Table 1. Phenotypic and related genetic traits of *V. cholerae* O1 strains isolated in Latin America

icddr,b

Country of Origin	Year of Isolation	No of Isolation	Serotype	Phenotypic properties				Genetic screening			Deduced Biotype
				CCA	PBS (50U)	Phage IV Classical	Phage V El Tor	MAMA PCR	*tcpA*	*rstR*	
Peru (49)	1991	1	Inaba	+	R	R	S	E	E	E	ET
	1992	4	Inaba	+	R	R	S	E	E	E	ET
		5	Ogawa	+	R	R	S	E	E	E	ET
	1993	1	Inaba	+	R	R	S	E	E	E	ET
		2	Ogawa	+	R	R	S	E	E	E	ET
	1994	2	Inaba	+	R	R	S	E	E	E	ET
		8	Ogawa	+	R	R	S	E	E	E	ET
	1995	1	Inaba	+	R	R	S	E	E	E	ET
		7	Ogawa	+	R	R	S	E	E	E	ET
	1996	3	Ogawa	+	R	R	S	E	E	E	ET
	1997	3	Ogawa	+	R	R	S	E	E	E	ET
	1998	8	Ogawa	+	R	R	S	E	E	E	ET
	1999	4	Ogawa	+	R	R	S	E	E	E	ET
Mexico (53)											
	1991	6	Inaba	+	R	R	S	E	E	E	ET
		2	Ogawa	+	R	R	S	C	E	C&E	Hyb-ET
		19	Ogawa	-	R	R	S	C	E	C&E	Hyb-ET
		2	Ogawa	-	S	R	S	C	E	C&E	Hyb-ET
	1992	3	Inaba	+	R	R	S	E	E	E	ET
		1	Inaba	+	S	R	S	E	E	E	ET
		1	Inaba	+	R	R	R	E	E	E	ET
		1*	Inaba	-	S	R	R	-	-	-	Hyb- Class (?)
	1993	6	Ogawa	-	R	R	S	C	E	C&E	Hyb-ET
		1	Ogawa	+	S	R	S	E	E	E	ET
		1	Ogawa	-	S	R	S	C	E	C&E	Hyb-ET
	1994	1	Inaba	+	R	R	S	E	E	E	ET
		1	Inaba	+	R	R	S	C	E	C&E	Hyb- ET
	1995	1	Inaba	+	R	R	S	E	E	E	ET
		2	Ogawa	-	R	R	S	C	E	C&E	Hyb- ET
		1	Inaba	-	R	R	S	C	E	C&E	Hyb- ET
		2	Inaba	-	S	R	S	C	E	C&E	Hyb- ET
		1	Inaba	-	R	R	R	C	C	C	Classical
		1	Ogawa	-	S	R	R	C	C	C	Classical
Guatemala (2)	1993	1	Inaba	+	S	R	S	E	E	E	ET
		1*	Inaba	-	S	R	R	-	-	-	Hyb- Class (?)
N16961		Control	Inaba	+	R	R	S	E	E	2	El Tor
O395		Control	Ogawa	-	S	S	R	C	C	1	Classical

* Serologically non-O1/non-O139

CCA, chicken cell agglutination; PBS, polymixin B; R, resistant; S, sensitive; E, El Tor; Hyb, hybrid; Class, classical

Table 2. Multi-locus genetic screening of *V. cholerae* O1 strains isolated in Latin America (1991- 1999) for vibrio pathogenecity island-related genes and gene clusters

Serial No.	Country/ Year	Serotype/ Deduced Biotype	HKG	VSP-I					MSHA				*hlyA*	VSP-II									VPI-I			*pilE*	RTX		RS1	CTX					
			mdh	175	178	180	183	185	398	400	403	406	489	490	493	498	502	504	512	514	516	517	*tcpA*	*toxT*	*acfB*	*pilE*	*rtxA*	*rtxC*	*rstC*	*rstA*	*orfU*	*zot*	*ctxAB*	*tlc*	[illegible]
Peru	1991	In-ET	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1991	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1993	Og-ET	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
	1996	Og-ET	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	E	+	+	+	+	+	+	+	+	+	-	+	+
	1997	Og-ET	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1999	Og-ET	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
Mexico	1991	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1991	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1991	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1991	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1991	Og-Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1991	Og-Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1992	Og-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1992	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1992	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1992	Non 01/0139	+	-	-	ND	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	+
	1993	Og-Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1993	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1994	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1994	In-Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1995	Og-Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1995	Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1995	In-Hyb-ET	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
		In-El Tor	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
		In-Classic	+	-	-	ND	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	C	+	+	+	+	-	-	+	+	+	+	+	+
		Og-El Tor	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
		Og-El Tor	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E	+	+	+	+	+	+	+	+	+	+	+	+
		In-Classic	+	-	-	ND	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	C	+	+	+	+	-	-	+	+	+	+	+	+
Guatemala	1993	In-ET	+	+	+	ND	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	E	+	+	+	+	+	+	+	+	+	+	+	+
	1993	Non 01/0139	+	-	-	ND	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	+
	N16961	El Tor	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	E†	+	+	+	+	+	+	+	+	+	+	+	+
	0395	Classical	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	C†	+	+	+	+	-	-	+	+	+	+	+	+

