Table 1. Variation rate of VNTR loci

VNTR Locus	Lengt of repeat	No. isolate of variant	variation rate	remarks
SS01	7	9	14.5%	
SS02	9	1	1.6%	
SS03	7	6	9.7%	
SS04	7	2	3.2%	
SS05	7	3	4.8%	
SS06	7	10	16.1%	
SS07	7	1	1.6%	
SS08	60	1	1.6%	
SS09	6	4	6.5%	
SS10	6	6	9.7%	
SS11	6	3	4.8%	
SS12	9	3	4.8%	
SS13	6	2	3.2%	
SS14	9	2	3.2%	
SS15	6	1	1.6%	
SS16	17	1	1.6%	
SS17	6	1	1.6%	
SS18	5	3	4.8%	
SS19	5	3	4.8%	
SS20	40	2	3.2%	
SS21	18	1	1.6%	
SS22	11	2	3.2%	
SS23	16	4	6.5%	
SS24	168	2	3.2%	
SS25	135	1	1.6%	
SS26	101	1	1.6%	No amplicon

3) Comparison of discriminatory power

62 tested isolates were classified into 18 *XbaI*-PFGE type (fig. 2). Major pattern was SZNX01.011, 13 isolates were included in this pattern. Next prevailed pattern was SZNX01.013 and SZNX01.004, 8 and 6 isolates were included into these patterns respectively. Many isolates included in PFGE patterns SZNX01.011 are not related

epidemiologically each other, but their PFGE pattern was indistinguishable. Similar result was shown in XbaI-PFGE pattern SZNX01.013 and SZNX01.004.

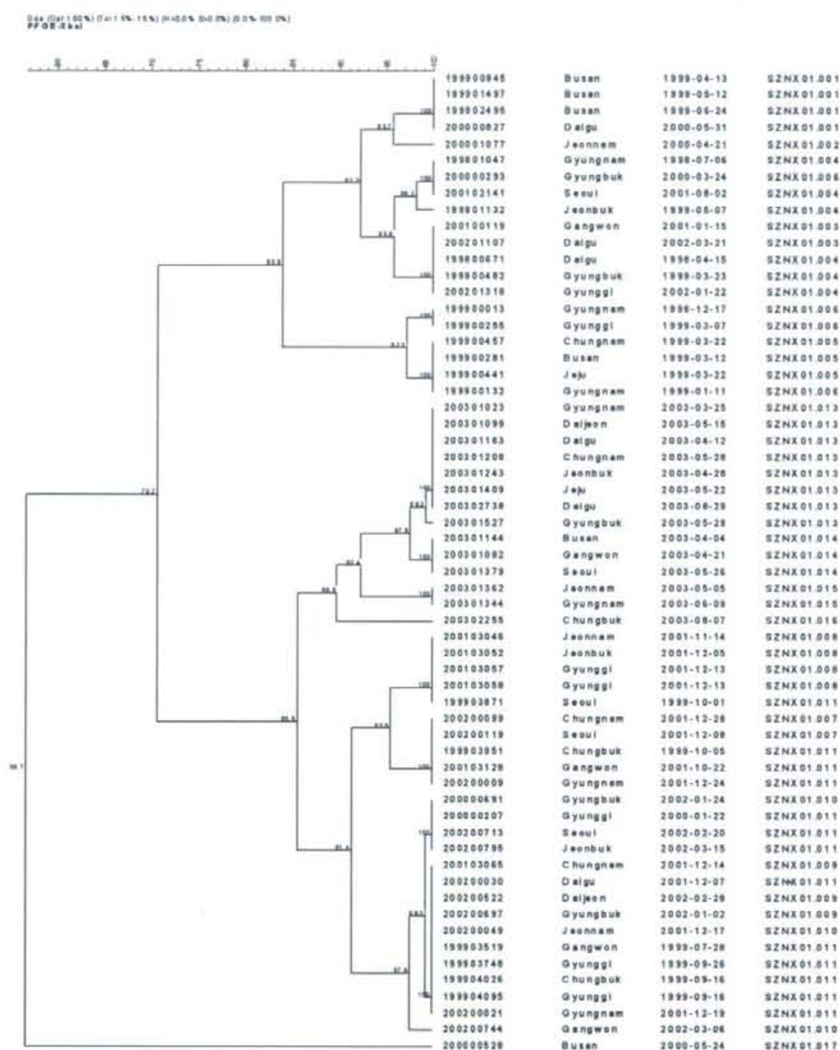

Figure 2. Dendrogram constructed with *XbaI*-PFGE pattern of 62 S. sonnei isolates in this study

The discriminatory power of MLVA was higher than that of PFGE. From the fragment analysis result of 26 VNTR loci (table 2), MLVA typing with the four VNTR loci (SS01, SS03, SS06, and SS09; 4 loci MLVA typing), 62 isolates are classified into 27 patterns. MLVA typing with another 4 VNTR loci (SS01, SS03, SS06, and

SS10; 4-1 loci MLVA typing) identified 33 patterns in the 62 isolates. MLVA typin with eight variable VNTR loci (SS01, SS03, SS06, SS09, SS10, SS11, SS12, and SS13 ; 8 loci MLVA typing) discriminated the 62 isolates into 37 patterns. MLVA typing with 26 VNTR loci classified 62 tested isolate into 46 patterns (fig. 3, 4).

