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SHORT REPORT
Biochemical fingerprinting of *Vibrio parahaemolyticus* by the PhenePlate system: comparison between pandemic and non-pandemic serotypes

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SUMMARY

During recent years a pandemic clone of *Vibrio parahaemolyticus* has emerged. Isolates of this clone are distributed among several serotypes, but are genotypically related. In the present study, a phenotyping method (biochemical fingerprinting) was used to characterize pandemic and non-pandemic isolates belonging to *V. parahaemolyticus*. It was found that the pandemic isolates showed a high level of phenotypic homogeneity and a majority of the pandemic isolates belonged to the same biochemical phenotype, whereas non-pandemic *V. parahemolyticus* isolates were more heterogeneous. In conclusion, biochemical fingerprinting of *V. parahaemolyticus* can be used as a first screening method to differentiate between pandemic and non-pandemic isolates of *V. parahaemolyticus*.

Vibrio parahaemolyticus, a halophilic Gram-negative marine bacterium belonging to the Vibrionaceae family has attracted much attention as the causative agent of human gastroenteritis in the recent past. *V. parahaemolyticus* is associated with foodborne disease in Japan and throughout Asia since 1950 [1], and is the most frequently isolated *Vibrio* species associated with gastroenteritis in the United States [2]. The incidence of *V. parahaemolyticus* infection has increased around the world since 1996, including several large outbreaks in the United States, Southeast Asia, Canada and Mexico [2–5]. Clinical manifestations of *V. parahaemolyticus* infections include diarrhoea, abdominal cramps, nausea, vomiting, headache, fever and chills, with an incubation period ranging from 4 to 96 h [6]. Human *V. parahaemolyticus* gastroenteritis is often due to consumption of seafood, including raw and improperly cooked shellfish [7–9]. *V. parahaemolyticus*is widely disseminated in marine and estuarine environments [9, 10]. Most clinical strains of *V. parahaemolyticus* produce known virulence factors such as the thermostable direct haemolysin (TDH) encoded by the *tdh* genes [11], the TDH-related haemolysin (TRH) encoded by the *trh* genes, and epidemiological evidence supports the view that *V. parahaemolyticus* strains carrying the *tdh* and/or the *trh* gene are virulent [12, 13].

Serotyping is applied for epidemiological investigations of *V. parahaemolyticus* and more than 75 combinations of O and K serotypes have been described [14]. However, recent studies indicates that a few specific serotypes are more often associated with gastroenteritis [15]. Serotype O4:K12 has been associated with foodborne outbreaks in the United States and Mexico [16], and serotype O3:K6 was recognized as the dominant cause of gastroenteritis in Calcutta, India in 1996 [17]. Since 1996, serotype O3:K6 strains have been responsible for pandemic

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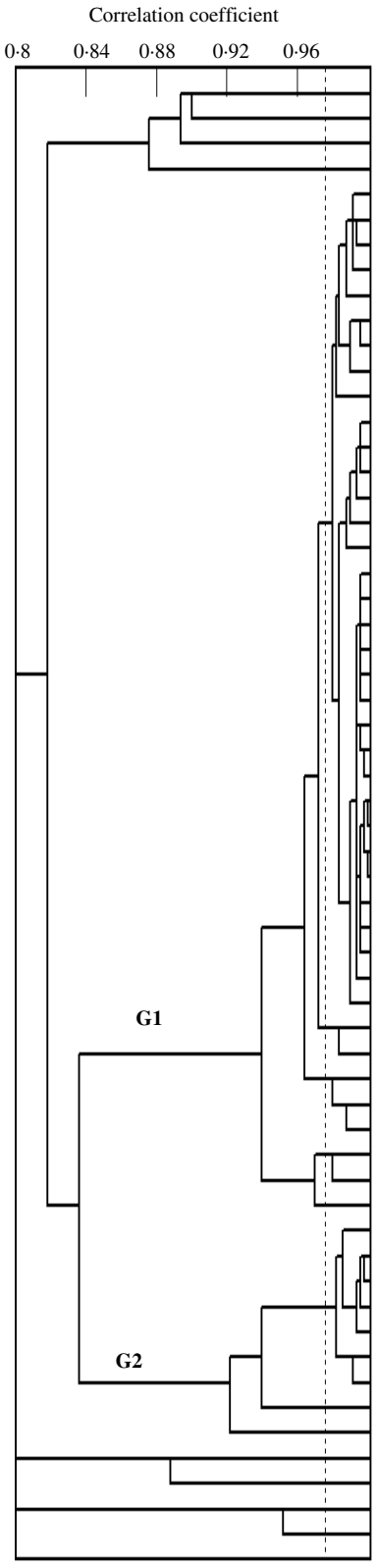