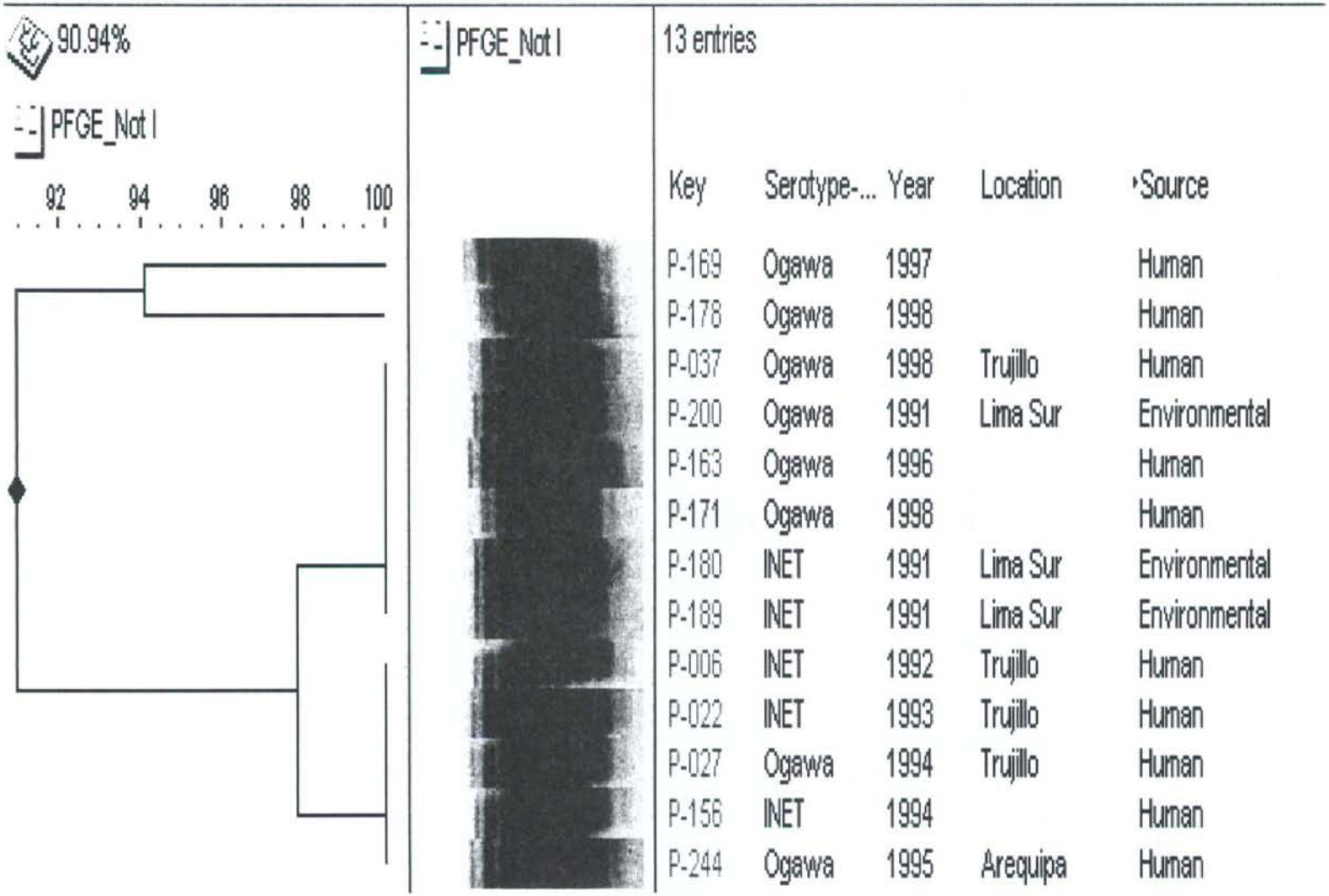

Fig. 1. PFGE analysis of Peruvian *V. cholerae* O1 EL Tor strains (1991 – 1999)

