B. Analysis of PFGE Gel (TIFF), Linking Lanes, and Adding Text Data 1 2 3 4 5 1 2 3 4 5 TS S R L TL

C. Exercise 1: Analyze a PFGE Gel (TIFF) and Link Entries to a Database 1 2 3 4 5 1 2 3 4 5 TS S R L TL

D. Creation and File Location of PulseNet Bundle Files 1 2 3 4 5 1 2 3 4 5 TS S R L TL

E. Exercise 2: Prepare and Create a PulseNet 1 2 3 4 5 1 2 3 4 5 TS S R L TL

Bundle File for Distribution

Date: Feb 8, 2012

Subject Matter Presentation Time Allotted

A. Data Importing into and Exporting from BioNumerics 1 2 3 4 5 1 2 3 4 5 TS S R L TL

B. Exercise 3: Analyze a PFGE Gel Image; 1 2 3 4 5 1 2 3 4 5 TS S R L TL

Data Import into and from BioNumerics

C. Querying and Performing Comparisons 1 2 3 4 5 1 2 3 4 5 TS S R L TL
with Exporting Data from BioNumerics

D. Exercise 4: Performing Queries and and Performing Comparisons 1 2 3 4 5 1 2 3 4 5 TS S R L TL

E. Advanced Tools 1 2 3 4 5 1 2 3 4 5 TS S R L TL

F. Exercise 5: Query the Database Using the Advanced Query Tool 1 2 3 4 5 1 2 3 4 5 TS S R L TL

G. Identify the Problems 1 2 3 4 5 1 2 3 4 5 TS S R L TL

Date: Feb 9, 2012

Subject Matter Presentation
Time Allotted

A. Database Management Tools 1 2 3 4 5 1 2 3 4 5 TS S R L TL

B. Exercise 6: Database Settings and Layout, Pick List 1 2 3 4 5 1 2 3 4 5 TS S R L TL
Printing Reports

C. Working with subsets 1 2 3 4 5 1 2 3 4 5 TS S R L TL

D. Exercise 7: Create Subsets for serotype in Salmonella Database 1 2 3 4 5 1 2 3 4
5 TS S R L TL

E. Naming Patterns and Creating Local Unique Pattern Lists 1 2 3 4 5 1 2 3 4
5 TS S R L TL

F. Exercise 8: Identifying and Naming Unique Patterns in the database 1 2 3 4 5 1 2 3 4
5 TS S R L TL

G. Using the Chart and Statistics Tool and Groups 1 2 3 4 5 1 2 3 4
5 TS S R L TL

H. Exercise 9: Create Charts and Graphs to Create Reports 1 2 3 4 5 1 2 3 4
5 TS S R L TL

I. Composite Data Sets 1 2 3 4 5 1
2 3 4 5 TS S R L TL

J. Exercise 10: Cluster analysis using a composite data set 1 2 3 4 5 1 2 3 4
5 TS S R L TL
for *Salmonella*

Date: Feb 10, 2012

Subject Matter Time Allotted — Presentation

A. Demo on PFGE protocols 1 2 3 4 5 1 2 3 4
5 TS S R L TL

B. Troubleshooting PFGE Gels 1 2 3 4 5 1
2 3 4 5 TS S R L TL

C. Practical Session on BioNumerics 1 2 3 4 5 1 2 3 4
5 TS S R L TL

8. Do you have suggestions for any topics that were not included in this course that should be included in future courses?
   1. **Not answered**
   2. **N/A**
   3. **The courses are overall (comprehensive)**
   4. **MLVA/DNA Sequence**
   5. **Protocols of MLVA and analysis**
   6. **How to join PulseNet A.P.**
   7. **Not answered**
   8. **How to deal with the MLVA information with BioNumerics**[5]

9a. What activities did you find most helpful in the computer laboratory?
   1. **Not answered**
   2. **ALL**
   3. **Naming patterns and creating unique pattern lists, composite data sets, and working with subsets**
   4. **Not answered**
   5. **Not answered**
   6. **Hands on**
   7. **Sorting entries in database especially useful when there's huge amount of strains & info.**

8. **All is valuable**

9b. What activities did you find least helpful in the computer laboratory?

