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Molecular characterization of *Vibrio cholerae* O1 and non-O1 from human and environmental sources in Malaysia

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SUMMARY

A total of 31 strains of *Vibrio cholerae* O1 (10 from outbreak cases and 7 from surface water) and non-O1 (4 from clinical and 10 from surface water sources) isolated between 1993 and 1997 were examined with respect to presence of cholera enterotoxin (CT) gene by PCR-based assays, resistance to antibiotics, plasmid profiles and random amplified polymorphic DNA (RAPD) analysis. All were resistant to 9 or more of the 17 antibiotics tested. Identical antibiotic resistance patterns of the isolates may indicate that they share a common mode of developing antibiotic resistance. Furthermore, the multiple antibiotic resistance indexing showed that all strains tested originated from high risk contamination. Plasmid profile analysis by agarose gel electrophoresis showed the presence of small plasmids in 12 (7 non-O1 and 5 O1 serotypes) with sizes ranging 1·3–4·6 MDa. The CT gene was detected in all clinical isolates but was present in only 14 (6 O1 serotype and 8 non-O1 serotype) isolates from environmental waters. The genetic relatedness of the clinical and environmental *Vibrio cholerae* O1 and non-O1 strains was investigated by RAPD fingerprinting with four primers. The four primers generated polymorphisms in all 31 strains of *Vibrio cholerae* tested, producing bands ranging from < 250 to 4500 bp. The RAPD profiles revealed a wide variability and no correlation with the source of isolation. This study provides evidence that *Vibrio cholerae* O1 and non-O1 have significant public health implications.

INTRODUCTION

Vibrio cholerae is an important cause of cholera in humans causing in its severe forms, profuse diarrhoea, vomiting and muscle cramps. Transmission of this organism is associated with consumption of contaminated foods and often with contaminated water and person-to-person transmission [1–3]. The pathogenicity of cholera is mainly associated with their ability to produce a cholera enterotoxin (CT), encoded by two contagious genes that form the *ctxAB* operon [4]. Since not all *Vibrio cholerae* strains are toxigenic, regular examination of isolates for their potential to produce CT are needed to obtain a better understanding of the public health hazard caused by toxigenic strains. Differentiation of *V. cholerae* will be required to ascertain the incidence, prevalence and diversity of strains. Epidemiologic investigation of cholera requires the characterization of *V. cholerae* isolates by typing systems which allow determination of isolates relatedness. It is common to use phenotypic and genotypic techniques for the characterization of organisms, and among them plasmid profiles, anti-

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biotic resistance patterns and random amplification of polymorphic DNA (RAPD) has been described. The latter has been shown to be a rapid and powerful technique that can be applied for strain differentiation within species [5–7]. In the work reported here, *V. cholerae* O1 and non-O1 strains isolated from surface water and clinical strains isolated from patients as well as from outbreak cases between 1993 to 1997 were characterized by antibiotic resistance, plasmid profiling and random amplified polymorphic DNA analysis and detection of the cholera toxin (CT) gene by using specific primer in PCR.

MATERIALS AND METHODS

Bacterial strains

Seventeen *V. cholerae* strains isolated from surface water from a location which received sewage drainage from a hospital within Peninsular Malaysia were investigated. The samples were collected in pre-sterilized bottles and brought to the laboratory in an ice container and processed within 2 h of collection. One ml of water sample was transferred into 99 ml of alkaline peptone water (APW) and incubated at 37 °C for 24 h, and then appropriate dilutions were plated onto thiosulphate–citrate–bile salts–sucrose agar (TCBS: Oxoid) for selective isolation of *V. cholerae*. After overnight incubation at 37 °C, identification of isolates were performed as described by Sakazaki and Shimada [8]. We also studied 14 *V. cholerae* strains from clinical specimens. Four were isolated from epidemiologically unrelated patients in a hospital in 1993 and 1994. The remaining 10 were recovered from symptomatic patients associated with the outbreaks of *V. cholerae* diarrhoea in 1995 and 1997 in Kuala Lumpur, Malaysia.