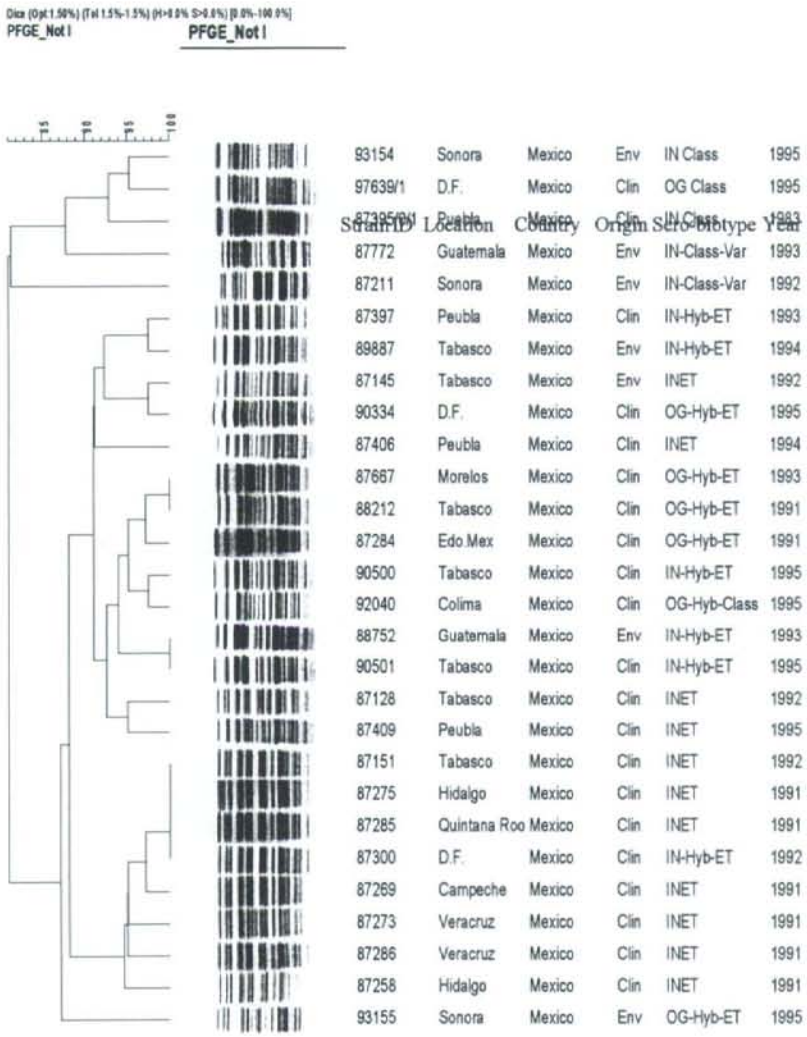

Fig. 2. PFGE analysis of Mexican *V. cholerae* O1 biotype EL Tor and classical strains

