Epidemiol. Infect. (1995), **115**, 435–446
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An epidemiological study of *Vibrio cholerae* O1 in the Australian environment based on rRNA gene polymorphisms

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(*Accepted 2 July 1995*)

SUMMARY

Since 1977, *Vibrio cholerae* O1 has been isolated from the Australian aquatic environment and periodically cholera cases have occurred following exposure to these environments. To study the relationships between clinical isolates and environmental isolates from rivers and aquatic life, widely distributed throughout the country, a wide range of molecular typing methods were employed. In this paper we report the analysis of the 180 Australian isolates (10 clinical and 170 environmental) using ribotyping. Seven ribotype patterns were observed among the Australian inaba isolates, 2 of which included all clinical inaba isolates and 84% environmental inaba isolates collected from 9 rivers and creeks in eastern Australia during an 8-year period. Isolates from epidemiologically related clinical cases, asymptomatic household contacts and sewage were indistinguishable. The ogawa isolates were more diverse, with 9 ribotypes observed among 24 isolates from 8 rivers during the same period. Ribotype patterns were not shared between the serotypes with the exception of one ogawa isolate which could be distinguished using PFGE. Ribotyping has been useful in confirming an association between epidemiologically related clinical isolates and the aquatic environment and the persistence of several clones of the O1 serovar in the Australian environment during an 8-year period.

INTRODUCTION

The first culture confirmed case of cholera due to *Vibrio cholerae* O1 in Australia was reported in 1969 when a patient had returned via air from a cholera endemic area 2 days prior to the onset of symptoms [1]. Prior to this, cholera had not been reported on the Australian mainland. During the early days of white colonization, ships entering en route from cholera endemic countries were quarantined off shore when passengers and/or crew were reported to have cholera like symptoms [2]. The advent of air travel now allows the transport of infected persons inter-continentally, with the manifestation of symptoms after disembarkation at great distance from the site of infection. Periodically, travellers return to Australia with cholera; however, there is no evidence for secondary local spread of the disease following the importation of these cases [3].

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https://doi.org/10.1017/S0950268800058593 Published online by Cambridge University Press

Table 1. *Details of the* Vibrio cholerae *O1 strains used in the study and their ribotype patterns*

Place of isolation	Source	Year of isolation	Serotype	Ribotype: No. of strains	Ribotype: R1	Ribotype: R2	Ribotype: Other types
			Rockhampton area				
Eight Mile Creek	Water	1980	Inaba	1		1	
Limestone Creek	Water	1983	Inaba	2		2	
Lion Creek	Water	1983	Inaba	1		1	
Prospect Creek	Water	1985	Ogawa	1			R11, 1
Unknown	Water	1983	Ogawa	1			R10, 1
		1986	Ogawa	1		1	
Mt Morgan	Faeces	1983	Ogawa	1			R12, 1
Rockhampton	Faeces	1983	Inaba	1		1	
			Albert–Logan Rivers area				
Albert River	Water	1977	Inaba	20	19	1	
		1978		1		1	
		1980		8	7	1	
		1981		8	5	2	R6, 1
Logan River	Water	1977	Inaba	8	8		
		1980		5	1	4	
		1981		7	1	6	
	Fish	1977	Inaba	2		2	
Albert–Logan Rivers	Water	1979	Inaba	32	7	24	R4, 1
			Tributaries of Albert River				
Canungra Creek	Water	1979	Inaba	1		1	
		1981		4	2	1	R5, 1
Palen Creek	Water	1981	Inaba	2		2	
			Ogawa	1			R8, 1
Albert–Logan Rivers area	Sewage	1981	Inaba	3	2	1	
Beaudesert	Sewage	1981	Inaba	1		1	
Beenleigh	Sewage	1977	Inaba	2	1		R3, 1
		1981	Inaba	1		1	
Beenleigh	Blood	1977	Inaba	1	1		
	Faeces	1977	Inaba	2	2		
North Pine Dam	Water	1980	Inaba	1	1		
			Brisbane River area				
Brisbane River	Water	1978	Inaba	3	2	1	
		1980		10	10		
			Tributaries of Brisbane River				
Bulimba Creek	Water	1978	Ogawa	1			R10, 1
		1980	Inaba	1		1	
Stanley Creek	Water	1981	Inaba	1	1		
Warrill Creek	Water	1981	Inaba	1		1	
			Water treatment works on Brisbane River				
Holts Hill	Water	1980	Inaba	7	5	2	
Mt Crosby	Water	1980	Inaba	8	7	1	
Mt Gravatt	Sewage	1978	Ogawa	6			R11, 6
Ipswich	Faeces	1980	Inaba	2		2	