Serotyping

Strains were grown at 37 °C on plate count agar plates, and serological reactions were determined by slide agglutination with polyvalent O1, mono-specific Ogawa–Inaba antisera and with specific anti-O139 antisera obtained commercially (Denka Seiken, Tokyo). Non-O1 strains described here did not react with both of anti-O1 and anti-O139 antisera. A positive reaction was recorded when complete clumping of the bacterial cells against a clear background was observed.

Antibiotics and antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed by the disk diffusion method according to National Committee for Clinical Laboratory Standard [9]. The *V. cholerae* strains were tested against the following antibiotics disks (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA) (on Mueller–Hinton agar): ampicillin, 10 μg; bacitracin, 10 μg; chloramphenicol, 30 μg; ceftazidine, 30 μg; carbenicillin, 100 μg; cephalothin, 30 μg; erythromycin, 15 μg; furazolidone, 100 μg; gentamicin, 10 μg; kanamycin, 30 μg; moxalactam, 30 μg; nalidixic acid, 30 μg; penicillin, 10 μg; rifampicin, 30 μg; streptomycin, 10 μg; sulphafurazole, 100 μg; tetracycline, 30 μg; and trimethoprim, 5 μg. The control strain was *Escherichia coli* ATCC 25922, and isolates were deemed resistant if the zone of inhibition around the disk was < 3 mm radius or the zone was > 3 mm smaller than the control zone. The multiple antibiotic resistance index of isolates is defined as a/b where 'a' represents the number of antibiotics to which the particular isolates was resistant and 'b' the number of antibiotics to which the isolate was exposed [10].

Extraction of genomic and plasmid DNA

Genomic DNA isolation was done as described by Ausubel and colleagues [11]. Plasmid profile analysis were performed on all *V. cholerae* O1 and non-O1 clinical and surface water isolates according to the method of Birnboim and Doly [12]. The plasmids were separated on a 0·8% agarose gel and visualized by ethidium bromide staining. The approximate molecular mass of each plasmid was determined by comparison with plasmids of known molecular mass of *E. coli* V517 [13].

PCR assays for CT gene

All isolates were tested for the presence or absence of the CT genes with a set of primer described by Shangkuan and colleagues [14]. The primer pair, C2F (5′-AGGTGTAAAATTCCTTGACGA-3′) and C2R (5′-TCCTCAGGGTATCCTTCATC-3′) amplifies a 385-bp fragment of *ctxA2-B* gene were obtained from Genosys Biotechnologies, Inc., PCR amplification was performed in a reaction volume (25 μl) containing template DNA (10 ng), 2·5 μl 10 × reaction buffer, 1 mM (final concn) each of dNTP, 1 μm of each primer, 2·0 mM (final concn) $MgCl_2$ and 2·5 U Taq poly-

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merase. The PCR was done in the Thermal cycler (Perkin–Elmer Cetus 2400). The samples were subjected to denaturation at 95 °C for 2 min (one cycle) followed by 35 cycles, each consisting of 60 s at 95 °C, 90 s at 60 °C and 90 s at 72 °C. The synthesis was completed at 72 °C for 10 min. Strains NIH41 (*V. cholerae* O1) and MC1061 (*E. coli*) were used as positive and negative controls for the CT gene.

RAPD-PCR

In preliminary experiments, ten 10-mer random primers with 50% GC content (Genosys Biotechnologies Inc.) were investigated for RAPD-PCR analysis of multiple isolates on serotypes O1 and non-O1. Based on the results obtained, primer Gen15003 (5′-AGGATACGTG-3′), Gen15005 (5′-CGGATAACTG-3′), Gen15007 (5′-TCCGACGTAT-3′) and Gen15008 (5′-GGAAGACAAC-3′) were used for RAPD-PCR analysis of all *V. cholerae* strains. Amplification reactions were performed in 25 μl volume containing 2·5 μl 10 × reaction buffer, 1 mM (final concn) each of dNTP, 5 pmol primer, 2·0 mM (final concn) magnesium chloride, 1 unit Taq polymerase and 10 ng of genomic DNA. Amplifications were carried out in the thermal cycler (Perkin–Elmer Cetus 2400) for 1 cycle at 94 °C (2 min) for denaturation, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C. A negative control without DNA was included in each RAPD run. The PCR amplification products were fractionated by electrophoresis through 1·2% agarose gel and detected by staining with ethidium bromide.