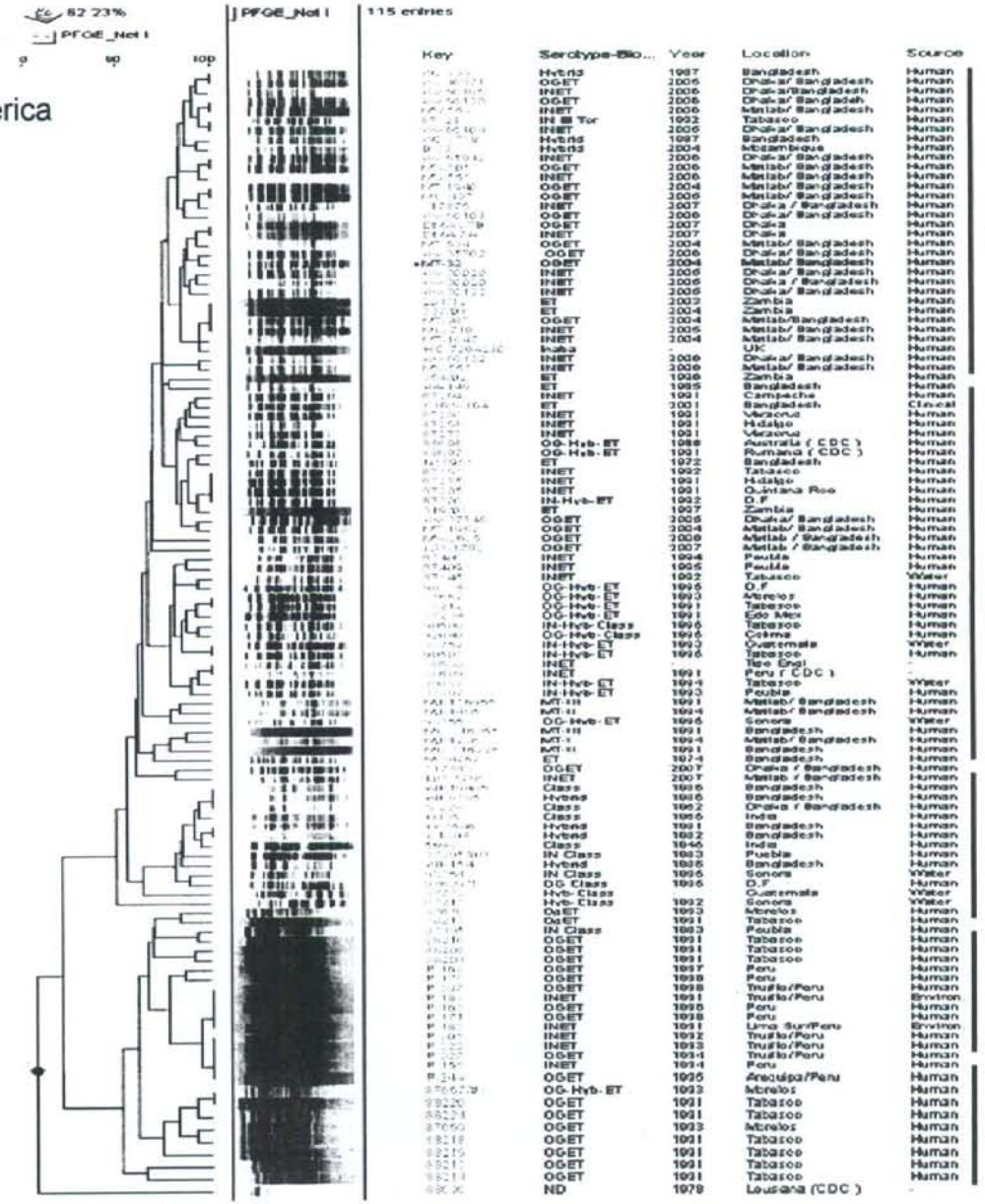

Fig. 3. *V. cholerae* strains of Latin America vs Asia & Aferica

STUDY TITLE:

"Genotyping of *Vibrio cholerae* O1, *Vibrio parahaemolyticus* and *Campylobacter* spp"

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STUDY DIRECTOR: (Name of the Research Director) **T. Ramamurthy,** Deputy Director

Report Date: March 13, 2009

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1. **Objectives:**

   (i) Tracking the spread of molecular types of hybrid strains of *Vibrio cholerae* O1 in the Asia-Pacific region

   (ii) Epidemiology and molecular characterization of *Campylobacter* spp in Kolkata, India

   (iii) Multilocus sequence typing (MLST) of pandemic strains of *Vibrio parahaemolyticus* isolated from clinical and environmental sources

2. **Study Design:**

(i) Tracking the spread of molecular types of hybrid strains of *Vibrio cholerae* O1 in the Asia-Pacific region

The epidemic and pandemic cholera is caused by toxigenic *Vibrio cholerae* is known for its dynamicity, as the organism often changes its biotypic features as well as molecular configuration. *V. cholerae* belonging to serotype O1 has two well established biotypes, namely, classical and El Tor, that are differentiated based on number of phenotypic traits like susceptibility to polymyxin B, chicken cell (erythrocytes) agglutination (CCA), hemolysis of sheep erythrocytes, Voges-proskauer (VP) test, which measures the production of acetylmethylcarbinol, and phage susceptibilities (1, 2). Biotype is a sub specific taxonomic classification of *V. cholerae* O1.

Till date, seven cholera pandemics were assigned, of which, the first 6 pandemics were caused by the classical biotype of *V. cholerae* O1. The seventh pandemic that started in 1961 and continuing till date is associated with El Tor biotype of *V. cholerae*. Differentiation of *V. cholerae* strains into biotype is not directly related to the process of clinical management of cholera but is of immense public health and epidemiological importance in identifying the source and spread of infection, particularly when *V. cholerae* is first isolated in a country or geographic area. Conventionally, at least two or more of the phenotypic tests mentioned above should be included to determine the biotype, since results can vary for individual isolates.

Comparative genetic analyses have recently revealed a high degree of conservation among diverse strains of *V. cholerae* but have also shown genes that

differentiate classical biotype from El Tor biotype (3). Molecular biotyping of *V. cholerae* O1 using multiplex PCR targeting the *ctxA-tcpA* gene complex exploits the nucleotide sequence differences of the major subunit protein of the toxin co-regulated pilus (TCP) gene (*tcpA*) to differentiate between classical and El Tor biotypes (4). Only in toxigenic *V. cholerae* O1 El Tor and O139 strains, cholera toxin prophage region (CTXΦ) is often flanked by an element termed RS1 containing *rstC* gene (5). The only difference between RS1 and RS2 is the presence of *rstC* gene in RS1 alone (5, 6). Another virulence associated protein known as repeat in toxin (RTX) encoded by a cluster of genes of 10kb size, comprising four ORFs, *rtxABCD*, of which the *rtxC* gene has been observed only in El Tor biotype (7). Nucleotide sequence comparison of hemolysin encoding *hlyA* gene from classical and El Tor strains reveal the presence of an 11-base-pair deletion in classical strains that results in a truncated protein product of 27 kilodaltons in classical strains rendering it non-hemolytic, whereas in El Tor strains the HlyA is intact 82-kilodalton and biologically active (8). On the basis of differences in the sequences of *hlyA* genes, a 19-base-pair oligodeoxynucleotide probe has been developed to distinguish between the two biotypes of *V. cholerae* serogroup O1 (9). This gene marker was found to be very useful to differentiate the biotypes than the other commonly used methods, which are less reliable and often difficult to interpret (9). Recently, comparative genomic studies using a *V. cholerae* DNA microarray on 11 epidemic isolates identified two regions, *Vibrio* seventh pandemic island I (VSP-I), encompassing VC0175 to VC0185 and VSP-II, encompassing VC0490 to VC0497, that were found exclusively among El Tor biotype isolates (3). Subsequently, it was shown that the VSP-II region actually encompassed a 26.9 kb region (VC0490–VC0516) in *V. cholerae* biotype El Tor and O139 serogroup isolates (10). Besides these phenotypic and genotypic differences, there are also dissimilarities in the infection pattern of disease caused by the two biotypes (11). Epidemiological studies proved occurrence of more asymptomatic carriers of El Tor strains that outnumber active cases by a ratio of up to 50:1 (12), better survival of El Tor strains in the environment and in the human host, and more efficient host-to-host transmission of El Tor strains than of classical strains (13).