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Table 1 (*cont.*)

Place of isolation	Source	Year of isolation	Serotype	Ribotype: No. of strains	R1	R2	Other types
			Other areas				
Boyne River	Water	1981	Inaba	1		1	
Mary River	Water	1981	Inaba	1			R9, 1
Condamine River	Water	1980	Inaba	2	2		
Clarence River	Water	1984	Ogawa	1			R8, 1
Lismore	Sewage	1981	Inaba	1		1	
Lismore	Faeces	1981	Inaba	2		2	
Brisbane Water	Water	1982	Ogawa	2			R7, 1; R13, 1
Georges River	Water	1981	Ogawa	4			R7, 3; R6, 1
		1982	Ogawa	3			R6, 3
	Oysters	1981	Ogawa	1			R7, 1
NW Australia	Water	1986	Ogawa	1			R14, 1
Burdekin River	Faeces	1984	Inaba	1		1	
			Isolates from other endemic areas				
Malaysia	Faeces	1992	Ogawa	1			R18, 1
Malaysia	Faeces	1987	Inaba	1			R15, 1
Malaysia	Faeces	1989	Ogawa	1			R16, 1
Peru	Faeces	1991	Inaba	1			R15, 1
Middle East	Faeces	1987	Inaba	1			R17, 1

In 1977, a case of indigenous cholera occurred on the mid-east coast of the country [4] and the subsequent public health investigations revealed that *V. cholerae* O1 was widespread in the local aquatic environment [5]. Since this time indigenous cholera cases have periodically occurred in eastern Australia and *V. cholerae* O1 has been isolated from river systems both in the east and north west of the country [3, 5, 6].

V. cholerae O1 isolates from Australian cases and environmental sources have been extensively studied taxonomically [6–8], for the possession of cholera toxin (*ctx*) genes and CT production [9] and for epidemiological markers including zymovar or enzyme electropherotype (ET) [10] and RFLPs in the *ctx* genes [9]. From these studies it was demonstrated the Australian *V. cholerae* O1 isolates were diverse and distinct from the imported isolates available for study. Questions remain as to the clonality and evolution of the Australian isolates and their relationship to the current pandemic strains and to isolates from endemic areas such as the US Gulf Coast. We present here a study of the rRNA polymorphisms among a collection of 180 isolates of *V. cholerae* O1 from indigenous symptomatic and asymptomatic cholera patients, sewage and the Australian environment which aimed to further address these questions.

METHODS

Bacterial strains

One hundred and eighty strains of *V. cholerae* O1 biotype El Tor, isolated from patients with locally acquired infections, sewage, marine animals and from the