RESULTS AND DISCUSSION

We studied 4 strains of non-O1 *V. cholerae* isolated from patients in 1993 and 1994, and 5 strains each of *V. cholerae* O1 isolated in 1995 and 1997 associated with outbreaks of diarrhoea. In addition, strains of *V. cholerae* O1 (7 strains) and non-O1 (10 strains) isolated from surface water were also studied. The majority of *V. cholerae* strains from the surface water samples are non-O1 serotypes. All the strains of serotype O1 belong to biovar El Tor and were of subgroup Ogawa. The presence of *V. cholerae* serotype O1 in the surface water samples tested supported the findings of Nishikawa and colleagues [15] that O1 serotype has been reported in areas without a recent history of a cholera outbreak. In addition, the present study supports the findings of Colwell and Huq [16] that *V. cholerae* O1 (CT-positive) can become part of the aquatic flora. Hence, the presence of *V. cholerae* O1 (CT-positive) in the environment may represent a potential public health hazard in the study area.

All 31 strains of *V. cholerae* were resistant to 9 or more antibiotics tested (Table 1). However, none were resistant to chloramphenicol, furazolidone, nalidixic acid and trimethoprim. Of great concern is the observation of the high level of antibiotic resistance among the *V. cholerae* O1 strains which most often show less resistance than non-O1 strains. The same problems has been described in studies performed in Thailand, Russia, India, Africa and South America [17–21]. Taken together, these data clearly illustrate the changing antibiograms of *V. cholerae* isolates worldwide. That the antibiotic resistance patterns of the *V. cholerae* O1 and non-O1 from both sources were similar, indicates that they may share a common environment and a common mode for developing antibiotic resistance in the study area. All isolates of *V. cholerae* O1 and non-O1 from clinical and environmental sources used in this study had multiple antibiotic resistance (MAR) indices of 0·44–0·72, indicating that all strains originated from high risk sources of contamination like swine, poultry, cattle and human environments where antibiotics are often used [10]. Although antibiotic resistance is not considered a virulence factor, it may assist in the persistence and establishment of the organism in the host [22] and may contribute to the dissemination of pathogenic *V. cholerae* strains.

The plasmid profiles of the *V. cholerae* O1 and non-O1 clinical and environmental isolates are shown in Table 1. The analysis of plasmid profiles showed that strains of both serotypes had a characteristic plasmid profile with plasmids of 1·3 and 1·5 MDa found in most of the plasmid-containing strains of both serotypes. It is interesting to note that small plasmids less than 4·6 MDa occur in 4 and 8 *V. cholerae* O1 and non-O1 strains respectively. These observations are in general agreement with several studies that have cited the lower incidences of plasmids among *V. cholerae* O1 serotype, and that plasmids of low molecular weight were frequently found in *V. cholerae* [23–25]. The phenotypic traits of these small plasmids detected in this study have not been determined, however, a recent study by Rubin and colleagues [26] reported on the possible role of small plasmids (designated as pTLC for toxin-linked cryptic) in virulence of *V. cholerae*. Attempts to increase the sensitivity of our procedure by concentrating the plasmid extracts failed