1. **Not answered**
2. **None**
3. **Not answered**
4. **None**
5. **Not answered**
6. **Not answered**
7. **Composite data – Not very applicable**
8. **None**

10. Was the time allotted for each topic or practice session appropriate? Yes__8__ No_____

a. For which activities should more time be allowed?

1. **Not answered**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **BioNumerics hands on**
7. **Not answered**
8. **Not answered**

b. For which activities should less time be allowed?

1. **Not answered**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Not answered**
8. **Not answered**

11. In your opinion, should we have this course again for other PulseNet participating Laboratories?
Yes7 No_____ Not answered **1**______

1. **Not answered**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Not answered**
8. **Not answered**

12. Other comments about course:

1. **Not answered**
2. **I think duration of the training should be longer that is 2 weeks instead of a week.**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Not answered**
8. **Not answered**

*Name (Optional):*____
Date: ____________________

Identifying improvement opportunities

1. For Q4b, participant no. 2 rated the quality and usefulness of the practices as "Good" and stated in Q12 that the duration of the training should be 2 weeks instead of a week.
2. For Q6, participant no. 7 commented that "There's more room for further improvement in terms of protocols/techniques." It could be taken as one of the objectives to participate in future workshops.
3. For Q7, the time allotted for all lecture and exercise sessions on day 3 and day 4 was rated too long by participant no. 7. However, the same participant rated in Q10 that the time allotted for each topic or practice session as appropriate.
4. For Q7, the time allotted for all sessions on day 4 was rated short by participant no. 6. Since the overall feedback on time allotment was at the right amount, review of time allotment is considered not necessary.
5. For Q8, 3 participants mentioned the use BioNumerics for MLVA analysis. Inclusion of lecture and exercise for BioNumerics MLVA Plugin module would be considered in future workshops.
6.

WORKSHOP EVALUATION

Course name: The Ninth PulseNet Asia Pacific PFGE Workshop

Location: Public Health Laboratory Centre (PHLC), 382 Nam Cheong Street
Shek Kip Mei, Kowloon, Hong Kong

Dates: February 7-10, 2012

Offered by:
- Public Health Laboratories Centre (PHLC), Department of Health, Hong Kong
- Association of Public Health Laboratories (APHL)
- National Institute of Infectious Diseases (NIID), Department of Bacteriology, Japan
- PulseNet Program, Enteric Diseases Laboratory Branch (EDLB), Centers for Disease Control and Prevention (CDC), USA

Please complete this evaluation so that we can improve this workshop when it is given again.

1. What is your overall evaluation of this course?

Excellent_8___ Good_____ Satisfactory_____ Unsatisfactory_____

2. Were the objectives of the course clearly defined? Yes_8__ No____

3. Were the objectives of the course met? Yes__8__ No____

4a. Please rate the quality and usefulness of handouts.

Excellent_8____ Good_____ Satisfactory_____ Unsatisfactory_____

4b. Please rate the quality and usefulness of the practices.

Excellent__7___ Good__1[1]___ Satisfactory_____ Unsatisfactory_____

5. Please rate how this course will influence your ability to perform and interpret molecular subtyping of *Salmonella* serotypes, *E. coli, Shigella, Vibrio* and other organisms in the future.

Very positively__6___ Positively__2__ Not much_____ Not at all_____

6. Would you recommend this course to others in public health laboratories? Yes__7___ No__1___

Please explain:

1. **It was excellent**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Very useful for users in the laboratory. Allows exchange of information/technique. There's more room for further improvement in terms of protocols/techniques.[2]**
8. **Not answered**

7. Please rate each of the following lectures:

"Subject Matter": **1 = material was not at all pertinent; 5 = it was very pertinent**
"Presentation": **1 = material was not at all clear; 5 = it was very clear**
"Time Allotted": **TS = lecture was too short; S = short; R = right amount of time; L = long; TL = lecture was too long**