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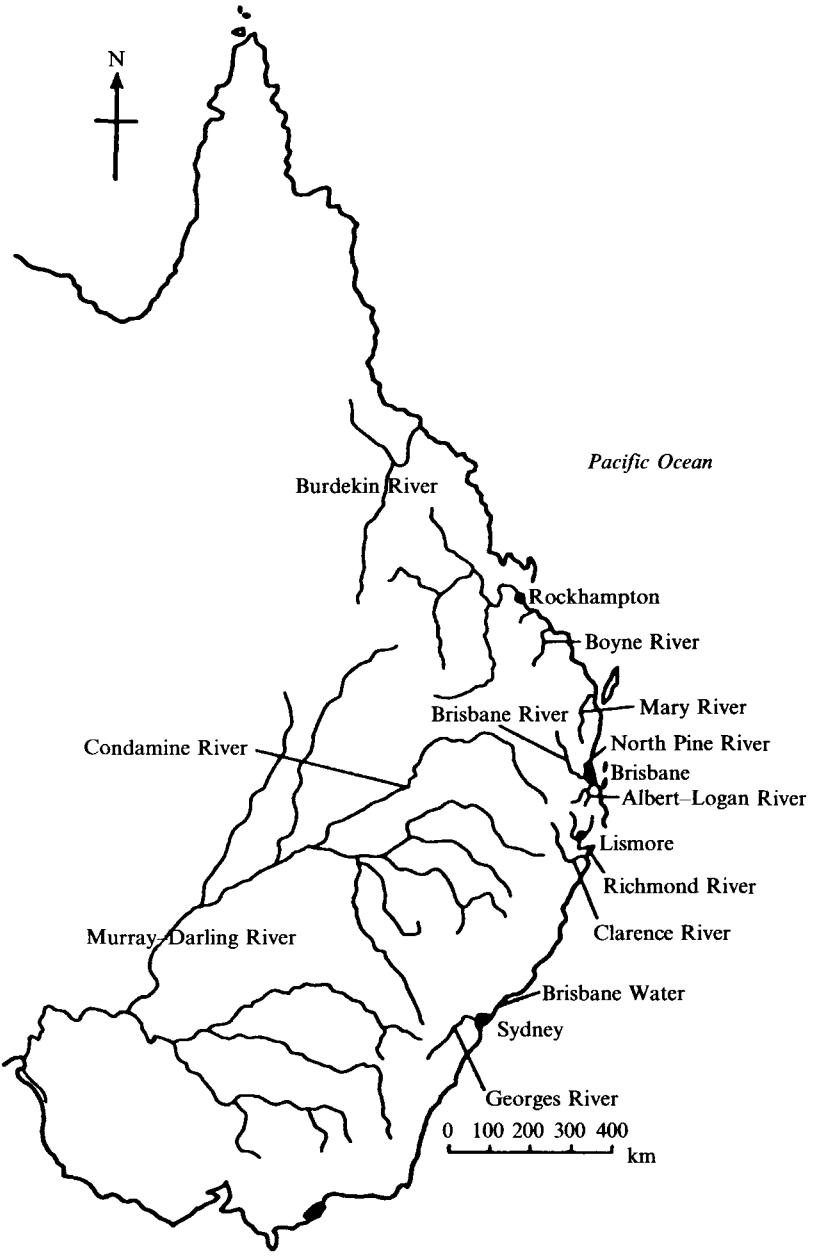

Fig. 1. Map of the east coast of Australia showing the location of rivers and patients which were the source of the *Vibrio cholerae* O1 included in this study.

Australian aquatic environment were used in this study. A collection of epidemiologically unrelated clinical and environmental isolates from other continents was included for comparison. Details of the isolates are presented in Table 1 and the geographical locations in Figure 1. The isolates were isolated originally by P. M. Desmarchelier or received from other laboratories, after which they were maintained in the lyophilized state. Working stock cultures were held

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in 0·4% semi-solid nutrient agar (Oxoid) and subcultured onto nutrient agar (Oxoid) when required. Serotyping was performed using polyvalent and monovalent antisera (Wellcome).

Isolation of genomic DNA

Genomic DNA was prepared using the rapid extraction procedure with guanidium thiocyanate, based on the method of Pitcher and colleagues. 1989 [11], using 2–3 loopsful of growth harvested from nutrient agar cultures grown at 37 °C overnight. Prior to restriction digest analysis DNA was further treated with proteinase K and extracted with phenol/chloroform/isoamyl alcohol [12]. Spooled DNA was dissolved in 100 μl Tris–EDTA buffer and stored at 4 °C. The DNA concentrations were calculated from absorbances at 260 nm determined in a Spectrophotometer (Hitachi 150-20).

