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Molecular evidence of clonality amongst *Vibrio cholerae* O1 biotype El Tor during an outbreak in Malaysia

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SUMMARY

Forty-three clinical strains of *V. cholerae* O1 biotype El Tor were isolated between 3 May and 10 June 1998 during an outbreak in the metropolitan area of Kuala Lumpur and its suburbs. With the exception of three Inaba strains that were restricted to three members of a family, all the others belonged to the Ogawa serotype. The strains were analysed for clonality using ribotyping and pulsed-field gel electrophoresis (PFGE). Two ribotypes, V/B21a and B27, were identified among 40 Ogawa isolates using *Bgl*I restriction endonuclease. Ribotype V/B21a has been described previously from Taiwan and Colombia and several Asian countries while B27 has been reported among isolates from Senegal. The three Inaba strains belonged to one ribotype, designated type A, not previously reported. PFGE analysis using *Not*I revealed that all isolates within a ribotype had identical profiles demonstrating clonality amongst the strains. Dice coefficient analysis of the two Ogawa genotypes revealed 89% similarity on ribotype patterns and 91.3% on PFGE profiles. Ribotype V/B21a isolates were associated with cases from dispersed areas of Kuala Lumpur and its suburbs while ribotype B27 was restricted to cases from one particular area suggesting a common-source outbreak.

INTRODUCTION

Although there are at least 140 serotypes of *Vibrio cholerae* identified to date, strains belonging to the O1 serotype have been the exclusive cause of cholera in the last three pandemics. The O1 serotype can further be divided into the classical and El Tor biotypes on the basis of phenotypic differences. The current ongoing pandemic that began in 1961 has been attributed solely to the El Tor biotype in contrast to the two previous pandemics, which were due to the classical biotype. The emergence of the O139 serogroup and of antibiotic-resistant strains has caused major concern worldwide.

Cholera due to *V. cholerae* O1 biotype El Tor is endemic in Malaysia and poses a public health problem as sporadic outbreaks occur periodically. In addition, due to the recruitment of foreign labour-force from cholera-endemic countries, there is concern regarding the importation of strains from these countries into Malaysia. Traditional serotyping and biotyping methods used in past epidemiological studies of cholera have been limited by the lack of discrimination provided by phenotypic characteristics of *V. cholerae* O1 [1, 2]. Molecular characterization by multilocus enzyme electrophoresis (MEE), used in the past to analyse stable 'house-keeping' enzymes, could not demonstrate differences between strains which were widely distributed in endemic areas [3].

Newer approaches in molecular biology used to type strains of *V. cholerae* O1 have been used effectively to monitor strains from outbreaks or epidemics. These include techniques such as Southern hybridization with a cholera toxin gene probe [4], and DNA fingerprinting with an insertion sequence [5].

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However, ribotyping using labelled *Escherichia coli* rRNA to probe polymorphic rDNA and DNA fingerprinting or detection of genomic polymorphism using pulsed-field gel electrophoresis (PFGE) have been found to be more useful in distinguishing strains in the epidemiological surveillance of cholera as reported by other workers [6–9]. All these studies are comparable as ribotyping was performed using *Bgl*I restriction digestion of genomic DNA. Popovic and co-workers [10] developed a standardised ribotype scheme for *V. cholerae* O1 and defined 27 ribotype patterns in a collection of 214 strains isolated from 35 countries over the past 60 years. In the subsequent 2 years, further reports by other workers demonstrated the emergence of new ribotype patterns, which were different to those in the foregoing scheme and unique to various geographical regions. It was also possible to trace the transmission of a particular ribotype to its original location [9, 11]. In addition, PFGE studies were also found to be useful in distinguishing strains from various geographical regions [12].

To the best of our knowledge to date, in Malaysia, there has only been one report on the epidemiological analysis of multiple *V. cholerae* isolates obtained during two defined outbreaks and sporadic cases using PFGE [13]. In that study, DNA macro-restriction analysis using *Not*I demonstrated clonality among strains isolated within the defined outbreaks whereas isloates from sporadic cases demonstrated varied profiles. Based on these findings it was concluded that the PFGE was far superior to other methods in producing stable and reproducible restriction endonuclease analysis patterns that were easy to interpret. In the present report, we present molecular evidence on the clonality of strains of *V. cholerae* isolated from a recent outbreak in Malaysia which occurred during a period of water shortage in the metropolitan area of Kuala Lumpur and its suburbs. The analysis was performed in a hierarchical fashion in which ribotyping was first performed and strains belonging to the same ribotype were then compared for genomic polymorphism using PFGE.