Table 1. Vibrio cholerae *strains used in this study*

Strain-year*	Antibiotic resistance†	Plasmid(s) size (MDa)	RAPD-type with primers			
			Gen15003	Gen15005	Gen15007	Gen15008
C1(non-O1)-93	ApBCazCbErKfMoxSfTe (0·50)	—‡	A	A	UT*§	A
C2(non-O1)-93	ApBCazCbErKfMoxRdSfTe (0·55)	—	B	A	UT	A
C3(non-O1)-93	ApBCazErKfMoxPRdSf (0·50)	1·3, 1·5	B	B	UT	A
C4(non-O1)-94	ApCazCbErKfMoxPSmSf (0·50)	—	C	C	UT	A
C5(O1)-95	ApBCazCbErKfKmPRdSmSfTe (0·67)	—	D	D	A	C
C6(O1)-95	ApBCazCbErGmKfKmMoxPSmSfTe (0·72)	—	E	D	A	B
C7(O1)-95	ApBCazCbErkfPRdSmSf (0·55)	—	E	A	A	B
C8(O1)-95	ApBCazCbErKfMoxPRdSmSf (0·61)	1·3, 1·5	E	D	A	C
C9(O1)-95	ApBCazCbErKfMoxPSmSf (0·55)	—	E	D	A	UT
C10(O1)-95	ApBCazCbErKfMoxPRdSfTe (0·61)	—	F	E	B	C
C11(O1)-97	ApBCazCbErKfMoxPRdSfTe (0·61)	—	E	F	C	C
C12(O1)-97	ApBCazCbErMoxKfPRdSfTe (0·61)	—	E	F	C	C
C13(O1)-97	ApBCazCbErKfMoxPRdSfTe (0·61)	—	G	F	C	C
C14(O1)-97	ApBCazCbErKfMoxPRdSmSfTe (0·67)	—	E	F	C	C
E6(non-O1)-97	BCazCbErGmKfKmMoxPRdSmSf (0·67)	1·3, 1·5	H	G	D	D
E7(non-O1)-97	ApBCazCbKfKmMoxPRdSmSf (0·61)	1·3, 1·5	UT	G	E	D
E8(non-O1)-97	BCazCbErKfKmMoxPRdSmSf (0·61)	1·3, 1·5	UT	G	E	D
E9(non-O1)-97	ApBCazCbErKfKmMoxPRdSm (0·61)	—	I	G	E	D
E10(non-O1)-97	BCazCbErKfKmMoxPRdSmSf (0·61)	1·3, 1·5	I	G	E	G
E11(non-O1)-97	BCazCbErGmKfKmMoxPRdSmSf (0·67)	1·3, 1·5	I	G	E	D
E12(non-O1)-97	ApBCazCbErKfKmMoxPRdSmSf (0·67)	—	UT	G	E	D
E13(non-O1)-97	BCazCbErGmKfKmMoxPRdSmSf (0·67)	1·3, 1·5	J	G	E	D
E14(O1)-97	BCbErGmKfKmRdSf (0·44)	—	L	J	F	E
E15(O1)-97	BCazCbErKfKmRdSf (0·44)	—	M	K	F	E
E16(non-O1)-97	ApBCazCbErGmKfKmMoxPRdSmSf (0·72)	—	K	H	H	F
E17(non-O1)-97	BCazCbErKfKmMoxPRdSmSf (0·61)	1·3, 1·5	O	I	E	H
E18(O1)-97	BCbErKfKmMoxRdSmSf (0·50)	1·3, 1·5, 4·6	N	L	G	F
E19(O1)-97	BCbErGmKfKmPRdSmSf (0·55)	4·6	L	UT	F	I
E20(O1)-97	BCbErKfKmRdSmSf (0·44)	1·3, 1·5	L	M	F	J
E21(O1)-97	BCazCbErkfKmPRdSmSf (0·55)	—	L	N	E	J
E22(O1)-97	BCazCbErGmKfKmPRdSf (0·55)	—	UT	O	D	J

* Only strains E10, E12 and E22 were CT-negative. C designates clinical isolates and E designates environmental isolates.
† Number in parentheses indicates the multiple antibiotic resistance (MAR) index values. Tested for ampicillin (Ap), bacitracin (B), carbenicillin (Cb), ceftazidine (Caz), cephalothin (Kf), chloramphenicol (Cm), erythromycin (Er), furazolidone (Fu), gentamicin (Gm), kanamycin (Km), moxalactam (Mox), nalidixic acid (Na), penicillin (P), rifampicin (Rd), streptomycin (Sm), tetracycline (Te), sulphafurazole (Sf), and trimethoprim (Tmp).
‡ Non detected.
§ UT, untypable.

to reveal larger plasmids in the *V. cholerae* strains examined. There is remarkable uniformity of plasmid profile through the period 1993–7 within the plasmid-containing isolates of both serotypes. In addition, plasmid of 4·6 MDa was found in two strains of serotype O1. Hence, the limited variability in plasmid sizes and plasmid patterns observed may be of useful marker for epidemiological investigation of these strains.