Table 2. Number of pattern identified various typing method in this study

Methods	VNTR loci	No. of patterns
Xbal-PFGE		18
4 loci MLVA	SS01, 03, 06, 09	27
4 loci MLVA-1	SS01, 03, 06, 10	33
8 loci MLVA	SS01, 03, 06, 10~13	37
26 loci MLVA	SS01~26	46

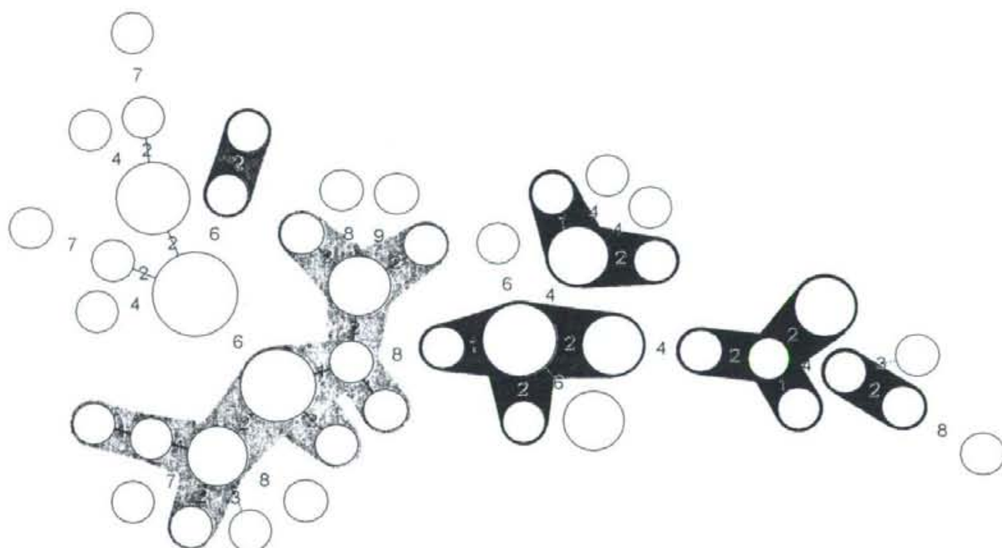

Figure 3. Minimum spanning tree constructed with 26 VNTR loci profiles for 62 *S. sonnei* isolates.

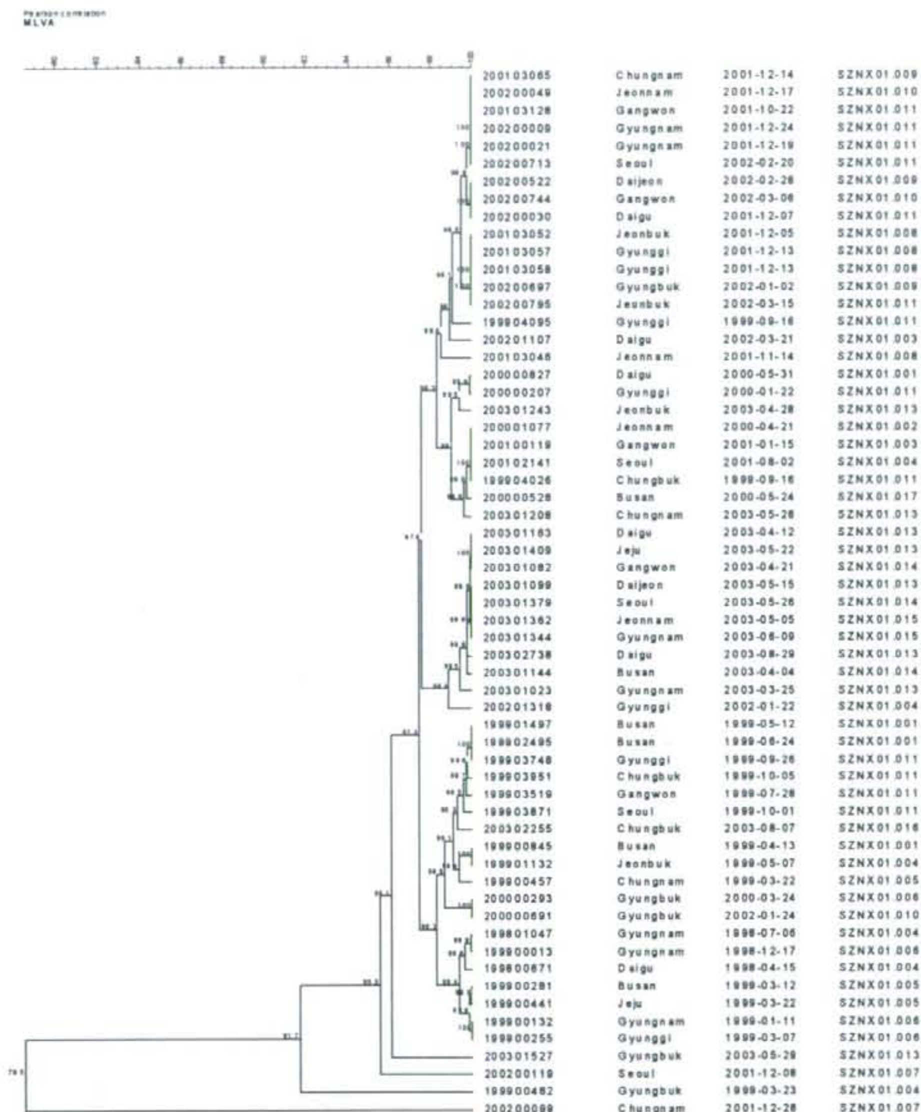

Figure 3. Dendrogram constructed with 26 VNTR loci MLVA patterns 62 *S. sonnei* isolates.

