cholera. Conventional culture methods and detection of *ctx* gene by polymerase chain reaction technique were carried out on 80 water samples. Only one sample was positive by culture methods while 8 were positive by PCR. DNA finger printing by pulsed-field gel electrophoresis showed that the clinical isolates in Daro and Bintulu were genetically identical while the environmental isolate was closely related. Recovery of *Vibrio cholerae* by culture method is poor and newer methods of detection should be developed.

Key Words: Cholera, Aquatic environment, *ctx* gene, Pulsed-field gel electrophoresis

Introduction

An outbreak of cholera occurred in the district of Daro, Sarawak at the end of May 1998, two months after the last cholera outbreak in Bintulu district.

Epidemiological studies suggested that the source of this year's cholera outbreak in certain areas of Sarawak could possibly be the rivers or streams which were the only alternative sources of water for washing, bathing or other water activities during a prolonged drought season. Unfortunately, no *Vibrio cholerae* 01 was isolated from these suspected rivers, streams or canals by the local health authority.

At the request of the Sarawak State Department of health, this study was undertaken to detect *Vibrio cholerae* 01 from the aquatic environment in the affected areas in Daro district, where the cholera outbreak was still ongoing and Bintulu where the cholera outbreak had just ended by applying conventional and molecular techniques. DNA fingerprinting by PFGE was also be carried out on the strains isolated from clinical and the environment to determine the strain relatedness.

Table I
Sampling sites from Daro and Bintulu.

District	Sampling Sites	Method for detection (no.)			Positive samples	
		E	M	D	culture	PCR
Daro	Canal near Nyawai Longhouse	3	2	0	-	+(1)
	Canal near Kg. Nangar	3	2	0	-	+(2)
	Nangar river	3	2	0	-	-
	Pond in Kg. Nangar	2	0	0	-	+(2)
	Parit China river at Kg. Nangar	3	2	0	-	-
	Kuala Matu river	3	2	0	-	+(1)
	Canal near Kuala Matu	2	2	0	-	-
Bintulu	Kg Sebuan Besar river	3	2	0	-	+(1)
	Pond in Rumah Nyuak	2	0	0	-	-
	Stream BDA Pasir Putih	3	2	0	-	-
	Sibiu river	2	2	4	+	+(1)
	Kemena river	3	2	0	-	-
	Anau river near Rumah Lunga	2	2	3	-	-
	Anau river near Rumah Muking	2	2	4	-	-
	Jelai river	3	2	4	-	-

Key: E = enrichment in APW, M= Moore swabs, D = Direct plating on Monsur's agar

Materials and Methods

Sampling sites

Seven sampling sites in Daro and eight from Bintulu, which consist of aquatic environments such as ponds, canals and rivers were chosen (Table I). The sites were identified based on where cases of cholera and contacts were isolated. A total of 80 samples were collected from these sites which consisted of 42 water samples for direct enrichment method, 23 Moore swabs and 15 water samples for direct plating.

Methods

Enrichment in alkaline peptone water

A total of 39 samples were collected for enrichment in alkaline peptone water. Fifty mls of concentrated alkaline peptone water (10x strength APW) in sterile containers (50 mls sterile conical tubes Blue Max MPS 2073 Becton Dickinson) and sterile plastic bags (Nasco Whirl-Pak®) were taken to the field for the collection of water samples. At the sampling site, about 450 mls of water samples were collected and poured into Whirl-Pak plastic bag containing 50 ml 10x strength APW.

After 6 hours of incubation at 37°C, two loopfuls of the sample were taken just below the surface of the water and plated out on two Monsur's agar. The plates were then incubated overnight at 37°C.

Moore swabs

Moore swabs were used only for sampling of running river or canal water. The swabs were suspended by the nylon line in the water at the sampling site and left in place for 1 day. A total of 26 swabs were removed and transferred into sterile Whirl-Pak plastic bags after cutting the nylon line. About 250 ml of single strength APW (single strength) was poured into the bags to submerge the swabs and the samples incubated at 37°C for 6 hours before plating out on two plates of Monsur agar as described above.