Pandemic status	Strains name	Year of isolation	Source of isolation	Serotypes O:K	*toxR*	*tdh*	*thr*	GS-PCR	ORF8	Ref. no
NP	VP 364	2000	Calcutta, India	1:56	+	+	–	–	–	
NP	VP3	1994	Calcutta, India	2:3	+	+	–	–	–	
NP	VP33	1995	Calcutta, India	1:1	+	+	–	–	–	
NP	VP25	1994	Calcutta, India	8:21	+	+	–	–	–	
P	VP193	1997	Calcutta, India	3:6	+	+	–	+	+	
P	VP342	2000	Calcutta, India	1:25	+	+	–	+	+	
P	VP272	1998	Calcutta, India	4:68	+	+	–	–	–	
P	VP354	2000	Calcutta, India	3:6	+	+	–	+	+	
P	VP164	1997	Calcutta, India	3:6	+	+	–	+	+	
P	VP301	1999	Calcutta, India	4:68	+	+	–	+	+	
P	VP363	2000	Calcutta, India	1:25	+	+	–	+	+	
P	VP 376	2000	Calcutta, India	1:25	+	+	–	+	+	
IP	KX-V-568	1998	To Kansai- F. Vietnam	4:68	NT	NT	NT	NT	NT	
P	VP250	1998	Calcutta, India	1:UT	+	+	–	+	+	[17]
P	VP343	2000	Calcutta, India	1:25	+	+	–	+	+	
IP	AN-8373	1998	Bangladesh	3:6	NT	+	–	+	NT	[17]
P	VP356	2000	Calcutta, India	1:UT	+	+	–	+	+	
IP	DOH95815	1997	Taiwan	3:6	NT	+	–	+	NT	[17]
NP	VP95	1996	Calcutta, India	K6	+	+	–	+	+	
P	VP325	1999	Calcutta, India	3:6	+	+	–	+	+	
P	VP331	1999	Calcutta, India	1:25	+	+	–	+	+	
IP	KX-V225	1996	To Kansai- F Thailand	3:6	NT	+	–	+	NT	[16–18]
IP	AN13938	1998	ICDDRB, Bangladesh	3:6	NT	NT	NT	NT	NT	[17]
IP	KX-V226	1996	To Kansai- F Thailand	3:6	NT	+	–	NT	NT	[17, 18]
IP	OP419	1998	Osaka, Japan	3:6	NT	NT	NT	NT	NT	[17]
IP	AN5034	1998	Bangladesh	4:68	NT	+	–	+	NT	[16, 17]
P	VP262	1998	Calcutta, India	4:68	+	+	–	+	+	
IP	KX-V231	1996	To Kansai- F Thailand	3:6	NT	+	–	NT	NT	[16]
P	VP346	2000	Calcutta, India	1:25	+	+	–	+	+	
IP	KX-V138	1995	To Kansai-F Indonesia	3:6	NT	+	–	NT	NT	[17]
IP	OP424	1998	Osaka, Japan	4:68	NT	+	–	+	NT	[17]
IP	KX-V563	1998	To Kansai-F Thailand	4:68	NT	NT	NT	NT	NT	[17]
IP	KX-V508	1997	To Kansai-F Nepal	4:68	NT	NT	NT	NT	NT	[17]
IP	1(1926)	1998	Kyoto, Japan	3:6	NT	NT	NT	NT	NT	[17]
IP	OP411	1997	Osaka, Japan	3:6	NT	NT	NT	NT	NT	[17]
IP	be98-2062	1998	USA	3:6	NT	+	–	+	NT	[17]
IP	FIHES98VI-32-4	1998	Fukuoka,JAPAN	3:6	NT	–	–	+	–	[17]
P	VP207	1997	Calcutta, India	1:UT	+	+	–	+	+	
P	VP234	1998	Calcutta, India	4:68	+	+	–	+	+	
P	VP165	1997	Calcutta, India	3:6	+	+	–	+	+	
IP	KX-V740	1999	To Kansai-F Thailand	4:68	NT	NT	NT	NT	NT	[17]
IP	AN-16000	1998	Bangladesh	1:UT	NT	+	–	+	NT	[16, 17]
P	VP62	1996	Calcutta, India	3:6	+	+	–	+	+	
NP	VP184	1997	Calcutta, India	4:13	+	+	–	–	–	
P	VP165	1997	Calcutta, India	3:6	+	+	–	+	+	
NP	VP136	1996	Calcutta, India	1:1	+	+	–	–	–	
IP	AQ3732	1982	Unknown	3:6	NT	–	+	–	–	[18]
IP	AQ4733	1992	Singapore	3:6	NT	–	–	–	–	[18]
IP	AQ4901	1993	Thailand	3:6	NT	–	+	–	–	[18]
IP	AQ3794	1983	Singapore	3:6	NT	–	+	–	–	[18]
IP	AQ4235	1987	Thailand	3:6	+	–	+	–	–	[18]
IP	AQ4019	1985	Maldives	3:6	NT	–	+	–	–	[18]
IP	AQ4037	1985	Maldives	3:6	NT	–	–	–	–	[18]
IP	AQ4093	1986	Maldives	3:6	NT	–	–	–	–	
NP	VP 337	2000	Calcutta, India	4:9	+	+	–	–	–	
NP	VP 287	1999	Calcutta, India	13:UT	+	+	+	–	–	
NP	VP26	1995	Calcutta, India	4:55	+	+	–	–	–	
NP	VP51	1996	Calcutta, India	2:3	+	+	–	–	–	