All the MLVA typing (4, 4-1, 8, and 26 loci MLVA typing) were variable in the indistinguishable *XbaI*- PFGE pattern (table 3). For example, *XbaI*- PFGE pattern SZNX01.011, 13 isolates included in this pattern, discriminated 7 different patterns by

4 loci MLVA typing, discriminated 8 patterns by 4-1 loci MLVA typing method, discriminated 10 patterns by 8 loci MLVA typing method, and also discriminated 26 loci MLVA typing method. *XbaI*- PFGE pattern SZNX01.013 showed similar result, 8 isolates included in this indistinguishable pattern discriminated 6 to 7 patterns by each MLVA typing methods.

Table 3. Molecular patterns classified by various moleculartyping methods of 62 *S. sonnei* isolates used in this study

Isolate No.	Source	Date of isolation	PFGE-XbaI	4-MLVA	4-1MLVA	8-MLVA	26-MLVA
199800671	DG	1998-04-15	SZNX01.004	4M23	41M29	8M33	SZNM.039
199801047	GN	1998-07-06	SZNX01.004	4M17	41M19	8M21	SZNM.025
199900013	GN	1998-12-17	SZNX01.006	4M23	41M28	8M32	SZNM.038
199900132	GN	1999-01-11	SZNX01.006	4M12	41M14	8M16	SZNM.020
199900255	GG	1999-03-07	SZNX01.006	4M12	41M14	8M16	SZNM.020
199900281	BS	1999-03-12	SZNX01.005	4M10	41M12	8M14	SZNM.018
199900441	JJ	1999-03-22	SZNX01.005	4M10	41M11	8M13	SZNM.017
199900457	CN	1999-03-22	SZNX01.005	4M02	41M03	8M03	SZNM.002
199900482	GB	1999-03-23	SZNX01.004	4M16	41M18	8M20	SZNM.024
199900845	BS	1999-04-13	SZNX01.001	4M02	41M02	8M02	SZNM.003
199901132	JB	1999-05-07	SZNX01.004	4M02	41M02	8M02	SZNM.003
199901497	BS	1999-05-12	SZNX01.001	4M05	41M06	8M06	SZNM.007
199902495	BS	1999-06-24	SZNX01.001	4M05	41M06	8M06	SZNM.008
199903519	GW	1999-07-28	SZNX01.011	4M05	41M06	8M06	SZNM.006
199903748	GG	1999-09-26	SZNX01.011	4M05	41M06	8M06	SZNM.007
199903871	SE	1999-10-01	SZNX01.011	4M13	41M15	8M17	SZNM.021
199903951	CB	1999-10-05	SZNX01.011	4M05	41M06	8M05	SZNM.009
199904026	CB	1999-09-16	SZNX01.011	4M22	41M27	8M31	SZNM.042
199904095	GG	1999-09-16	SZNX01.011	4M18	41M20	8M22	SZNM.026
200000207	GG	2000-01-22	SZNX01.011	4M26	41M32	8M36	SZNM.045
200000293	GB	2000-03-24	SZNX01.006	4M09	41M10	8M12	SZNM.016
200000528	BS	2000-05-24	SZNX01.017	4M21	41M25	8M29	SZNM.040
200000691	BS	2002-01-24	SZNX01.010	4M09	41M10	8M12	SZNM.016
200000827	BS	2000-05-31	SZNX01.001	4M25	41M31	8M35	SZNM.044
200001077	BS	2000-04-21	SZNX01.002	4M22	41M26	8M30	SZNM.041
200100119	BS	2001-01-15	SZNX01.003	4M22	41M27	8M31	SZNM.042

Table 3. Continued.