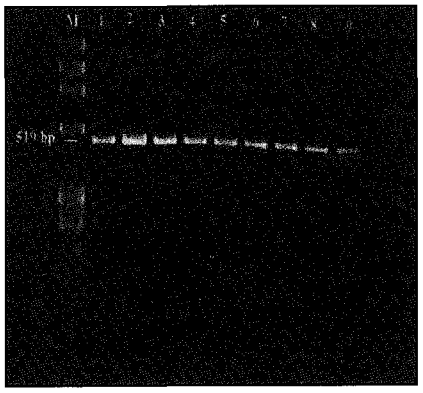

Fig.1: Detection of *ctx* gene in environmental samples. M = (X174/HaeIII marker; Lane1 = DNA from Vibrio cholerae 01; Lanes 2-9 = positive environmental samples.

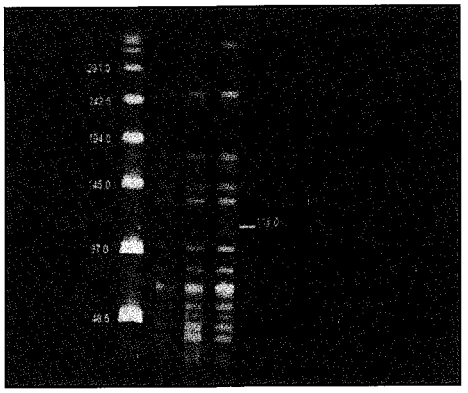

Fig. 2: Pulsed-field gel electrophoresis patterns of Vibrio cholerae 01. Lane 1 = Lambda ladder; Lane 2 = clinical isolate from Daro; Lane 3 = clinical isolate from Bintulu; Lane 4 = environmental isolate from Sibiu River.

Direct plating of fresh samples

In addition to enrichment method and swab sampling, 15 fresh water samples were taken in Bintulu district for direct plating on Monsur's agar without prior enrichment in APW. The samples were from Sibiu River (4), Anau river near Rumah Lunga (3) and Rumah Muking (4), and Jelai river (4). The agar plates were then incubated at 37°C overnight.

Screening

The Monsur agar plates were incubated at 37°C for 18 to 24 hours. Greyish, greyish-black or greyish with black centre colonies from Monsur agar were screened for oxidase activity. Oxidase positive colonies were serotyped with *Vibrio cholerae* 01 polyvalent antisera. Positive polyvalent 01 colonies were further serotyped with specific sera Inaba and Ogawa and confirmed using conventional biochemical tests.

Polymerase chain reaction

Polymerase chain reaction technique was carried out for the detection of toxin gene (*ctx* gene) of *Vibrio cholerae*. All the samples collected were tested for ctx gene. For samples that were enriched in APW, 1.5 mls of the water were taken just below the surface and transferred into sterile microfuge tubes. The tubes were centrifuged at 12000 rpm for 5 minutes and the pellet was resuspended in 500 µl sterile distilled water. After boiling for 10 minutes to release the DNA, the tubes were recentrifuged to remove cell debris.

Primers that amplify a 519-bp region spanning *ctx*A and *ctx*B cistrons were used (GTG GGA ATG CTC CAA GAT CAT CG, positions 1129 to 1151 and ATT GCG GCA ATC GCA TGA GGC GT, positions 1625 to 1647). Five µl of the supernatant were added to final volume of 50 µl reaction mixture consisting of 5µl 10X PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 50 (M of each of the four deoxynucleoside triphosphates and and 0.25 µM of each primer. PCR was carried out in a GeneAmp 2400 PCR System thermal cycler (Perkin Elmer Cetus, USA) with the following conditions: denaturation at 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 61°C and 2 min at 72°C and a final extension step at 72°C for 5 min. The PCR products were electrophoretically separated in 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and visualised under UV transilluminator.