Analysis of rRNA RFLPs

Ribotyping was performed essentially as previously described [12]. Genomic DNA was digested with the restriction enzyme, *Bgl*1 (BRL Laboratories) using the buffer supplied by the manufacturer and according to the manufacturer's instructions. Restriction fragments were separated by agarose gel electrophoresis, southern blotted onto nylon membrane and hybridized with a biotin-labelled cDNA probe [12]. A biotinylated *Hind*III digest of bacteriophage lambda (BRL Laboratories) was included in each gel. The restriction fragment patterns were compared visually.

Pulsed field gel electrophoresis

PFGE was performed essentially according to the method described by Cameron and colleagues [13] with a modification of the cell concentration used to prepare agarose plugs from an optical density of 0·6 to 0·3–0·4 at 610 nm. DNA was digested with *Not*I restriction endonuclease (New England Biolabs). The restriction fragments were separated by electrophoresis using a CHEF DR II system (Bio-Rad), ethidium bromide stained and analysed visually.

RESULTS

Among the 180 indigenous *V. cholerae* O1 isolates, 14 *Bgl*I rRNA RFLP patterns were observed with fragments ranging in size from approximately 11·2 to 1·0 kb. The ribotypes of individual strains are presented in Table 1 and a schematic diagram of the patterns in Figure 2. Types R1 and R2 (Fig. 3A) were the most common patterns overall, comprising 47% and 38% of the total isolates respectively. Single isolates belonged to types R3–5, 9, 10, 12, 13 and 14; five isolates to type R6 and R7. Both ogawa and inaba serotypes were present in some river systems while in others such as the Georges River, all isolates were ogawa. In the Albert–Logan Rivers system, inaba isolates could be ribotyped into 1–3 ribotypes within the summer season of any year during which *V. cholerae* are most frequently isolated [5]. The inaba isolates were predominantly R1 and R2 (97%) with four isolates from water (one from the Mary River, two from Albert–Logan Rivers, one from Canungra Creek) and one from sewage belonging to different

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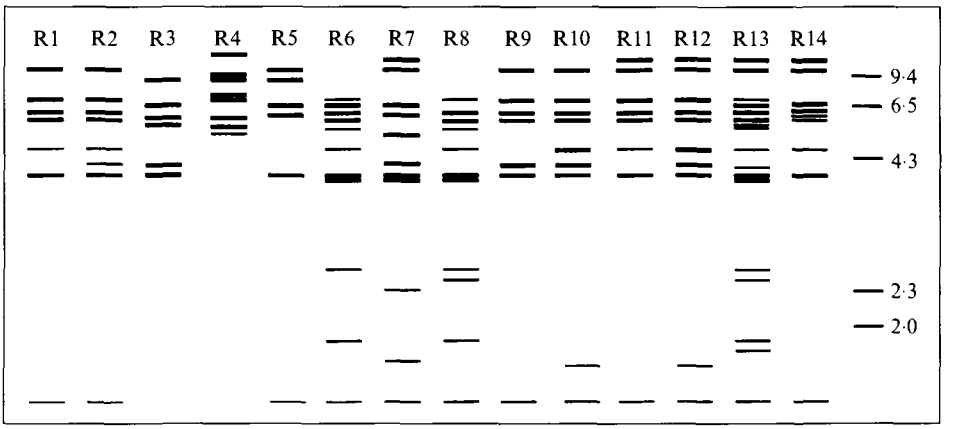

Fig. 2. Schematic diagram of the 14 *Bgl*1 ribotype patterns among 180 *Vibrio cholerae* O1 isolated from indigenous patients, marine animals and the aquatic environment in Australia. Molecular sizes are indicated by the markers in the right hand lane.

types. One culture isolated from water in the Rockhampton area in 1986 was the only ogawa isolate with a ribotype pattern indistinguishable from an inaba isolate.