Isolate No.	Source	Date of isolation	PFGE-XbaI	4-MLVA	4-1MLVA	8-MLVA	26-MLVA
200102141	BS	2001-08-02	SZNX01.004	4M22	41M27	8M31	SZNM.042
200103046	BS	2001-11-14	SZNX01.008	4M11	41M13	8M15	SZNM.019
200103052	BS	2001-12-05	SZNX01.008	4M19	41M22	8M25	SZNM.032
200103057	BS	2001-12-13	SZNX01.008	4M19	41M22	8M25	SZNM.034
200103058	BS	2001-12-13	SZNX01.008	4M19	41M22	8M25	SZNM.034
200103065	BS	2001-12-14	SZNX01.009	4M19	41M22	8M26	SZNM.036
200103128	BS	2001-10-22	SZNX01.011	4M19	41M22	8M26	SZNM.036
200200009	BS	2001-12-24	SZNX01.011	4M19	41M21	8M23	SZNM.030
200200021	BS	2001-12-19	SZNX01.011	4M19	41M21	8M23	SZNM.028
200200030	BS	2001-12-07	SZNX01.011	4M19	41M22	8M26	SZNM.035
200200049	BS	2001-12-17	SZNX01.010	4M19	41M22	8M26	SZNM.036
200200099	BS	2001-12-28	SZNX01.007	4M15	41M17	8M19	SZNM.023
200200119	BS	2001-12-08	SZNX01.007	4M01	41M01	8M01	SZNM.001
200200312	BS	2001-12-24	SZNX01.012	4M19	41M22	8M26	SZNM.036
200200522	BS	2002-02-28	SZNX01.009	4M19	41M21	8M23	SZNM.029
200200697	BS	2002-01-02	SZNX01.009	4M19	41M22	8M25	SZNM.033
200200713	BS	2002-02-20	SZNX01.011	4M20	41M24	8M28	SZNM.038
200200744	BS	2002-03-06	SZNX01.010	4M19	41M21	8M23	SZNM.027
200200795	BS	2002-03-15	SZNX01.011	4M20	41M23	8M27	SZNM.037
200201107	BS	2002-03-21	SZNX01.003	4M19	41M22	8M24	SZNM.031
200201318	BS	2002-01-22	SZNX01.004	4M03	41M04	8M04	SZNM.004
200203588	BS	2002-09-07	SZNX01.018	4M06	41M07	8M09	SZNM.011
200301023	BS	2003-03-25	SZNX01.013	4M06	41M07	8M08	SZNM.010
200301082	BS	2003-04-21	SZNX01.014	4M06	41M08	8M10	SZNM.014
200301099	BS	2003-05-15	SZNX01.013	4M06	41M07	8M09	SZNM.012
200301144	BS	2003-04-04	SZNX01.014	4M06	41M08	8M10	SZNM.013
200301163	DG	2003-04-12	SZNX01.013	4M06	41M08	8M10	SZNM.014
200301208	CN	2003-05-28	SZNX01.013	4M24	41M30	8M34	SZNM.043
200301243	JB	2003-04-28	SZNX01.013	4M27	41M33	8M37	SZNM.046
200301344	GN	2003-06-09	SZNX01.015	4M06	41M07	8M09	SZNM.012
200301362	JN	2003-05-05	SZNX01.015	4M06	41M07	8M09	SZNM.012
200301379	SE	2003-05-26	SZNX01.014	4M06	41M07	8M09	SZNM.012
200301409	JJ	2003-05-22	SZNX01.013	4M07	41M8	8M10	SZNM.014
200301527	GB	2003-05-29	SZNX01.013	4M14	41M16	8M18	SZNM.022
200302255	CB	2003-08-07	SZNX01.016	4M04	41M05	8M07	SZNM.005
200302738	DG	2003-08-29	SZNX01.013	4M08	41M09	8M11	SZNM.015

The subdivided MLVA patterns in *XbaI*- PFGE pattern SZNX01.011 and SZNX01.013 were related with region and time of isolation between same MLVA pattern isolates

On the contrary, some indistinguishable MLVA patterns were also discriminated by *XbaI* -PFGE typing. 14 isolates included 4M19 pattern of 4 loci MLVA were subdivided into 6 *XbaI*- PFGE patterns. In these patterns, some isolates showed relatedness with region or time of isolation, but, most of them in same pattern do not have any relatedness between each other.

Discussion

In Korea, from 1998 to 2003, unexpected abrupt increase of shigellosis in short period, made it difficult to identify the genetic relatedness between many small and large outbreaks and sporadic cases.

Accordingly, PFGE was introduced to investigate the genetic relatedness in many of shgellosis cases in this period, but in some cases, PFGE can not discriminated the cases those confirmed as different by epidemiologically and so on, the molecular typing using PFGE method reached in limit. Therefore the new molecular typing method can replace or complement the PFGE was required.

Among the next generation molecular subtyping methods, MLVA selected as next generation method for the PulseNet. MLVA already has been developed for several bacterial pathogens such as Entero-hemorrhagic *E. coli* O157, *Salmonella* and so forth, and its usefulness has reported in many studies.

In this study, we set up the MLVA method for the S. sonnei isolated in Korea, and evaluated its usefulness. Among the 26 VNTR loci those tested for variability, VNTR loci SS01, SS03, SS06, and SS10 were most variable for *S. sonnei* isolates tested in this study. This result is slightly different from previously reported study (Liang et. Al.) those used same VNTR loci. In that study, most variable VNTR loci were SS01, SS03, SS06, and SS09. Even though we tested only 62 isolates, we can prospect that those difference is from regional variance.

VNTR loci SS02, SS07, SS08, SS15, SS16, SS17, SS21, SS25, and SS26 showed no variability in this study. Only one kind of copy number of each repeat was amplified from 62 tested isolates. In the case of VNTR loci SS24,ss25, and SS26, the length of repeat is over 100 bases, and in this study, we used LIZ500 standard for fragment analysis, so multiple repeat of these VNTR loci was hard to detect.

Have nothing to do with number of selected VNTR loci, MLVA pattern for the tested 62 isolates are more than that of PFGE. Therefore we can conclude that the discriminatory power of MLVA is higher than that of PFGE for *S. sonnei* isolated in Korea.

In proportion to selected VNTR loci increase, discriminatory power of MLVA typing method is increased too. But, using only 4 or 8 VNTR loci MLVA typing method, showed enough discriminative ability for the *S. sonnei* isolates. Especially, the discriminative ability of 4-1 loci MLVA typing method, consist of VNTR loci SS01, SS03, SS06, and SS10 is equal to that of 8 loci MLVA typing.