Cholera toxin (CT), the primary toxin produced by *V. cholerae* O1 and O139, is responsible for most of the manifestations of the disease cholera. Based on the B subunit

of CT, two immunologically related but not identical epitypes have been designated: CT1 is the prototype elaborated by classical biotype strains and by U.S. Gulf Coast strains, while CT2 is produced by the El Tor biotype and O139 strains (14). Another classification identifies three types of *ctxB* genes based on three non-random base changes resulting in changes in the deduced amino acid sequence. Genotype 1 is found in strains of the classical biotype worldwide and in US Gulf Coast, genotype 2 is found in El Tor biotype strains from Australia, and genotype 3 is found in El Tor biotype from the seventh pandemic and the Latin American epidemic strains (15). Thus, the *V. cholerae* O1 El tor biotype of the ongoing seventh pandemic produces CT of the CT2 epitype and genotype 3, while the classical biotype CT belongs to the CT1 epitype and genotype 1.

Although the classical biotype of *V. cholerae* O1 is extinct, even in southern Bangladesh, the last of the niches where this biotype prevailed, Nair *et al.* (2002) (16) identified new varieties of *V. cholerae* O1, of El Tor biotype with traits of classical biotype, from hospitalized patients with acute diarrhea in Bangladesh. These strains could not be biotyped and were, therefore, designated as "hybrid type". The impact of such hybrids was emphasized when *V. cholerae* O1 isolated from Mozambique during an epidemic of cholera in early 2004 were found to carry the classical type CTX prophage but otherwise was identical to El Tor biotype (17, 18). Recently, a collection of *V. cholerae* O1 strains isolated in Bangladesh during the past four and a half decades were examined using monoclonal antibodies specific for classical and El Tor CT and the nucleotide sequence of the B subunit of CT of representative strains to determine the deduced amino acid sequence. This study revealed that all *V. cholerae* O1 El Tor strains isolated since 2001 produced CT subtype of the classical biotype indicating a cryptic change in the seventh pandemic El Tor biotype strains of *V. cholerae* O1 has occurred (19). Therefore, the epitype and genotype of CT of the El Tor strains currently associated with cholera in Bangladesh has shifted from epitype CT2 to epitype CT1 and from genotype 3 to genotype 1. The presence of classical CT in El Tor biotype *per se* is not novel and has been reported (16, 17, 20). In fact, the US Gulf Coast clone of *V. cholerae* O1 is El Tor strains that possess classical CT (15). The fact that El Tor strain producing classical CT has completely replaced the prototype seventh pandemic El Tor strains that produced the El Tor CT in Bangladesh is interesting. More recently, retrospective

analysis of *V. cholerae* O1 strains over a period of more than a decade established that hybrid CTX prophage with El Tor *rstR* and classical *ctxB* replaced El Tor type completely since 1995 in Kolkata, India (21).

Apart from classical and El Tor type biotypes, two new biotypes have been proposed, one possessing conventional phenotypic properties of both classical and El Tor thus designated as 'Hybrid biotype' and another which is similar to the El Tor biotype by conventional phenotypic traits, but produces classical type CT and thus designated as 'El Tor variant'. In the original publication (19), we had named these strains as altered El Tor but now renamed them as 'El Tor variant' (22). A recently developed mismatch amplification mutation assay (MAMA) PCR is useful in detecting El Tor or classical type *ctxB* (23). We believe that this amendment is essential in view of the current thought that some of these hybrids might cause a more severe kind of cholera and the evidence to this effect (24) is becoming available. There is also indication that the hybrid and El Tor variant type of strains are spreading to other parts of the world (25).