R1 was the most common ribotype amongst this collection. The isolates from the first indigenous cholera cases in 1977 had this pattern and R1 isolates were widespread in the water supply, sewage and river system in the south-east Queensland region in which the cases resided. R2 isolates were less common among isolates from this river system during 1977; however, R2 isolates were detected more frequently from river water both in this region and others from 1979. Clinical cases due to R2 occurred in Ipswich, Lismore and Rockhampton in 1980, 1981 and 1983, respectively. Both ribotypes were detected in municipal sewage from communities in the Albert–Logan Rivers area from 1977 to 1981. R2 was the only ribotype among the inaba strains isolated from rivers to the north, including the Boyne River and rivers in the Rockhampton area. This ribotype was also isolated from the faeces of a cholera case in Rockhampton and a case believed to have been acquired in the Burdekin River area further north.

Nine ribotypes were identified among the ogawa isolates from eight different river systems. They were detected less frequently than the inaba serotype, although both serotypes were detected in the Albert–Logan Rivers and Brisbane River systems. An isolate from the far north-west of Australia was ogawa ribotype R14 while isolates from rivers and oysters in the south-east were ogawa ribotypes R6, 7 and 13 (Fig. 3B). The specific sampling sites and the ribotypes isolated in the southern areas are shown in Table 2. R7 and 13 were isolated from brackish or estuarine waters and oysters collected from the Georges River and from Brisbane Water approximately 45 K north. A weir on the Georges River provides an unnatural barrier to tidal influences. R6 was detected in the fresh water, up-stream sites over 4 months and on one occasion from a site not far down stream from the weir during the same period.

Isolates from five cases of clinical cholera due to *V. cholerae* O1 inaba were ribotyped. Isolates from the blood and the faeces of the first recognized symptomatic patient from Beenleigh in 1977 were R1, as was the isolate from a

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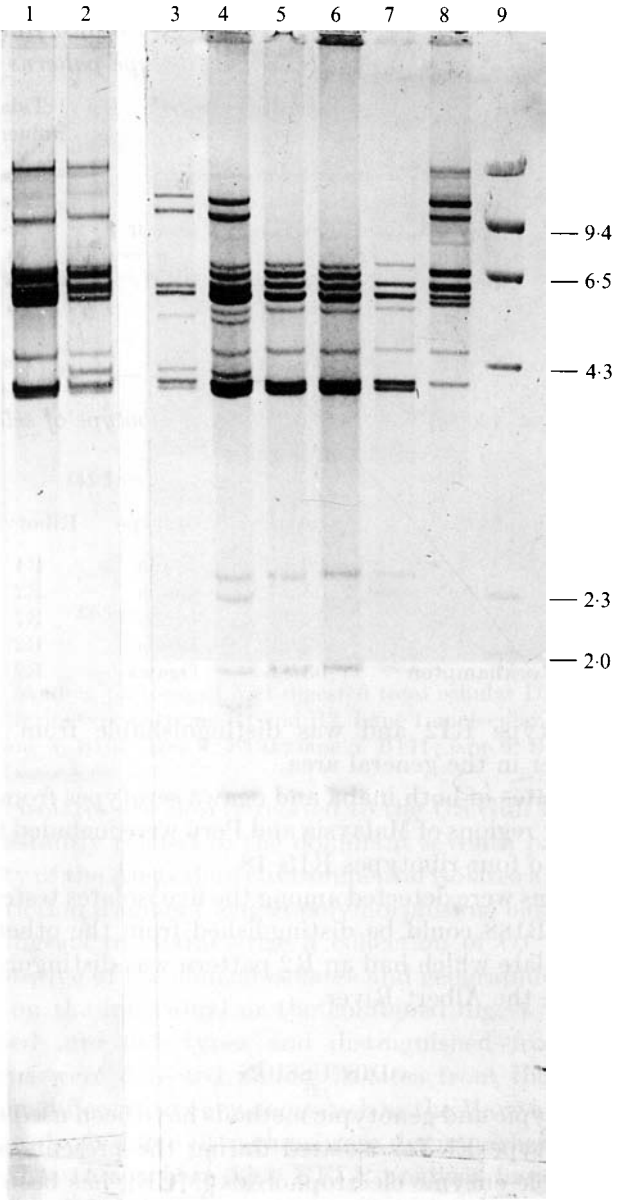