Date: Feb 7, 2012

	Subject Matter	Presentation	Time Allotted
A. Installation and Overview of BioNumerics/ PulseNet MasterScripts	1 2 3 4 5	1 2 3 4 5	TS S R L TL
B. Analysis of PFGE Gel (TIFF), Linking Lanes, and Adding Text Data	1 2 3 4 5	1 2 3 4 5	TS S R L TL
C. Exercise 1: Analyze a PFGE Gel (TIFF) and Link Entries to a Database	1 2 3 4 5	1 2 3 4 5	TS S R L TL
D. Creation and File Location of PulseNet Bundle Files	1 2 3 4 5	1 2 3 4 5	TS S R L TL
E. Exercise 2: Prepare and Create a PulseNet Bundle File for Distribution	1 2 3 4 5	1 2 3 4 5	TS S R L TL

Date: Feb 8, 2012

	Subject Matter	Presentation	Time Allotted
A. Data Importing into and Exporting from BioNumerics	1 2 3 4 5	1 2 3 4 5	TS S R L TL
B. Exercise 3: Analyze a PFGE Gel Image; Data Import into and from BioNumerics	1 2 3 4 5	1 2 3 4 5	TS S R L TL
C. Querying and Performing Comparisons with Exporting Data from BioNumerics	1 2 3 4 5	1 2 3 4 5	TS S R L TL
D. Exercise 4: Performing Queries and and Performing Comparisons	1 2 3 4 5	1 2 3 4 5	TS S R L TL
E. Advanced Tools	1 2 3 4 5	1 2 3 4 5	TS S R L TL
F. Exercise 5: Query the Database Using the Advanced Query Tool	1 2 3 4 5	1 2 3 4 5	TS S R L TL
G. Identify the Problems	1 2 3 4 5	1 2 3 4 5	TS S R L TL

Date: Feb 9, 2012

	Subject Matter	Presentation	Time Allotted
A. Database Management Tools	1 2 3 4 5	1 2 3 4 5	TS S R L TL
B. Exercise 6: Database Settings and Layout, Pick List Printing Reports	1 2 3 4 5	1 2 3 4 5	TS S R L TL
C. Working with subsets	1 2 3 4 5	1 2 3 4 5	TS S R L TL
D. Exercise 7: Create Subsets for serotype in Salmonella Database	1 2 3 4 5	1 2 3 4 5	TS S R L TL
E. Naming Patterns and Creating Local Unique Pattern Lists	1 2 3 4 5	1 2 3 4 5	TS S R L TL
F. Exercise 8: Identifying and Naming Unique Patterns in the database	1 2 3 4 5	1 2 3 4 5	TS S R L TL
G. Using the Chart and Statistics Tool and Groups	1 2 3 4 5	1 2 3 4 5	TS S R L TL
H. Exercise 9: Create Charts and Graphs to Create Reports	1 2 3 4 5	1 2 3 4 5	TS S R L TL
I. Composite Data Sets	1 2 3 4 5	1 2 3 4 5	TS S R L TL
J. Exercise 10: Cluster analysis using a composite data set for *Salmonella*	1 2 3 4 5	1 2 3 4 5	TS S R L TL

Date: Feb 10, 2012

	Subject Matter	Presentation	Time Allotted
A. Demo on PFGE protocols	1 2 3 4 5	1 2 3 4 5	TS S R L TL
B. Troubleshooting PFGE Gels	1 2 3 4 5	1 2 3 4 5	TS S R L TL
C. Practical Session on BioNumerics	1 2 3 4 5	1 2 3 4 5	TS S R L TL

8. Do you have suggestions for any topics that were not included in this course that should be included in future courses?

1. **Not answered**
2. N/A
3. **The courses are overall (comprehensive)**
4. **MLVA/DNA Sequence**
5. **Protocols of MLVA and analysis**
6. **How to join PulseNet A.P.**
7. **Not answered**
8. **How to deal with the MLVA information with BioNumerics[5]**

9a. What activities did you find most helpful in the computer laboratory?