Results obtained:

a) *V. cholerae* O1 strains

For molecular screening, nine *V. cholerae* O1 strains isolated from a cholera outbreak in Bihar (North-east part of India) were included in this study.

b) Identification of hybrid strains using MAMA-PCR

The test strains were initially subjected to several conventional tests (growth in thiosulphate-citrate-bile salts-sucrose agar, serogrouping using O1poly and mono-specific Ogawa, Inaba and O139 antisera). The hybrid strains of *V. cholerae* O1were confirmed by using the MAMA-PCR (23). Eight out of the nine strains harbored the *ctxB* of classical biotype (Fig. 1) and one strain had failed to amplify the *ctxAB* in the PCR assay.

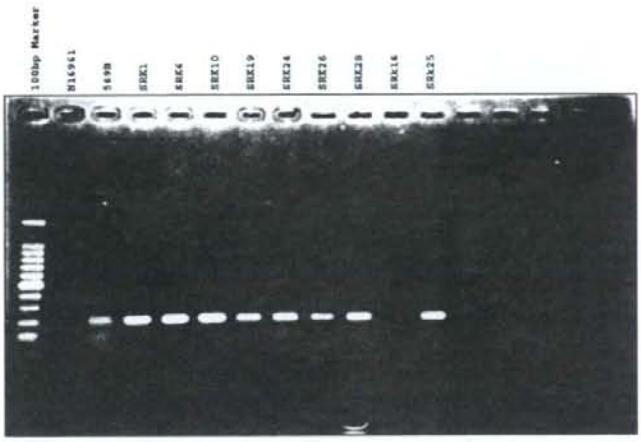

Fig. 1 MAMA PCR (Classical)

c) PFGE typing of *V. cholerae* O1

PFGE will be performed using the standardized protocol for international comparison (26). Of the 9 strains, 7 (SRK1, SRK10, SRK24, SRK26, SRK28, SRK19 and SRK6) were identical in the *Not*I PFGE profile (Fig. 2). The other two strains were different among themselves and from the rest of the other strains.

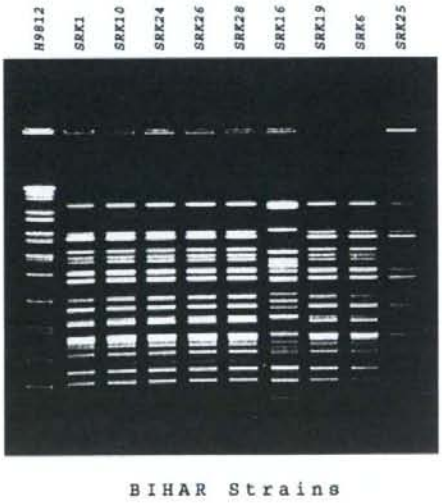

Fig. 2. *Not*I PFGE profiles of *V. cholerae* O1 strains from Bihar cholera outbrea

d) PFGE profiling and data analysis

Binumeric software was recently procured form the Applied Maths, Sweden. The staff from NICED were undergone a 5 days training (February 10-14, 2009) at the Public Health Laboratory Center, Kwaloon, Hong Kong for the use of this software. In the future, PFGE gel images will be digitalized and compared for designation of established/new profiles using Bionumeric software. Representative images of designated profiles will be displaced through the PulseNet Asia Pacific web site (http//www.PulseNet AsaiaPacific).

(ii) Epidemiology and molecular characterization of *Campylobacter* spp in Kolkata, India

During the past three decades, *Campylobacter* spp. have been the focus of great attention because of the increasing frequency with which they have been isolated from infected man and animals, as well as contaminated food and water. After its successful isolation from stools in the 1970s, *Campylobacter* has rapidly become the most commonly recognized cause of bacterial gastroenteritis in man. Although several *Campylobacter* spp. (*C. jejuni, C. coli, C. upsaliensis, C. lari, C. concisus, C. fetus subsp. fetus, C. jejuni* subsp. *doylei, C. hyointestinalis*) have been associated with diarrhea, *C. jejuni* is by far the most frequent species isolated from humans. Among several species of campylobacters, *C. jejuni* is a well known to cause morbidity, in both industrialized and developing countries, and represents a considerable drain on economic and public health resources. In the industrialized world, acute self-limiting gastrointestinal illness, characterized by diarrhea, fever and abdominal cramps, is the most common presentation of *C. jejuni* infection, but symptoms and signs are not so distinctive that the physician can differentiate this infection from illness caused by other organisms. Campylobacter enteritis, and occurs most often in patients whose immune system is severely compromised.

The epidemiology of *Campylobacter* infection in developing countries differs markedly from that of the developed world. In developing countries, *C. jejuni* is isolated more frequently but also the rates of carriage in healthy populations are often high (27).

There are also some reports from developing countries, where *C. jejuni* and *C. coli* have been isolated mostly from populations with diarrheal illness (27-29).Numerous studies from developed countries have demonstrated *C. jejuni* in 4—14% of patients with diarrhea and in fever than 1% of asymptomatic persons (29). Studies from the USA and other developed countries show that enteritis due to *Campylobacter* exceeds cases caused by *Salmonella* species, *Shigella* species or *Escherichia coli* O157:H7 (29, 30). It is estimated that true *Campylobacter* infection rates in the USA and UK are as high as 1% of the population per year (30).