Even though, it found in small cases, some indistinguishable MLVA patterns were subdivided by PFGE method, therefore, at least for the investigation of genetic relatedness of *S. sonnei*, MLVA should be used as complement method for PFGE not the replacement method for PFGE.

From the result of this study, 4 VNTR loci MLVA typing method using ss01, SS03, SS06, and SS10 can be used for the shigellosis surveillance and outbreak investigation.

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Publication list for this work:

1) **The Genetic relatedness and evolution of *Shigella sonnei* during prevalent shigellosis stage in Korea (in preparation).**

Pulse-Net Progress Report- 2009

ICDDR,B

1. *Vibrio:*

Prevalence and Diarrheal Link of *tdh+ Vibrio parahaemolyticus* Serogroup O8 : K21 in Estuarine Ecosystem of Bangladesh

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Objective.

Vibrio parahaemolyticus, a pandemic pathogen causing seafood-related gastroenteritis, occurs with high frequency in Bangladesh and India, although a very little is known about sero-distribution, virulence, or molecular traits of *V. parahaemolyticus* occurring in estuarine ecosystem of Bay of Bengal, where the pandemic serogroup, O3 : K6, was first reported from. In this study, we aimed at investigating virulence potential, as well as phenotypic and genetic traits of *V. parahaemolyticus* occurring in the estuarine ecosystem of Bangladesh.

Materials & Methods.

Forty-four *V. parahaemolyticus* strains, of which 42 isolated from Bay of Bengal estuaries and 2 from clinical sources of one of the areas, were analyzed for sero-distribution, Kanagawa phenomenon (KP), virulence and related genes by PCR, molecular fingerprinting by randomly amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), and PFGE to determine clonal relatedness.

Results.

TABLE 1. Characterizations of *V. parahaemolyticus* strains (n=44) isolated from the coastal aquatic ecosystem of the Bay of Bengal.

O:K Serotype	Place of Isolation	District	No. of Strains	Presence of following genes				Results of		KP
				toxR	*tdh*	*trh*	*tlh*	GS-PCR	ORF8 PCR	
O1:KUT	Karnaphooli estuary	Chittagong	1	+	-	+	+	-	-	+
O1:KUT	Karnaphooli estuary	Chittagong	4	+	-	-	+	-	-	-
O1:KUT	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	+
O1:K38	Karnaphooli estuary	Chittagong	1	-	-	-	+	-	-	-
O3:KUT	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	+
O3:KUT	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O3:K4	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	+
O3:K29	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O3:K30	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	+
O3:K30	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O3:K45	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O4:K34	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O5:KUT	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O8:K39	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
O10:KUT	Karnaphooli estuary	Chittagong	2	+	-	-	+	-	-	-
O11:KUT	Karnaphooli estuary	Chittagong	1	+	-	-	+	-	-	-
OUT:KUT	Karnaphooli estuary	Chittagong	2	+	-	-	+	-	-	-
O3:KUT	Bakergonj pond	Barishal	1	+	-	-	+	-	-	-
O8:K21	Bakergonj patient	Barishal	2	+	+	-	+	-	-	+
O8:K21	Mathbaria pond	Pirojpur	2	+	+	-	+	-	-	+
O4:K46	Kuakata beach*	Potuakhali	1	+	-	-	+	-	-	-
08:K21	Kuakata beach	Potuakhali	1	+	+	-	+	-	-	-
O8:K21	Kuakata beach	Potuakhali	1	+	+	-	+	-	-	+
O8:K21	Kuakata beach	Potuakhali	11	+	+	-	+	-	-	-
O9:KUT	Kuakata beach	Potuakhali	1	-	-	-	+	-	-	-
O9:KUT	Kuakata beach	Potuakhali	1	+	+	-	+	-	-	-
OUT:K33	Kuakata beach	Potuakhali	1	+	-	-	+	-	-	-

Serotyping indicated O8, O3, O1, and K21 to be the major O and K serogroups, respectively, and O8 : K21, O1 : KUT and O3 : KUT predominant. The K antigen(s) were untypable and pandemic serogroup O3 : K6 was not detected. Species-specific genes, *tox*R and *tlh,* were confirmed by PCR in all but two of the strains which also lacked *tox*R. A total of 18 (41%) strains possessed the virulence gene encoding thermostable direct haemolysin (TDH) and one had the TDH-related haemolysin (*trh*) gene, but not *tdh*. Ten (23%) strains exhibited KP surrogating virulence, of which six, including the two clinical strains, possessed

tdh. Of the 18 *tdh*+ strains, 17 (94%), including the two clinical strains, had sero-markers O8 : K21, one O9 : KUT, while the single *trh*+ strain was O1 : KUT. None had pandemic marker genes GS or ORF8. DNA fingerprinting employing RAPD, ERIC-PCR, and PFGE (*Sfi*I-digested DNA), and cluster analysis showed divergence among the strains. Dendrograms constructed using PFGE (*Sfi*I) images from a soft-database, including the pandemic and non-pandemic *V. parahaemolyticus* strains representing diverse geographic origin, however, showed that local strains formed a cluster, as did pandemic strains of diverse origin, i. e., "clonal complexes".