MATERIALS AND METHODS

Bacterial strains

Forty-three strains of *V. cholerae* O1 biotype El Tor were isolated from stools of infected individuals using thiosulphate-citrate bile salts sucrose (TCBS) agar (Oxoid, UK). Identification, serogrouping and biotyping were performed using standard protocols. With the exception of three Inaba strains, all other strains belonged to the Ogawa serotype. The strains were stored on nutrient agar slants supplemented with 0.5% sodium chloride at room temperature.

Ribotyping

DNA was extracted from overnight cultures on nutrient agar using the guanidium isothiocyanate method [14]. Five micrograms of purified DNA was digested with *Bgl*I (20 units; Promega, USA) at 37 °C for 18 h and electrophoretic separation of the digested DNA, Southern transfer, hybridization and development of the membrane was performed according to the method of Kaufmann and colleagues [15].

Pulsed-field gel electrophoresis (PFGE)

Bacterial DNA was immobilised in agarose plugs as previously described [16] and digested for 18 h at 37 °C with 20 units of *Not*I (Promega). DNA fragments were separated on a 1.2% agarose gel by PFGE in a CHEF-DR II system (Contour Clamped Homogenous Electric Field; Bio-Rad, USA) in 0.5 × Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer at 200 V for 28 h with the following initial and final pulsing times; 5 s for 8 h; 15 s for 10 h; and 25 s for 10 h. The separated DNA was stained with ethidium bromide and visualized under UV trans-illumination. DNA from *E. coli* strain MG1655 digested with *Not*I and lambda DNA ladder standards (Bio-Rad) were used as molecular size markers. DNA banding patterns were analysed for similarity using Dice coefficient with the aid of the BioImage Whole Band Analyzer software (BI Systems Corporation, USA).

RESULTS

All strains used in the study were obtained from an outbreak of cholera between 3 May and 10 June 1998 and belonged to the El Tor biotype. However, there were no strains isolated for 9 days between 17 and 25 May. During the first phase, 3–16 May, 11 isolates were recovered of which 8 belonged to serotype Ogawa and the remaining 3 to serotype Inaba. Ribotyping analysis of the Ogawa isolates using *Bgl*I demonstrated nine identical bands of 2.3, 4.1, 4.5, 5.7,

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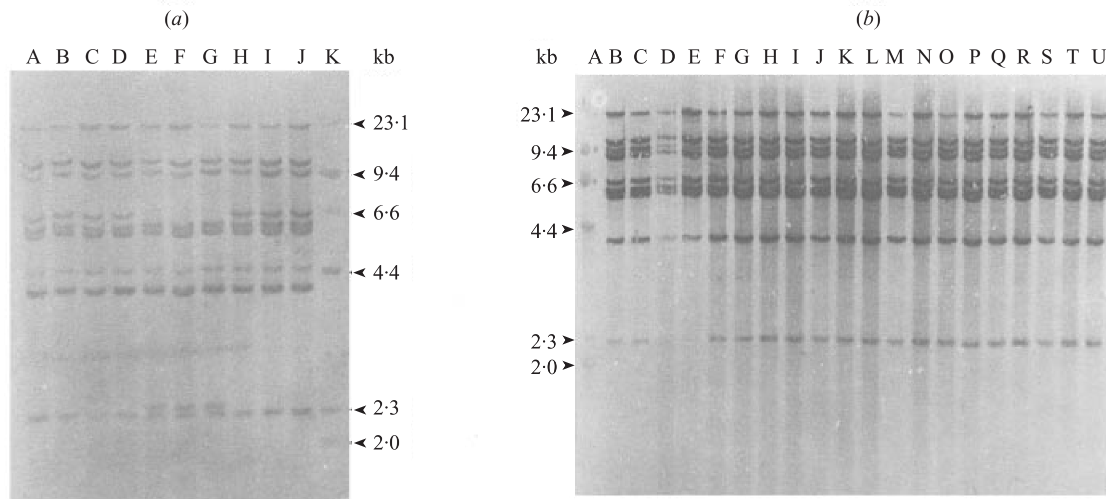

Fig. 1. *Bgl*I ribotype patterns of *V. cholerae* O1. (*a*) Lanes A-D and H-J, ribotype V/B21a (Ogawa); lanes E-G, ribotype A (Inaba); lane K, biotinylated lambda DNA *Hin*dIII marker. (*b*) Lane A, lambda marker; lanes B-U, ribotype B27 (Ogawa).