Most of the developing countries do not have surveillance systems to measure the disease burden of human campylobacteriosis and their association with diarrheal outbreaks. Antimicrobial resistance is an emerging problem globally. Recent research indicates emergence of fluoroquinolone resistant strains in many countries. Seasonality in the incidence of the fluoroquinolone resistant strains has also been reported in different countries. A possible link with indiscriminate use of antibiotics in poultry sector has been established.

Efforts have recently focused on determining important risk factors for *Campylobacter* infection to guide interventions aimed at reducing disease burden. Such risk factors are commonly determined for other pathogens through investigations of outbreaks; however, despite the large number of *Campylobacter* notifications, outbreaks are rarely detected. Case control studies to determine risk factors for infection have identified consumption of chicken, exposure to animals, and consumption of contaminated water as significant. A meaningful typing system that could be applied to *Campylobacter* isolates as they arrive in the public health laboratory could aid outbreak detection and help identify common sources of infection.

Numerous typing strategies, including pulsed-field gel electrophoresis (PFGE), PCR-restriction fragment length polymorphism analysis of flagellin genes (RFLP-*fla*), sequencing of the short variable region of the *fla* locus (SVR-*fla*), ribotyping, multilocus enzyme electrophoresis, multilocus sequence typing (MLST), randomly amplified polymorphic DNA, and amplified fragment length polymorphism have been employed to examine epidemiological relationships between isolates within the species *Campylobacter*.

Campylobacteriosis is considered to be a very potential zoonotic disease (animal to man infection and vice versa), which can causes significant morbidity and even mortality in adult and children particularly in developing countries like India. Recent surveys showed that people, in rural areas, who are mostly vulnerable to this disease due to their close association with farm animals and less hygienic precautions, do not have any knowledge and awareness regarding this common disease. In this scenario, molecular epidemiology of the *Campylobacter* species needs to be investigated in and around Kolkata which is most densely populated metro city in India, with special reference to drug resistance. Investigation of molecular epidemiology of *Campylobacter* species using different typing tools might be very much useful for – 1) Understanding routes of infection, 2) Identification of pathogenic strains, 3) Correlation between strains, separated by hosts and locations and 4) Suitable chemotherapy for *Campylobacter* mediated infections.

In this study, information on epidemiology of *Campylobacter* spp among hospitalized patients with acute diarrhea will be generated In addition, the species composition of campylobaters, their antimicrobial resistance and clonality will also be examined with the strains isolated from the diarrheal patients.

The objective of this aspect are subdivided as follows

a) Identification and speciation of campylobacters isolated from diarrheal patients admitted in the Infectious Diseases Hospital and children with diarrhea in urban slums of Kolkata.
b) Detection of antimicrobial resistance patterns of campylobacters isolated from diarrheal patients
c) PFGE profiling of campylobacters using standardized protocol and comparison of existing clones in other countries

Results obtained:

Campylobacter spp was isolated using standard procedures form the 1293 diarrheal patients admitted in the Infectious Diseases Hospital (IDH) and 962 children with diarrhea in urban slums of Kolkata. After initial isolation using selective media, the

suspected isolates were identified by staining and standard biochemical testes. Speciation was made using a PCR assay, targeting the *cdtB* gene that identifies *C. jejuni, C. coli* and *C. fetus* (XX). Campylobacters were detected less than 1.0% among acute diarrheal patients at the IDH as a sole pathogen (Table1). However, mixed infection was comparatively high with *C. jejuni*.

Table 1. Incidence of campylobacters among diarrheal cases in Kolkata, India.

Organism	Incidence (%) n=1293	
	Sole	Mixed
C. coli	3 (0.2)	10 (0.8)
C. jejuni	9 (0.7)	45 (3.5)

Among children with diarrhea in urban slums of Kolkata, the incidence trend was different as most of the children had mild diarrhea who needs no hospitalization. However, in 9 campylobacters positive cases other than the three targeted in the PCR might play a role in causing diarrhea (Table 2). In 19 cases (2.0%), the other campylobacters were identified as mixed pathogens along with the other enteric pathogens. Presently, we are in the process of doing PFGE with the *C. jejuni* and *C. coli* strains using the PulseNet protocol. We are also identifying the other camplylobacters using different biochemical testings.

Table 2. Incidence of campylobacters among diarrheal children <5 years of age in Kolkata, India.