Fig. UPGMA clustering of the PhP 48 typing data for 58 isolates of various serotypes of *Vibrio parahaemolyticus*, together with data on isolation source, isolation year, serotype and presence of toxin genes. First column shows pandemic status of the isolates, i.e. NP indicates non-pandemic, P indicates pandemic, IP indicates international pandemic strains. The last column shows the reference number for other studies that have used the same strains. NT indicates not tested. For comparative purposes, earlier presented data on the presence of *toxR*, *tdh* and *trh* genes by PCR assay [29, 30] and data on group-specific PCR (GS-PCR) and PCR for open reading frame ORF8 [19, 31] are shown.

spread into Southeast Asia, Japan and the United States through travellers and/or ships' ballast water [5, 18]. Furthermore, molecular typing has shown that this pandemic O3:K6 clonal group is distinguishable from O3:K6 strains isolated before 1996 and from other serotypes [17]. However, molecular and epidemiological studies indicate that the recently emerged serotypes O4:K68 and O1:KUT isolated from India and Bangladesh are clonally related to O3:K6 and also possess pandemic potential [19]. Thus, although those serotypes have different antigenic properties belonging to different serotypes, they seem to form one clonal group, recognized as the 'pandemic group'. Molecular typing methods such as PFGE, RFLP, AP–PCR, ribotyping, sequence determination of *tdh*, *toxR*, ORF8 PCR have been applied to discriminate and to find possible clues to the origin of the pandemic group of *V. parahaemolyticus* [20–22].

Previous studies on the pandemic strains of *V. parahaemolyticus* have mostly relied on serotyping and molecular typing techniques to discriminate between pandemic and non-pandemic strains. Identification and characterization of isolates below the species level is important in epidemiological studies, e.g. for tracing the source of infections and for identification of epidemic clones. Molecular typing methods which are highly discriminatory are often used for these purposes, but such methods are expensive for handling large numbers of isolates, especially for resource-poor countries. Biochemical fingerprinting using the PhenePlate (PhP) system is a phenotyping system based on kinetic measurements of the fermentation of selected reagents. It has previously been successfully used for identification of virulent clones of *Aeromonas* spp. [23, 24], *Vibrio anguillarum* [25] and *Escherichia coli* [26] in various ecological and epidemiological studies [27, 28]. In this study, we used the PhP biochemical fingerprinting system to understand the differences in phenotypic traits between the pandemic and non-pandemic strains of *V. parahaemolyticus*.

A total of 190 *V. parahaemolyticus* isolates including 161 collected from a diarrhoeal surveillance study performed during 1994–2000 at the National Institute of Cholera and Enteric Diseases (NICED), Calcutta, India, and 29 isolates from an international collection of Southeast Asian travellers' diarrhoeal strains were studied. All isolates were confirmed as being *V. parahaemolyticus* by standard culture and biochemical tests and serotyped by the commercially available *V. parahaemolyticus* serotyping kits (Denka Seiken Ltd, Tokyo, Japan). All strains had previously been examined for the presence of *toxR*, *tdh* and *trh* genes by PCR assay [29, 30]. Likewise, data on group-specific PCR (GS-PCR) and PCR for open reading frame ORF8 were available from previous investigations [19, 31].