DNA fingerprinting by pulsed-field gel electrophoresis (PFGE)

For DNA fingerprinting, two clinical isolates were obtained, one was from a patient in Daro Hospital and another from Bintulu hospital. An environmental isolate was obtained from Sibiu River.

DNA from these isolates was prepared following a modification of the method by Smith and Cantor[1]. Briefly, an overnight culture of the *Vibrio cholerae* was adjusted to a concentration of 1 X 10^9 cfu/ml. After washing in TE buffer (10mM Tris-HCl, 50mM EDTA; pH7.5), 200 µl of the bacterial suspension was added with an equal volume of 2% low-melting point agarose and mixed well before being allowed to solidify in a plug mould (Bio-Rad Laboratories). The gel plugs were incubated overnight at 37°C in 2 ml lysis buffer (6 mM Tris-HCl, 100 mM EDTA, 1 M NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% N-Laurylsarcosine, pH 7; lysozyme (100mg/ml) and Rnase (10 mg/ml)(with gentle shaking overnight. The lysis buffer was then replaced with proteolysis buffer (0.5 M EDTA, 1% N-Laurylsarcosine, pH 9.5; proteinase K 100mg/ml) and incubated at 50°C overnight with gentle shaking. The plugs were then washed on ice with TE buffer. A slice of the plug (2.5 mm) were cut and digested with 40 U of *Not*1 in recommended restriction enzyme buffer and incubated at 37°C overnight.

The digested DNA were then electrophoresed in 1.2% agarose (Bio-Rad Laboratories) using a contour-clamped homogeneous electric field (CHEF-DRIII) apparatus from Bio-Rad Laboratories. The following parameters were used 6 s for 8 hrs; 15 s for 8 hrs followed by 25 s for 8 hrs. This will result in migration of DNA. The gel was then stained with ethidium bromide and photographed under ultraviolet light using Gel Doc 1000 (Bio-Rad Laboratories).

The DNA digested by *Not*1 will appear as bands. Differences between isolates were determined by visual comparison of the DNA bands. Isolates were considered identical if there was complete concordance of the DNA fragment profiles[2].

Results

By the conventional culture methods only one sample was found to grow *Vibrio cholerae* 01. The positive culture was a sample taken from Sibiu river near the junction with Laru River at Bintulu District. This sample was prior enriched in alkaline peptone water before being subcultured onto Monsur's agar. All samples from Daro District were culture negative for *Vibrio cholerae* 01. The positive strain was serotyed Ogawa, biotyped El-Tor and confirmed using the usual array of biochemical tests.

A total of 8 out of 80 samples were found to be positive by PCR (Figure 1). Six of them were from water samples enriched in APW while 2 were from Moore swabs samples. All of the positive samples were prior enriched in alkaline peptone water before being tested for PCR of *ctx* gene. Six of the samples were taken from sites in Daro District while the other 2 were from Bintulu (Table I). This included the culture positive sample from Bintulu.

PFGE results of the clinical strains and the environmental strain are shown in Figure 2. In this study the DNA fragment profiles of the clinical isolates from Daro and Bintulu were indistinguishable. The environmental isolate from Sibiu River had an additional band at 115 base pair position.

Discussion

Several culture techniques and a molecular method were applied in this study with the main aim of detecting *Vibrio cholerae* 01 and not for comparison purposes.

In this study we managed to get only one culture positive for *Vibrio cholerae* 01. The diluting capacity of the flowing rivers makes it difficult to isolate *Vibrio cholerae*. Other environmental factors may also affect the presence and rate of isolation of the organism. Results of several studies have shown that various biological and physicochemical factors such as pH and temperature influence the growth, survival and distribution of *Vibrio cholerae* in aquatic environments[3,4].

It is much easier to isolate *Vibrio cholerae* from clinical cases because of the heavy organism load in the patient's samples without prior enrichment. Enrichment in alkaline peptone water is recommended in situations where low concentrations of organisms in the specimen are anticipated. *Vibrio cholerae* is at its optimal growth rate at 6 hours incubation and tend to concentrate near the surface of the alkaline peptone water. After 6 hours of incubation at 37°C a loopful of the sample taken just beneath the surface of the alkaline peptone water should be inoculated onto the selective media such as Monsur agar. During sampling of water samples it is advisable to immediately place the specimen into alkaline peptone water because *Vibrio* species are generally quite sensitive to drying, exposure to sunlight, and the development of an acid pH.