Organism	Incidence (%) n=962	
	Sole	Mixed
C. coli	-	8 (0.8%)
C. jejuni	8 (0.8%)	19 (2.0)
Other campylobacters	9 (0.9)	19 (2.0)

(iii) Multilocus sequence typing (MLST) of pandemic strains of *Vibrio parahaemolyticus* isolated from clinical and environmental sources

Vibrio parahaemolyticus is a natural inhabitant of coastal water all over the globe and is the leading cause of gastroenteritis. Until 1996, infections are generally caused by its diverse serotypes, which are sporadic in nature. Recent studies have shown the emergence of serotype O3:K6, a unique serotype, which is characterized by the potential to spread and to be associated with acute diarrhea often that the other serotypes. In February 1996, strains belonging to O3:K6 serotype were identified in Kolkata that accounted for about 80% of the strains isolated during that time (31). Since this first report, an increasing number of *V. parahaemolyticus* infections and large outbreaks caused by strains belonging to a pandemic clonal group have been reported throughout the world (32). The emergence and dramatic spread of pandemic strains of *V. parahaemolyticus* has raised public health concerns in both developing and developed countries.

Clinical strains of *V. parahaemolyticus* produce two major virulence factors; the thermostable direct hemolysin (TDH) encoded by *tdh*, and TDH-related hemolysin encoded by *trh*. In several studies it was proved that all the pandemic strains of *V. parahaemolyticus* harbors the gene *tdh* but not the *trh* (32). A number of genetic markers have been identified in pandemic strains of *V. parahaemolyticus* that include a unique *toxRS* sequence, a histone-like DNA-binding protein, additional type III secretion system encoding genes in the chromosome II and an open reading frame VP2905 (33-35). From 1998 to till date, the other potent serotypes such as O4:K68, O1:K25, O1:KUT were emerged and shown to be clonally related to progenerator pandemic strain of O3:K6 (32). It has been suggested that the new O3:K6 group of strains might have emerged as a result of the transfer of genetic elements.

Several molecular typing methods have been applied in the past for the determination of clonality among pandemic strains of *V. parahaemolyticus*. These methods are of limited value in elucidating the evolution of clonal groups/complexes of *V. parahaemolyticus*. A PFGE method was recently established for the universal application for strain typing of *V. parahaemolyticus* (36). In previous finding it was established that most of the pandemic strains belongs to O3:K6, O4:K68, O1:KUT, O1:K25 sertypes

were not discriminated in the PFGE (37). In order to have high discrimination power, the other methods such as multilocus sequence typing (MLST) and variable-number tandem-repeats (VNTR) analysis were recently established (37-39). The MLST is based on the sequence analysis of selective house-keeping (HK) genes of epidemiologically important pathogens. The advantage of MLST is that the submitted sequence profiles can be readily accessed via the Internet. The first MLST of *V. parahaemolyticus* was made with a set of four genes located in the chromosome I to investigate the evolution of pandemic strains (40). Following this investigation, the MLST was improved with high discrimination with seven HK genes, three from chromosome I and four from chromosome II (38). The sequences generated in this study using pandemic strains belonging to predominant serotypes O3:K6, O4:K68, O1:KUT, O1:K25 are available at http://pubmlst.org/vparahaemolyticus. It was found that pandemic strains of *V. parahaemolyticus* are genetically diverse with a semiclonal population structure and that frequent recombination events seem to play an important role in the clonal diversification.

The primary aim of this study was to compare the recently established MLST method to detect the genomic relatedness of newly emerging pandemic serotypes of *V. parahaemolyticus* in Kolkata.

The objective of this aspect are subdivided as follows

a) PCR amplification of *recA* (RecA protein) *dnaE* (DNA polymerase III, alpha subunit) *gyrB* (DNA gyrase, subunit B) in chromosome I and *dtdS* (Threonine dehyrogenase) *pntA* (Transhydrogenase alpha subunit) *pyrC* (Dihydroorotase) *tnaA* (Tryptophanase) in chromosome II

b) Comparison of sequences genes with data available in http://pubmlst.org/vparahaemolyticus

c) Identification of new clonal cluster among newly emerged pandemic serotype of *V. parahaemolyticus*

Results obtained:

Retrospective collection of *V. parahaemolyticus* strains isolated from the acute diarrheal patients was used in this study. The pandemic strains were confirmed using GS-PCR (33). The matching serotypes from the clinical and environmental samples will be considered for strain comparison. The MLST assay was based on the PCR amplification and comparison of DNA sequences (38). PCR targets *recA* (RecA protein) *dnaE* (DNA polymerase III, alpha subunit) *gyrB* (DNA gyrase, subunit B) in chromosome I and *dtdS* (Threonine dehyrogenase) *pntA* (Transhydrogenase alpha subunit) *pyrC* (Dihydroorotase) *tnaA* (Tryptophanase) in chromosome II. Comparison of sequenced genes were made with data available from the public database (http://pubmlst.org/vparahaemolyticus).

Of the 5 strains included in the analysis, there are other DnaE alleles in the pandemic *V. parahaemolyticus* strains (allele 17, 22, and 29). The other locus of the tested strains showed ST3. However, the analysis is still incomplete and further screening will be made with other 25 clinical and 5 environmental pandemic strains.

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