1. **Not answered**
2. **ALL**
3. **Naming patterns and creating unique pattern lists, composite data sets, and working with subsets**
4. **Not answered**
5. **Not answered**
6. **Hands on**
7. **Sorting entries in database especially useful when there's huge amount of strains & info.**
8. **All is valuable**

9b. What activities did you find least helpful in the computer laboratory?

1. **Not answered**
2. **None**
3. **Not answered**
4. **None**
5. **Not answered**
6. **Not answered**
7. **Composite data – Not very applicable**
8. **None**

10. Was the time allotted for each topic or practice session appropriate? Yes__8__ No_____

a. For which activities should more time be allowed?

1. **Not answered**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **BioNumerics hands on**
7. **Not answered**
8. **Not answered**

b. For which activities should less time be allowed?

1. **Not answered**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Not answered**
8. **Not answered**

11. In your opinion, should we have this course again for other PulseNet participating Laboratories?
Yes 7 No___ Not answered 1

1. **Not answered**
2. **Not answered**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Not answered**
8. **Not answered**

12. Other comments about course:

1. **Not answered**
2. **I think duration of the training should be longer that is 2 weeks instead of a week.**
3. **Not answered**
4. **Not answered**
5. **Not answered**
6. **Not answered**
7. **Not answered**
8. **Not answered**

Name (Optional):________________________ Date: ____________

Identifying improvement opportunities

1. For Q4b, participant no. 2 rated the quality and usefulness of the practices as "Good" and stated in Q12 that the duration of the training should be 2 weeks instead of a week.
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5. For Q8, 3 participants mentioned the use BioNumerics for MLVA analysis. Inclusion of lecture and exercise for BioNumerics MLVA Plugin module would be considered in future workshops.

Participants List for PulseNet Asia Pacific PFGE Workshop 2012 (7-10 FEB 2012)

		Name	Sex	Email	From	Arrival			Departure			Airport shuttle	Room no.
						Date	Time	Flight	Date	Time	Flight		
1	**Trainer**	Dr. Jun Terajima	M	terajima@nih.go.jp	Department of Bacteriology National Institute of Infectious Diseases Japan	6 Feb			11 Feb			No	Confirmed
2	Trainee	Mohammad Tarequl Islam	M	tareqislam62@yahoo.com	Laboratory Sciences Division ICDDR,B Bangladesh	6 Feb	1850	BG 78	10 Feb	2015	BG 79	No	Not in BP
3	Trainee	Mst. Mahmuda Akter	F	mahmuda@icddrb.org	Laboratory Sciences Division ICDDR,B Bangladesh	6 Feb	1850	BG 78	10 Feb	2015	BG 79	No	Not in BP
4	Trainee	Miss Cao Yanhong	F	delphinecao@gmail.com	Diagnostic Bacteriology Department of Pathology Singapore General Hospital Singapore	4 Feb			12 Feb			No	Self booking
5	Trainee	Dr. Phua Kia Kien	M	kkphua@kb.usm.my	Institute for Research in Molecular Medicine Universiti Sains Malaysia Malaysia	6 Feb	1920		11 Feb	1950		Yes	Confirmed
6	Trainee	Ying Huang（黄瑛）	F	huangying819@163.com	Beijing Centers for Diseases Control and Prevention, PR China							Yes	Confirmed
7	Trainee	Guirong Liu（刘桂荣）	F	lgr420@sohu.com	Beijing Centers for Diseases Control and Prevention, PR China							Yes	
8	Trainee	Yun Luo（罗芸）	F	amanda_ly@163.com	Zhejiang Provincial Center for Disease Control and Prevention, PR China	6 Feb			11 Feb				Confirmed
9	Trainee	Xiaoli Du（杜小莉）	F	duxiaoli@icdc.cn	National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PR China	6 Feb			11 Feb				Confirmed

Rating on the **time alloted** of lectures in the PulseNet Asia Pacific PFGE workshop 2012