Cholera toxin gene is only present in toxigenic *Vibrio cholerae*. Amplification of genes expressing cholera toxin subunits (*ctx*A) and B (*ctx*B) by polymerase chain reaction technique have been shown to be successful in distinguishing toxigenic from non-toxigenic *Vibrio cholerae* strains in pure cultures[5]. In a previous cholera outbreak, this technique has been successfully applied for early detection of cholera cases and contacts[6]. In this study we are able to detect cholera toxin genes in eight of the samples. All the positive samples were taken from samples enriched in alkaline peptone water. Prior enrichment in APW could have helped in multiplication of the organism to allow detection by PCR even though studies have shown that a DNA from a single bacterium can be detectable by PCR.

With conventional culture methods, serotyping for presumptive diagnosis of *Vibrio cholerae* can only be done if there is confluent growth; otherwise the suspected colonies need to be subcultured again on blood agar. Non-toxigenic strains or *Vibrio cholerae* non-01 colonies may appear similar to the toxigenic strains *Vibrio cholerae* 01. It would be labour intensive to screen all suspected colonies especially to less experienced laboratory personnel. The identification of cholera toxin is an important step in the diagnosis of cholera because only toxin producing strains have been associated with severe, watery diarrhoea and epidemics. The PCR test for cholera toxin can be adopted easily in a laboratory equipped with a thermal cycler.

Vibrio cholerae 01 can be isolated from patients and surface water during epidemics but not found in the environment during interepidemic periods. Surface water sources such as rivers, ponds and lakes have a very limited potential to act as a reservoir of *Vibrio cholerae*[7, 8]. Recovery of *Vibrio cholerae* from the environment by culture methods is usually poor even from sites where there are active cases[9]. *Vibrio cholerae* often persist in the environment as viable but non-culturable forms[10]. They were shown to persist for long periods in blue green algae and zooplankton thus these act as environmental reservoirs for *Vibrio cholerae*[9, 11]. This non-culturable *Vibrio cholerae* is in a dormant form and is not toxigenic and will revert to its toxigenic nature when exposed to conditions favourable for its growth. Fluorescent-monoclonal antibody technique has been shown to be useful in detecting viable but nonculturable *V. cholerae 01* in aquatic samples [9]. Further studies need to be carried out to determine whether the PCR test employed in this study can be used to detect the non-culturable forms. However this study showed that the technique is useful in detecting toxigenic *Vibrio cholerae* 01 in water samples during the outbreak and post-outbreak period. The results showed that the sensitivity of PCR technique could be exploited to determine the presence of toxigenic *Vibrio cholerae* in the aquatic environment. Information on contaminated water supplies can be given rapidly to public health personnel so that early control and preventive measures can be undertaken to prevent spread of cholera.

The aim of strain typing is to provide laboratory evidence that epidemiologically related isolates collected during an outbreak of disease are also genetically related and thus represent the same strain. The discriminative power and reproducibility of PFGE technique for molecular typing is well established [12]. In this study the DNA fragment profiles of the clinical isolates were indistinguishable, showing that they were genetically identical. This means that the *Vibrio cholerae* causing the outbreak in Daro is the same strain as that causing cholera in Bintulu. The environmental isolate from Sibiu River had an additional band at position 115 base pair. The additional DNA fragment seen in the environmental isolate means that this isolate is closely related to the strains obtained from clinical specimens, implicating that they are derived from the same clone.

An isolate is considered to be closely related to the outbreak strain if its restriction patterns (DNA fragment profiles) differs from outbreak pattern by changes consistent with a single genetic event i.e., point mutation or insertion of DNA [2]. This genetic event could have happened as the strain persists in the environment.

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