Fig. 3. *Bgl*1 ribotype patterns of some Australian isolates of *Vibrio cholerae* O1 serotype ogawa and inaba. Details of the isolates are shown in Table 1. Blot A serotype inaba: lane 1, patient faeces Beenleigh, 1977, R1; lane 2, Albert River water, 1977, R2. Blot B serotype ogawa: lane 3, NSW 10; lane 4, NSW 11; lane 5, NSW 12; lane 6, NSW 13; lane 7, Clarence River; lane 8, NW Australia; land 9, molecular weight markers (kb). Details of isolates in Blot B are provided in Table 2.

household contact. Isolates from both a symptomatic case and a household contact in Ipswich in 1980 were both R2. In 1981, in Lismore, isolates from two household members and from sewage from their septic tank were R2. The faecal isolate from a patient from Mt Morgan in the Rockhampton area in 1983 was

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Table 2. *Details of the isolation of* Vibrio cholerae *O1 ogawa from the Georges River and Brisbane Water and the ribotype patterns*

Strain no.	Date of isolation	Site and sample	Tidal influence	Ribotype
NSW 4	05/81	Oysters, Georges River estuary	Yes	7
NSW 5–7	05/81	Water, Georges River estuary	Yes	7
NSW 8	12/81	Water, Cabramatta Creek, d/s weir	Yes	6
NSW 9	01/82	Water, u/s Liverpool weir	No	6
NSW 12	02/82	Water, Glenfield Br., u/s weir	No	6
NSW 13	03/82	Water, Bunburycurran Creek, u/s weir	No	6
NSW 10, 11	01/82	Water, Brisbane Water	Yes	7, 13

Table 3. *Pulsed-field gel electrophoresis patterns and ribotype of selected isolates of* Vibrio cholerae *O1*

Strain no.	Place of isolation	Date of isolation	Serotype	Ribotype	PFGE pattern
B11	Albert River	2/1977	Inaba	R1	a
B162	Albert River	03/81	Inaba	R2	a
B141	Logan River	04/80	Inaba	R2	a
B188	Limestone Creek	02/83	Inaba	R2	b
B191	Creek near Rockhampton	03/86	Ogawa	R2	c

serotype ogawa ribotype R12 and was distinguishable from ogawa isolates detected in river water in the general area.

Representative isolates of both inaba and ogawa serotypes from other endemic areas in three different regions of Malaysia and Peru were included for comparison. These were assigned to four ribotypes R15–18.

Three PFGE patterns were detected among the five isolates tested (Table 3, Fig. 4). Inaba R2 isolate B188 could be distinguished from the other R2 strains as shown. The ogawa isolate which had an R2 pattern was distinguishable from the inaba R2 isolate from the Albert River.

DISCUSSION

A variety of phenotypic and genotypic methods have been used to study strains of *V. cholerae* O1 biotype El Tor isolated during the present, seventh cholera pandemic [14]. Multiple enzyme electrophoresis (MEE), has been used to divide El Tor strains from diverse global regions into four major clonal groups representing broad geographical areas [10, 15, 16]. Australian *V. cholerae* O1 isolates were assigned to 9 zymovars based on 13 structural loci by Desmarchelier, Momen and Salles, 1988 [10]. Clinical isolates and *ctx*+ environmental isolates were the same zymovar, Z14. This study included the clinical inaba isolates and some of the inaba environmental isolates included in the zymovar study. Wachsmuth and colleagues, 1991 [16] included five Australian isolates in a MEE study and assigned these to electropherotype or zymovar, ET1. The specific source or site of isolation of their isolates was not reported and it is not possible to relate the isolates to our study. These studies together with a further study of Salles and Momen, 1991, including the Australian data, were used to demonstrate the