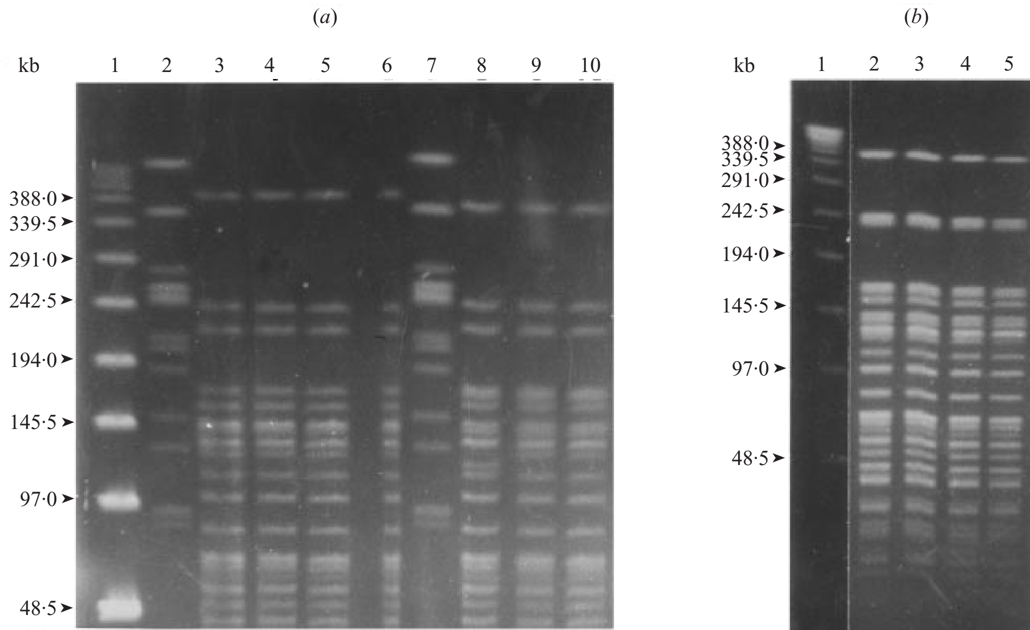

Fig. 2. PFGE profiles of *Not*I digested total DNA of *V. cholerae* O1 isolates of various ribotypes. (*a*) Lane 1, lambda marker; lanes 2 and 7, *Not*I digested DNA of *E. coli* MG1655; lanes 3–6, ribotype V/B21a; lanes 8–10, ribotype A. (*b*) Lane 1, lambda marker; lanes 2–5, ribotype B27.

6.0, 6.6, 9.6 and 11.1 and 25.5 kb (Fig. 1*a*). This profile was identical to ribotype V of Shangkuan and colleagues [6] and ribotype B21a of Tamayo and colleagues [7] and was designated ribotype V/B21a. DNA fingerprinting by PFGE revealed identical genomic profiles demonstrating clonality amongst these eight Ogawa isolates (Fig. 2*a*). The three Inaba isolates were all of a single ribotype designated A, not previously reported. Ribotype A of the Inaba strains differed from the Ogawa ribotype by the absence of a 6.6 kb band and the presence of an additional 2.4 kb band (Fig. 1*a*). The PFGE profiles demonstrated a difference in one band amongst the three Inaba isolates. Two isolates had a band of 134.0 kb which was absent from the third isolate but a band of 130.2 kb in the latter isolate was absent from the other two isolates (Fig. 2*a*).

During the second phase of the outbreak, 26 May

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to 10 June, all 32 isolates similarly belonged to serotype Ogawa and 4 of them were of ribotype V/B21a and had PFGE profiles identical to the Ogawa isolates collected during the first phase of the outbreak. The remaining 28 isolates demonstrated a second *Bgl*I ribotype profile identical to ribotype B27 of Aidara and colleagues [8] defined by nine bands of 2.3, 4.1, 5.7, 6.1, 6.6, 8.9, 9.8, 11.3, and 26.0 kb (Fig. 1*b*). DNA fingerprinting using *Not*I revealed identical banding profiles (Fig. 2*b*).

The relatedness of the two Ogawa ribotypes, B27 and V/B21a and their PFGE profiles using Dice coefficient analysis demonstrated similarities of 89 and 91.3% respectively. The PFGE patterns differed between strains belonging to ribotype B27 and V/B21a by three bands at 255.0, 178.5 and 139.8 kb amongst type B27 and a 222 kb band amongst type V/B21a (Fig. 2).

In retrospect, it was found that all the Ogawa strains of ribotype V/B21a were isolated from various areas in Kuala Lumpur and its suburbs. However, all the type B27 strains isolated during the second phase originated from one particular area, Kampung Kerinci, a residential district of Kuala Lumpur and probably from the consumption of hawker food sold in the same area. The Inaba strains were restricted to a father and his two daughters and appeared to be unrelated to the outbreak.