Biochemical fingerprinting of the isolates were performed using the PhP system (PhPlate Microplate Techniques, Stockholm, Sweden) according to the manufacturer's instructions [32]. PhP-RV (a rapid screening method using 11 dehydrated reagents) was initially used for typing 161 isolates, and PhP-48 (using 48 reagents) was used to further discriminate among 58 isolates. According to the data obtained from typing with the PhP-RV system, the 161 isolates were distributed into eight PhP types (data not shown). Among the isolates, 65% clustered into PhP type 1. Within this type, 46% of the isolates belonged to serotype O3:K6, 12% to O4:K68, 8% to O1:K25, 9% to O1:KUT and 25% to different serotypes. Serotype O3:K6 was distributed among seven different PhP types. Thus, the PhP-RV typing system did not allow consistent discrimination of the pandemic group strains from others.

A total of 58 isolates were further typed using the more discriminatory PhP-48 system. Thirty-one of these were randomly selected isolates from NICED Calcutta patients [11 non-pandemic and 20 pandemic strains (seven of O3:K6, six O1:K25, four O4:K68, and three O1:KUT Calcutta patients)] and the other 27 were from Southeast Asia (19 isolated in 1996 or later, and eight isolated before 1996). UPGMA cluster analysis of the PhP data revealed two distinct clusters (G1 and G2) and eight single isolates (Fig.). Except for two isolates, cluster G1 included only isolates belonging to the pandemic serotypes (O3:K6, O4:K68, O1:KUT, O1:K25). Pandemic serotypes O3:K6 and O4:K68 strains isolated from Southeast Asian travellers were not found to be different from the strains isolated in India. Cluster G2 contained only strains isolated before 1996; all but one of which were serotype O3:K6. The other non-pandemic isolates showed a high diversity and each belonged to a unique PhP type. For comparative purposes, earlier presented data on the presence of *toxR*, *tdh* and *trh* genes by PCR assay [29, 30] and data on group-specific PCR (GS-PCR) and PCR for open reading frame ORF8 [19, 31] are also presented in the Figure. It was shown that the non-pandemic isolates are mostly negative for GS-PCR and ORF8, compared to pandemic isolates.

In concurrence with the phenotyping data, molecular typing with PFGE of 139 *V. parahaemolyticus* isolates of O3:K6 serotype showed that strains isolated after 1996 from different Asian countries were closely related (strains KX-V225, 226, 231 are included in this study) and that they were distinct from O3:K6 strains isolated before 1996 (strains AQ3732, AQ4733, AQ3794, AQ4019 are included in this study) [18]. Another study by Yeung *et al*. (2002) also showed similar PFGE patterns of O3:K6 isolates originating from different countries (strains KX-V225, AN5034, AN16000 are included in this study) but that the pattern was distinct from non-O3:K6 strains [16]. Similarly, it has been shown that strains of the pandemic serotypes O3:K6, O4:K68 and O1:KUT exhibit a unique profile by arbitrarily primed PCR profiling, ribotyping and PFGE analysis (O3:K6 serotype strains KX-V225, KX-V226, KX-V231; O1:KUT serotype strains VP250, AN16000; O4:K68 serotype strains AN5034, KX-V508, KX-V563 are included in this study) [21]. The biochemical fingerprinting in this study also showed that *V. parahaemolyticus* pandemic clonal serotypes have a similar phenotypic pattern as demonstrated by molecular typing methods. The results from our study again indicate that isolates belonging to serotypes O3:K6, O4:K68, O1:KUT and O1:K25 are related in both phenotypic and genotypic characteristics and may belong to the pandemic clonal group of *V. parahaemolyticus*. The high phenotypic and genotypic homogeneity may indicate that the strains have diverged from a common ancestor by the alteration of genes associated with a few O:K antigens and a few single clones may be responsible for the emergence of the pandemic serotypes.

Biochemical fingerprinting, PhP typing, is a phenotyping method that is easy to learn and does not require access to expensive equipment and reagents. It can thus be used as a tool for epidemiological surveillance in laboratories where there is a lack of genotyping resources. A main advantage is that it is simple to perform and data processing and presentation are automated. In general less than 8 h of labour is required to assay 200 isolates. Furthermore, preliminary results may be obtained after overnight incubation. Thus, the PhP typing method may be suitable as a screening method for surveillance purposes and/or to study outbreaks of *V. parahaemolyticus* gastroenteritis, especially for large numbers of isolates, for which it can be used to select those isolates that require further typing by molecular typing methods. In conclusion, biochemical fingerprinting using the PhP system showed good agreement with molecular characterization of pandemic and non-pandemic isolates of *V. parahaemolyticus*.

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DECLARATION OF INTEREST

None.

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