Cholera toxin (CT) has been considered a major virulence factor of *V. cholerae*. Hence, the detection for the presence of CT genes is important in epidemiological studies of this bacteria. Minami and colleagues [27] reported that production of CT was detected in all CT gene-positive strains, indicating that there was no silent CT gene in their test strains. PCR-based assay have been widely used for the detection of cholera enterotoxin gene [14, 28–31]. In this study, two of the *V. cholerae* non-O1 strains and a single strain of O1 isolated from surface water were negative for CT gene. Elsewhere, Tamayo and colleagues [32] showed that all *V. cholerae* clinical strains were positive for CT gene. The present study also showed the presence of CT gene in all clinical

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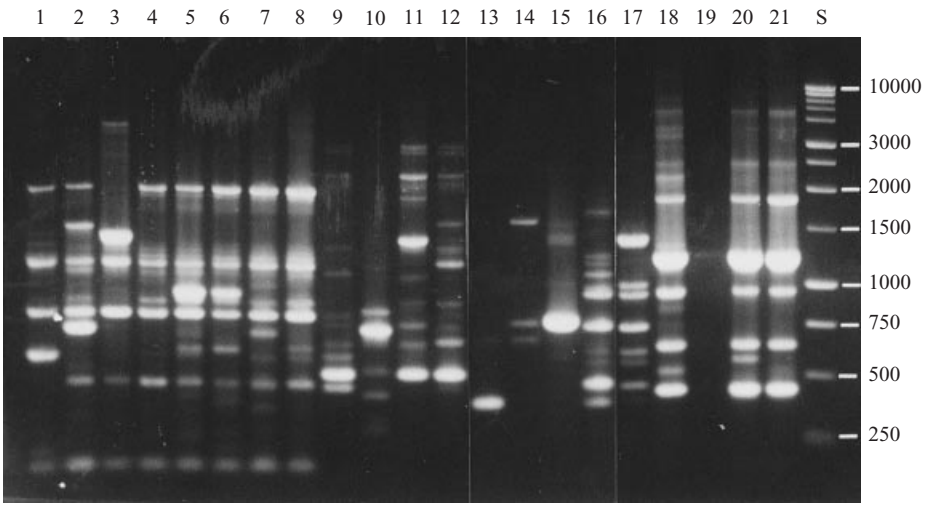

Fig. 1. RAPD fingerprints of *V. cholerae* isolated from patients and outbreaks cases obtained with primers Gen15003 (Lanes: 1 [A], 2 [B], 3 [C], 4 [D], 5 [E], 6 [E], 7 [F], 8 [G]); Gen15005 (Lanes: 9 [A], 10 [B], 11 [C], 12 [D], 13 [E], 14 [F]); Gen15007 (Lanes: 15 [A], 16 [B], 17 [C]); and Gen15008 (Lanes: 18 [A], 19 [empty], 20 [B], 21 [C]). Lane S contain lambda ladder DNA molecular weight markers (in bp).