DISCUSSION

Despite progress in the economic and health sectors in many developing countries, the resurgence of cholera remains uncontrolled and of great concern in many of these countries. In order to execute intervention strategies such as vaccines there is a need to have an extended knowledge of the type of strains that are predominant in various parts of the world. Although newer methods such as Southern hybridization with the cholera toxin probe [4] and insertion sequence elements [5] have been developed, ribotyping and PFGE have been found to be stable, reproducible and discriminatory. The development of a standardized ribotyping scheme using *Bgl*I for *V. cholerae* O1 by Popovic and colleagues initiated a database for a limited number of strains from 35 countries and various parts of the United States [10]. However, there is still a paucity of documentation on isolates in Southeast Asia where the disease is endemic.

In this study, ribotyping and PFGE were used in a hierarchical fashion to determine the clonality of strains within the outbreak and to ascertain if transmission of strains had occurred from other parts of Asia into this region. Although there is information on strains available from different parts of the world, this study differs from others in that the strains were all collected during an outbreak as opposed to over a period of time. In Malaysia, sporadic outbreaks of cholera occur all year round. In this outbreak, there appeared to be two phases with an interval of 9 days when no cases were seen at the University Hospital. But it is highly likely that it was a single continuous outbreak with patients presenting themselves at other hospitals and health centres. Two *Bgl*I ribotypes, V/B21a and B27 were identified among the 40 Ogawa isolates. Shangkuan and colleagues [6] described the presence of ribotype V amongst strains from cases of imported cholera during an outbreak in Taiwan between 1993 to 1995 and others isolated from imported seafood from in various Asian countries. Furthermore, two strains of ribotype B21a which was identical to ribotype V (Shangkuan and colleagues' scheme), were isolated in Colombia and this ribotype has also been reported from Turkey (1980), Romania (1991–4), Pakistan (1993), Lebanon (1993), Cambodia (1993) and Italy (1994) [7]. Interestingly, this ribotype profile was distinct from the ribotype patterns proposed in the standardized ribotyping scheme for *V. cholerae* [10].

The second ribotype, B27, was recently described amongst strains from Senegal [8] and was also recovered from an outbreak in Guinea-Bissau thought to have originated from Calcutta [9]. However, due to slight differences in the molecular weights of the banding profiles in the above two studies, it was difficult to speculate on the origin of these strains. Interestingly, ribotype B5a which was reported to be the most common type identified in the seventh cholera pandemic was not found in the present study [10].

The high percentage similarity of the two Ogawa ribotypes suggests that variations in the rDNA loci of a single parent strain due to DNA rearrangements or mutations may have occurred. PFGE demonstrated clonal profiles of DNA amongst isolates within each ribotype, thus providing evidence that the outbreak resulted from a common source and then spread from person-to-person. The relatedness of the PFGE patterns (91.3% similarity) between strains of the two Ogawa ribotypes also supports the conclusion that these strains were clonally related [17].

Most cases when interviewed reported the con-

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sumption of hawker food and drinks. Ribotype V/B21a was found amongst cases from rather dispersed areas of Kuala Lumpur and its suburbs suggesting that this strain may already be quite widespread and ubiquitous in Malaysia. However, ribotype B27 was restricted to one area suggesting a common-source outbreak and this strain may have been imported into the country more recently than type V/B21a. At the same time it was found that during the water shortage that affected the capital, tankers delivered potable water for consumption which may have been contaminated. Secondly, water obtained from taps, during intermittent supply may also have been contaminated due to negative pressure in the water pipes.

A third strain belonging to serotype Inaba was isolated from members of the same household demonstrating person-to-person transmission and/or a common-source outbreak. There were no other reports of this strain and therefore it may be concluded that this strain was responsible for a family outbreak.

The clonality of strains and the ribotypes identified suggest the predominance of certain ribotypes in Malaysia as well as perhaps the ability of these genotypes to survive, transmit and cause disease in man. It is evident from this study that cholera is prevalent in Kuala Lumpur and its suburbs, and an interruption in the supply of safe potable water may give rise to an outbreak of cholera. Mahalingam and colleagues [13] reported that isolates obtained from two outbreaks were clonal within the outbreak and isolates from sporadic cases were heterogeneous at the DNA level. Their results also suggested that multiple clones did coexist simultaneously giving rise to sporadic cases. Therefore in the present study it is not surprising that there were three different clones present during the same phase.

In summary, ribotyping and PFGE are discriminatory and useful in the molecular epidemiology of *V. cholerae*. This study has also demonstrated the reproducibility of the *Bgl*I ribotype scheme as the profiles could be compared with published patterns. The use of PFGE also allowed the identification of clonal lines within ribotypes. However, there is a need to establish a universally accepted, stable and reproducible typing scheme and centres for this should be set up at national and international levels. This will facilitate study of the emergence of different genotypes and their transmission worldwide so that appropriate vaccine and interventions may be designed and developed.

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