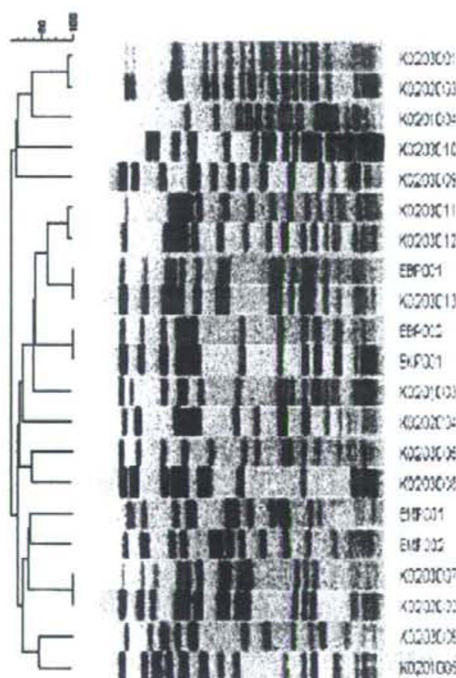

Fig 1. PFGE patterns of *Not*I-digested genomic DNA of selected (n=26) *V. parahaemolyticus* isolates from the estuarine ecosystem of Bangladesh. Strain identification number, sero-marker and the place of isolation of the strains are indicated. The dendrogram was established by the Bionumeric Software

Fig 2. Dendrogram constructed with PFGE images of *Not*I-digested genomic DNA of selected *V. parahaemolyticus* strains isolated from the estuarine ecosystem of Bangladesh to determine clonal relatedness with *V. parahaemolyticus* strains from different geographical regions, including different serogroups representing pandemic, non-pandemic

Conclusions.

The demonstrated prevalence of *tdh*+ and diarrheagenic serogroup O8:K21 strains, which are unlike clonally but form regional clonal complext, in drinking water sources of the coastal villages of Bangladesh indicates a significant human health risk for inhabitants.

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2. *Campylobacter.*

Fluoroquinolone resistant *Campylobacter jejuni* from Bangladesh show high degree of heterogeneity and are associated with a single point mutation in *gyrA*

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Introduction: Campylobacteriosis is one of the most common human bacterial intestinal disorders occurring worldwide (Tauxe R.V., 2001). *Campylobacter* species, are the major cause of human bacterial gastroenteritis, and may be responsible for as many as 400–500 million cases worldwide each year (Friedman *et al.*, 2000). Most *Campylobacter* infections cause gastroenteritis, but invasive disease (e.g., bacteremia and meningitis) may also occur. Whilst gastroenteritis is normally self-limiting and antibiotic therapy not usually required, treatment is indicated in young children, pregnant woman, immunocompromised patients and in extraintestinal infections (Nachamkin, 1992). In such cases, fluoroquinolones, particularly ciprofloxacin has increasingly been considered as the treatment of choice as most of the enteric pathogens are susceptible to these agents and high concentrations can be attained in the lumen (DuPont *et al.,* 1987). However, since the early 1990's there has been a dramatic increase in the prevalence of fluoroquinolone resistance among campylobacters isolated from both man and poultry (Endtz *et al.* 1991). The purpose of this study was to investigate the type and frequency of any possible mutation in the *gyrA*, *gyrB* and *parC* genes of

fluoroquinolone resistant *C. jejuni* isolated in Bangladesh and to investigate the clonal relationships among the strains using Pulsed-Field Gel Electrophoresis (PFGE).

Methodology: Forty *C. jejuni* strains were isolated during a systemic surveillance study conducted in 2002 in ICDDR, B. Of these, 12 strains were sensitive (zone diameter ≥21), 26 strains were resistant (zone diameter ≤15) and two strains were Intermediate (zone diameter=19) to ciprofloxacin. From these, ten representative strains comprising of two sensitive, two intermediate and six resistant strains were included in this study to investigate the presence of any possible mutation in the Quinolone Resistance Determining Region (QRDR) of *gyrA*, *gyrB* and *parC* genes of these isolates. All these strains were analyzed by pulsed-field gel electrophoresis (PFGE) to reveal the clonal relationship among the strains. Intact agarose-embedded chromosomal DNA of *C. jejuni* was prepared according to the standardized PulseNet protocol for *C. jejuni* as described earlier (Ribot et al, 2001) and digested with SmaI restriction enzyme (Invitrogen, USA). *Salmonella* Braenderup H9812 strain restricted with XbaI was used as the size standard (Hunter *et al.*, 2005). The restriction fragments were then resolved by electrophoresis using CHEF-MAPPER® system apparatus (Bio-Rad Laboratories, Richmond, CA, USA) using 0.5x TBE buffer (45 mM Tris-HCl, 45 mM boric acid, 1.0 mM EDTA, pH 8.0). Run conditions were generated by the autoalgorithm mode of the CHEF Mapper PFGE system for the sizes ranging between 50 and 400 kb, and PFGE was performed for 18 h in 1% pulsed-field certified agarose in 0.5X TBE (Tris/ borate/EDTA) buffer. Analysis of the tagged image file format (TIFF) images was carried out with BioNumerics software (Applied Maths, Belgium) using the dice coefficient and UPGMA to generate dendrograms with 1.0% tolerance values.