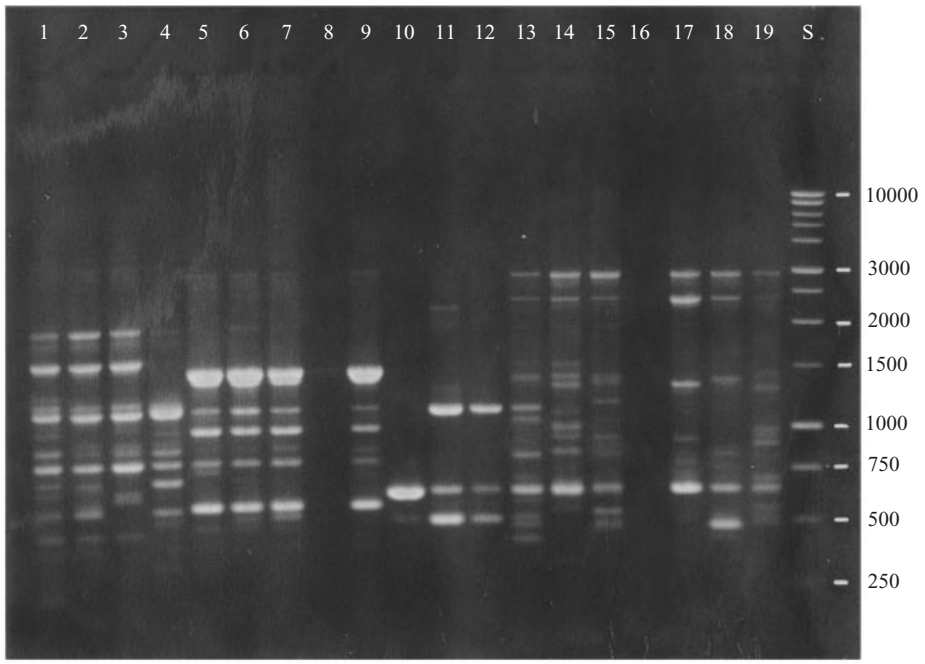

Fig. 2. RAPD fingerprints of *V. cholerae* isolated from surface water obtained with primers Gen15003 (Lanes: 1 [H], 2 [I], 3 [J], 4 [K], 5 [L], 6 [M], 7 [N], 8 [empty], 9 [O]) and Gen15005 (Lanes: 10 [G], 11 [H], 12 [I], 13 [J], 14 [K], 15 [L], 16 [empty], 17 [M], 18 [N], 19 [O]). Lane S contain lambda ladder DNA molecular weight markers (in bp).

strains examined. Though non-O1 serotypes are seldom associated with harbouring of cholera toxin [14, 27, 33, 34], most of the non-O1 strains in this study (12 of 14) were CT positive. This is not surprising as *V. cholerae* O1 and non-O1 were reported to possess an identical *ctx* gene. Toxin production does not correlate with serotype as *V. cholerae* non-O1 may be enterotoxigenic and generally associated with sporadic cause of gastroenteritis and extraintestinal infections with no epidemic potential [35, 36]. In addition, some strains of *V. cholerae* O1 may not produce CT [37]. Though two of the *V. cholerae* non-O1 and a single O1 serotype isolated from surface water were CT-negative, it should be noted that the enteropathogenicity of *V. cholerae* involves various virulence factors such as CT-like

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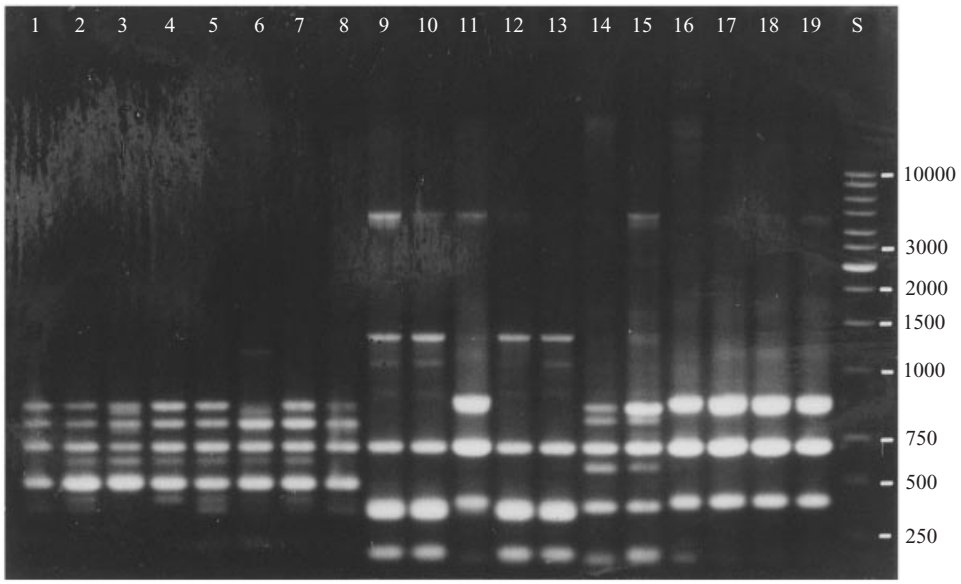