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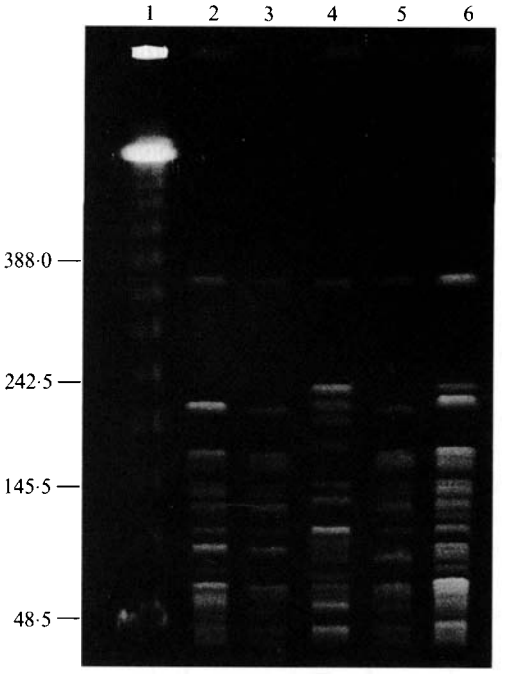

Fig. 4. PFGE banding patterns of *Not*I-digested total cellular DNA from selected *V. cholerae* O1 with ribotype patterns R1 and R2. Lane 1, molecular weight marker (kb): lane 2, B11; lane 3, B162; lane 4, B191; lane 5, B141; lane 6, B188.

Australian *ctx*+ isolates are closely related to the US Gulf Coast strain although they are more distantly related to the dominant seventh pandemic clone.

As the majority of the Australian environmental isolates are *ctx*+, we have used *ctx* RFLPs (restriction fragment length polymorphisms) based on *Bgl*II, *Hind*III, *Pst*I and *Xba*I digests to characterize a collection of O1 isolates including 15 strains, representative of the clinical isolates and geographical locations included here [9]. Based on the individual or the combined digest patterns, the isolates could be grouped into five types and distinguished from imported strains. Common patterns were detected among isolates from the 1977 investigations while isolates from different river systems such as the Mary and North Pine Rivers and faecal isolates from different regions were distinguishable.

Other approaches to southern blot RFLP analysis have proven to be more discriminatory for *V. cholerae* O1. Koblavi and colleagues, 1990, first used ribotyping to study clonal diversity among *V. cholerae* O1 of both classical and El Tor biotypes from the seventh and previous pandemics [17]. A subsequent study by Popovic and colleagues, 1993 further developed the technique and they proposed a standardized ribotyping scheme [18]. These studies have demonstrated the presence of a dominant El Tor clone or clones within a country and evidence of inter-continental spread of the current pandemic. Locally ribotyping is useful in establishing the epidemiological relationship among strains. Popovic and colleagues included three isolates from Australia in their ribotyping study [18]. These were designated ribotypes 9 and 10 within their scheme and were unique to Australia. We have not used the typing scheme proposed by Popovic and

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colleagues. While we agree in principle with the proposal for a standardized scheme for the comparison of strains inter-continentally, it could not be justified economically for our localized studies. In our experience, the only confident way to compare isolates would be to test the type strains of Popovic and colleagues in our facility as we have not found it adequate to compare published photographic and schematic representations. This requires the purchase of the entire typing set from the appointed culture collection and restrictive importation processing.

Another approach to RFLP analysis is PFGE which uses restriction endonucleases that cut DNA infrequently and obviates the need for southern blotting. PFGE when applied in the epidemiological typing of *Vibrio cholerae* O1 further distinguished common electropherotypes and ribotypes [13]. Cameron and colleagues [13] typed two Australian isolates designated ribotype 9 and 10 by Popovic and colleagues [18] in their PFGE study and found minor differences in the respective patterns.

Our ribotype analysis of the Australian environmental isolates, has demonstrated the presence of 14 ribotype patterns among the *V. cholerae* O1, some of which are temporally and geographically restricted. The majority of the Australian serotype O1 El Tor isolates are sub-serotype inaba which we assigned to seven ribotypes of which two, R1 and R2, were predominant. The patterns of R1, R2 and R9, patterns R11 and R14, and R2 and R10 respectively, are distinguished by one restriction fragment only. This is insufficient to assume different clones are present, leaving 11 possible clonal groups. PFGE of a limited number of inaba isolates further supported the persistence of specific clones in the local environment. In addition, PFGE was able to distinguish R2 isolates from the Albert–Logan Rivers system from those from rivers further north. The analysis of a large number of isolates using PFGE might identify further clones present in this environment.