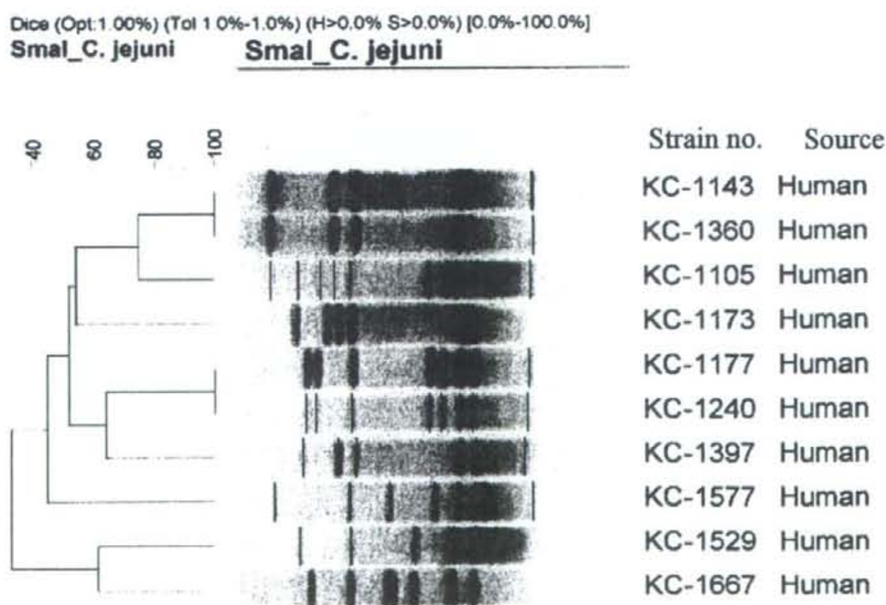
Results and Conclusion: The QRDR's of the *gyrA* from all the ciprofloxacin resistant and reduced susceptible strains contained a single point mutation at codon 86 (ACA→ATA) resulting in the incorporation of isoleucine instead of threonine. In addition, some silent mutations were also observed. PFGE analysis showed that most of the fluoroquinolone - resistant strains isolated in Bangladesh were heterogenous (Figure 1). However, two isolates showing intermediate susceptibility to ciprofloxacin showed identical PFGE patterns.

Table 1. Disc diffusion test, MIC and amino acid changes in *gyrA* in *C. jejuni* strains isolated from Bangladesh.

Strain	Antibiotic susceptibility						Substitutions in QRDR[c]	
	Zone diameter (mm)		MIC (mg/L)				*gyrA*	
	Nal	Cip	Nal	Cip	Of	Nor	Thr-86 (wt)[a]	
KC-1173	30	50	2	0.016	0.094	0.094	ACA	-[b]
KC-1529	30	48	1.5	0.47	0.125	0.032	ACA	-
KC-1177	7	19	>256	1.0	24	8	ATA	Ile
KC-1240	7	19	>256	1.5	24	8	ATA	Ile
KC-1397	16	15	64	>32	>32	48	ATA	Ile
KC-1577	13	14	64	>32	>32	64	ATA	Ile
KC-1105	7	9	>256	>32	>32	64	ATA	Ile
KC-1143	7	7	>256	>32	>32	48	ATA	Ile
KC-1360	7	7	>256	>32	>32	48	ATA	Ile
KC-1667	7	7	>256	>32	>32	256	ATA	Ile

NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; OF, ofloxacin
[a]wt, Wild type, [b]– indicates no substitution

FIG. 1. Dendrogram generated by BioNumerics software, showing the distance calculated by the dice similarity index of PFGE SmaI profiles for 10 representative *C. jejuni* isolates isolated from human diarrheal samples. The degree of similarity (%) is shown on the scale.



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Running Title: Hybrid-El Tor co-exists with El Tor & classical since 1991 in Latin America

El Tor (ET) in Peru, Classical, ET, and Hybrid Variants of Both Biotypes in Mexico Constitute Two Independent Pools of *Vibrio cholerae* O1 Causing Endemic Cholera in Latin America: Asia-Independent Emergence of Hybrid-ET, in 1991

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Running Title: Two Different Clones of El Tor with Classical and Hybrid Variants of both Biotypes in Latin America

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ABSTRACT

Vibrio cholerae serogroup O1 biotype El Tor (ET), causing current 7th cholera pandemic by globally replacing 6th pandemic biotype classical (CL), has been replaced recently by a hybrid (hyb)-ET having classical traits in Asia and Africa. The 1991-epidemic that devastated Latin America (LA) was believed to be extension of 7th pandemic ET from Asia, after Africa, although detail understanding of the evolutionary trend of *V. cholerae* causing endemic cholera in LA is crucial for global intervention and preventive measures. This study, a retrospective one, on microbiological, phenotypic, molecular and phylogenetic analyses of *V. cholerae* (n=104) isolated in Peru (n=49; 1991 - 1999), Mexico (n=53; 1991 – 1995), and Guatemala (n=2; 1993), shows the 1991-epidemics and subsequent endemic cholera to be caused by *V. cholerae* O1 ET in Peru, while ET and CL including hyb-ET in Mexico, confirming two regional pools of *V. cholerae* O1, and also, Asia-independent emergence of hyb-ET from co-existing progenitors of both biotypes in LA. All *V. cholerae* tested had consistent ET attributes with high degree of genetic fidelity of 1991 prototype in Peru. Conversely, *V. cholerae* O1 strains tested in Mexico included both ET and CL, including pre-1991 CL, concurrently with the predominant hyb-ET since 1991, showing 1991 to be the year of transition from EL to hyb-ET, which occurred in the co-presence of both progenitors, ET and CL that were found coexisting in Mexico until 1995. The ET and CL variants had 1-4 phenotypic markers of each other, while all hyb-ET possessed $CTX^{CL}\Phi$-encoded genes, $ctxB^{CL}$ and $rstR1^{CL}$ in pair, suggesting possible incorporation of the $CTX^{CL}\Phi$ into ET in the emergence of hyb-ET. The data of multi-locus genetic screening, involving 11 major virulence and related genes and gene clusters, which covered >165 kb of *V. cholerae* genome, appeared concordant with the phenotype and genotype-based primary sero-biotype categorizations, excepting prototype ET of Peru and Mexico, which had consistent regional signatures in VSP-II ORFs (512, 514, and 516), showing the 1991-epidemics in Mexico to be a local event, not an extension of Peru. Finally, not too distant but distinct DNA fingerprints for ET of Peru and Mexico, and their clonal disparity with that of Asia, as confirmed by pulsed-field gel electrophoresis (PFGE) and cluster analyses by dendrograms of the *Not*I-digested genomic DNA, support us to conclude that 1991-epidemics and subsequent endemic cholera including the emergence of hyb-ET were local, Asia-independent events that involved locally existent *V. cholerae* O1 in LA.