Fig. 3. RAPD fingerprints of *V. cholerae* isolated from surface water obtained with primers Gen15007 (Lanes: 1 and 8 [D], 2 and 5 [E], 3 [F], 4 and 7 [G], 6 [H] and Gen15008 (Lanes: 9 and 10 [D], 11 [E], 12 and 13 [F], 14 [G], 15 [H], 16 [I], 17, 18 and 19 [J]. Lane S contain lambda ladder DNA molecular weight markers (in bp).

enterotoxin, El Tor-like hemolysin, Zot, shiga-like toxin, fimbrial genes, invasiveness, haemagglutination and thermostable direct hemolysin similar to that of *V. parahaemolyticus* [35, 38–44]. Though none of the CT-positive *V. cholerae* O1 and non-O1 clinical and environmental strains were examined for disease potential using animal challenge model, it is safe to assume that they are pathogenic as cholera enterotoxin (CT) has been considered a major virulence factor of *V. cholera* [45].

Of the ten primers tested in this study, four of the primers (Gen15003, Gen15005, Gen15007 and Gen15008) generated the best amplification patterns for strains differentiation (Table 1). The representative profiles of the reproducible bands for the isolates used in this study from these four primers are presented in Figs 1–3. The number of RAPD bands produced for a given primer ranged from one to ten, with molecular sizes ranging from < 250 to 4500 bp. Several of the isolates were not typable using four primers Gen15003, Gen15005, Gen15007 and Gen15008, respectively (Table 1). These results could be interpreted as the loss of specific sites for primer binding in the chromosomal DNA of these isolates. Fifteen, 15, 7 and 10 RAPD-types were differentiated using primers Gen15003, Gen15005, Gen15007 and Gen15008 (Table 1). If the primers were to be judged on discriminatory power, then primers Gen15003 and Gen15005 were most suitable for typing. The combination of the results with the four primers increased the number of different RAPD-types to 26 which support the findings of Rath and colleagues [46] that strains with identical RAPD patterns generated by a few primers could be different when investigated with more primers or primer combination. Previously described methods for typing of *V. cholerae* such as multilocus enzyme electrophoresis (MEE), ribotyping and pulsed field gel electrophoresis (PFGE) were reported to be time-consuming and labour-intensive [14, 32, 47–49]. We report here the use of RAPD to detect DNA sequence diversity among *V. cholerae* isolates, and that the technique was found to have many advantages from the viewpoints of economics, simplicity, reproducibility and time as reported elsewhere [5, 50]. When RAPD is applied to study the clonal relatedness between isolates of the same bacterial species, the possibility exist that plasmid(s) DNA influences the chromosomal RAPD pattern as short primers are used, which may hybridize well with plasmid DNA. This may result in amplification of extra fragments leading to erroneous conclusion with regards to the clonal relatedness of the isolates. Testing selected strains, our results showed that the RAPD pattern did not change regardless of the presence or absence of plasmids. Our observation is in general agreement with that of Elaichouni and colleagues [51] who reported that the presence of larger plasmids or small plasmids does not interfere with the RAPD fingerprint from different strains.

In conclusion, the present study has highlighted the

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high prevalence of CT genes in *V. cholerae* non-O1 (12 of 14) strains from clinical and environmental sources. In addition, *V. cholerae* O1 and non-O1 strains examined were resistant to nine or more of the antibiotics tested. Our data from the RAPD assays showed the presence of a wide heterogeneity within clinical and environmental strains of *V. cholerae* isolated from a geographically restricted area, and lack of a correlation between genetic pattern relatedness and sources where the strains were isolated.

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