Koblavi and colleagues, 1990, ribotyped isolates from the seventh pandemic representing diverse geographical areas and temporal changes within endemic areas [17]. They proposed that the current pandemic is collectively a number of localized epidemics due to distinct cones which, with the succession of epidemic waves results in the emergence of new clones. There are no epidemics and insufficient sporadic cases of symptomatic cholera in our communities to observe such evolution of strains; however, the patterns of inaba isolates could be followed at intensively monitored riverine sites such as the Albert–Logan Rivers. During 1977 and 1978, 93% inaba isolates were R1 and 7% R2. This pattern changed in 1979 when R2 represented 77% isolates. Karaolis and colleagues, 1994 estimated from a ribotype study of *V. cholerae* O1 isolated during the seventh pandemic that there has been a restriction site change approximately every 6 years [19]. The distribution of ribotypes has to be interpreted with some caution as the number of isolates detected from a specific site are different and the duration of monitoring is uneven between sites, reflecting the public health response to human cases.

The ogawa isolates had a greater variation in the position of restriction sites than the inaba isolates with 8 patterns among the 24 isolates tested. Some of the types were geographically and temporally specific while others were common to aquatic systems 650 km apart. Isolates from up-stream and down-stream in the Georges River during a 9-month period were clearly distinguishable while in the

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estuary, common isolates were detected in oysters and their growing waters. The detection of the same ribotype 7 months later in similar estuarine oyster farm waters approximately 45 K due north suggests specific strains may be adapted to freshwater and marine habitats. These may be indigenous types or may be translocated with the frequent movement of marine life or shipping transport along this part of the coast. In contrast, isolates could not be related from a sewage outfall at Mt Gravatt and isolates from the receiving waters. As multiple ribotypes were detected in some river systems such as the Albert–Logan Rivers, the lack of correlation may have been due to either lack of epidemiological association or the testing of insufficient colonies from isolation plates. This emphasizes the need to select multiple colonies from isolation plates from environmental samples in epidemiological investigations.

A curiosity of the Australian cholera experience is the origins of the bacterium detected since 1977. The importation from an endemic area is a possibility but unlikely given the few severe imported cases notified, the widespread distribution of and diversity of O1 strains in our environment and the lack of similarity between the imported and local strains [9, 10]. The ogawa strains are infrequently isolated and have an unexplained distribution further south than the inaba serotype, in spite of extensive monitoring of these environments for extended periods. It can be postulated that the ogawa strains which lack *ctx* and related toxin genes have both acquired these genes and seroconverted. Stroeher and colleagues, 1992, demonstrated seroconversion within O1 sub-types is the result of a rare mutational event in the *rfbT* gene, with respective serovars subsequently emerging within human populations through the selective pressures of human immune responses [19]. In the event of such mutations occurring, the ribotypes would remain indistinguishable or closely related. We found only one ogawa strain with a ribotype pattern indistinguishable from inaba strains in the Rockhampton riverine environment; however, this isolate was distinguishable by PFGE.

Ribotyping has proven to be a discriminatory tool for the study of the relationship among strains of *V. cholerae* O1 in the Australian environment. Evidence is provided for the persistence of several clones belonging to both the inaba and ogawa serotype in our riverine environment over several years and this has resulted in occasional sporadic human infections. While the origins of the *V. cholerae* O1 in our environment remains unexplained, MEE, *ctx*RFLP and ribotyping to date have suggested the Australian *V. cholerae* O1 isolates are not related to the current seventh pandemic strains.

ACKNOWLEDGEMENTS

We thank the microbiologists in many private and government laboratories who have supplied *V. cholerae* strains in the past. We thank Mrs A. Lew and Dr J. Vickers who have assisted in the ribotyping of *V. cholerae* in the past.

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