Introduction

Historically cholera is endemic in Asia for centuries and remains a major killer disease for the people particularly of the flood plains of the Ganges delta of Bay of Bengal (**Glass et al.**, 1982). Although as many as seven cholera pandemics have been recorded, cholera epidemics, until 1992, were caused by *Vibrio cholerae* serogroup O1, biotype classical or El Tor. The classical biotype is believed to have caused the first six

pandemics, which occurred in South-east Asia, and subsequently introduced into the other parts of the world, between 1817 and 1923 (**Dziejman** et al., 2002; 9, **Politzar** et al., 1959; 27). Although the biotype El Tor was first reported in 1905 (**Sack 2004**; 31), it was not until the early 1960s that *V. cholerae* biotype El Tor emerged as the cause of the 7th pandemics by displacing the classical biotype (**Faruque 2003**; 11, **Siddique 1991**; 32). The classical biotype that maintained its low profile in southern Bangladesh in the early 1980s underwent slow extinction from its last niche in the coastal ecosystem of Bay of Bengal since 1980s (**Siddique** 1991). However, the emergence in 1992 of yet another non O1 serogroup, designated *V. cholerae* O139 synonym Bengal, as the cause of massive cholera in coastal villages of Bangladesh (**Albert 1992**; 2, **Cholera Working Group, ICDDR, B., 1993** 4) and India (**Ramamurthy** 1993; 28) and its subsequent emergence and reemergence by displacing *V. cholerae* O1 El Tor was considered another significant event in the history of cholera (**Siddique** 1994; 33). Although little is known about the geographic distribution of *V. cholerae* O139, this bacterium still maintains its low profile (**Alam** et al, 2006a; 2006b;) wile *V. cholerae* O1 El Tor continues to cause cholera in the flood plains of Bay of Bengal **Siddique** 1996; 34), where *V. cholerae* O1 classical biotype co-existed with El Tor until 1980s (**Siddique** 1994).

Over the past few years, remarkable advancements in our understanding of the molecular genetics and the pathogenesis of *V. cholerae* causing Asiatic cholera have taken place. Recent molecular analysis of *V. cholerae* O1 El Tor strains isolated from patients with acute watery diarrhea in Bangladesh shows them to be hybrids because they possessed phenotypic and genotypic traits of classical under El Tor background (**Nair**, 2002; 25). Subsequent retrospective study showed that all of the El Tor strains of *V. cholerae* O1 isolated in Bangladesh since 2001 were hybrids of both classical and El Tor biotypes, while those isolated before 2001 contained all the El Tor attributes associated with the 7th pandemic *V. cholerae* O1 (**Nair**, 2006; 26). Although the consequence of such continued genetic shifts among the cholera bacteria is not fully understood, the severe dehydration caused by these *V. cholerae* El Tor hybrid strains was shown to be increasingly significant in Bangladesh (**Alam** et al., unpublished data; **Siddique**, 2007; 35).

In 1991, cholera entered into Latin America after more than a century simultaneously at different sites along the coastline of Peru. Once introduced into the coastal communities, cholera spread fast through contaminated water and foods (**Seas C**). Microbiological analysis of stool specimens yielded toxigenic *V. cholerae* O1 biotype El Tor. Following the massive appearance in Peru, the epidemic spread rapidly from country to country covering in three years all the countries of Latin America except Uruguay and the Caribbean, affecting more than million people and resulting almost 9000 deaths between January 1991 and December 1993 (**Guthmann, JP J Trop Med Hyg. 1995**). Since *V. cholerae* O1 biotype El Tor entered into the Latin America, efforts have been made to understand the origin of these strains. Limited molecular genetic studies have indicated that the Latin American isolates from the 1991 epidemic were clonal and may have been an extension of the seventh pandemic strain of El Tor biotype in the Western hemisphere (**Wachsmuth** IK, 1993; 5). More recent studies have documented relatively high rate of genetic changes as shown by changing serotypes, electrophoretic types (ET) (**Beltran, 1999**), ribotypes and PFGE types among the Latin American strains (**Dalsgaard**, 1997; **Popovic T** , 1993; 3, **Evins GM** , 1995; 7). Despite different molecular types of *V.*