No.	Lecture Title	Participants 1	2	3	4	5	6	7	8
A	Installation and Overview of BioNumerics/MasterScripts	NA	NA	R	R	R	R	R	R
B	Analyzing of PFGE Gel (TIFF) and Linking Lanes, and Adding Text Data	NA	NA	R	R	R	R	R	R
C	Exercise 1: Analyze a PFGE Gel (TIFF) and Link Entries to a Database	NA	NA	R	R	R	R	R	R
D	Creation and File Location of PulseNet Bundle Files	NA	NA	R	R	R	R	R	R
E	Exercise 2: Prepare and Create a PulseNet Bundle file for Distribution	NA	NA	R	R	R	R	R	R

No.	Lecture Title	Participants 1	2	3	4	5	6	7	8
A	Data Importing into and Exporting from BioNumerics	NA	NA	R	R	R	R	R	R
B	Exercise 3: Analyze a PFGE Gel Image; Data Import into and from BioNumerics	NA	NA	R	R	R	R	R	R
C	Querying and Performing Comparisons with Exporting Data from BioNumerics	NA	NA	R	R	R	R	R	R
D	Exercise 4: Performing Queries and Performing Comparisons	NA	NA	R	R	R	R	R	R
E	Advanced Tools	NA	NA	R	R	R	R	R	R
F	Exercise 5: Query the Database Using the Advanced Query Tool	NA	NA	R	R	R	R	R	R
G	Identifying the Problems	NA	NA	R	R	R	R	R	R

No.	Lecture Title	1	2	3	4	5	6	7	8
A	Database Management Tools	NA	NA	R	R	R	R	TL[3]	R
B	Exercise 6: Database Settings and Layout, Pick List, Printing Reports	NA	NA	R	R	R	R	TL	R
C	Working with Subsets	NA	NA	R	R	R	R	TL	R
D	Exercise 7: Create Subsets for serotype in Salmonella Database	NA	NA	R	R	R	R	TL	R
E	Naming Patterns and Creating Local Unique Patterns Lists	NA	NA	R	R	R	R	TL	R
F	Exercise 8: Identifying and Naming Unique Patterns in the database	NA	NA	R	R	R	R	TL	R
G	Use the Chart and Statistics Tool and Groups	NA	NA	R	R	R	R	TL	R
H	Exercise 9: Create Charts and Graphs to Create Reports	NA	NA	R	R	R	R	TL	R
I	Composite Data Sets	NA	NA	R	R	R	R	TL	R
J	Exercise 10: Cluster analysis using a Composite Data Set for Salmonella	NA	NA	R	R	R	R	TL	R

No.	Lecture Title	Participants 1	2	3	4	5	6	7	8
A	Demo on PFGE protocols	NA	NA	R	R	R	S[4]	TL	R
B	Troubleshooting PFGE Gels	NA	NA	R	R	R	S	TL	R
C	Practical Session on BioNumerics	NA	NA	R	R	R	S	TL	R

Note: NA= Not answered; TL= lecture was too long; R=Right amount of time; S = short and TS=lecture was too short

プロジェクト６：バングラデシュ

"PROGRESS REPORT-2012"

Drug Resistance, Virulence, and Genetic Traits of *Vibrio cholerae* Causing Cholera in Dhaka, 2006 -2011

Shah M. Rashed[1], Shahnewaj B. Mannan[1], Fatema-tuz Johura[1], Abdus Sadique[1], Haruo Watanabe[2], R. Bradley Sack[3], Anwar Huq[4], Rita R. Colwell[2,4,5], Alejandro Cravioto[1], and Munirul Alam[1].

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Running Title:

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ABSTRACT

Vibrio cholerae O1 biotype El Tor (ET), causing 7th cholera pandemic, was replaced recently in Bangladesh by a hybrid ET variant possessing *ctxB* allele (genotype 1) of classical biotype that originally caused the first six cholera pandemics. In the present study, *V. cholerae* associated with endemic cholera in Dhaka during 2006 - 2011 were analyzed for major phenotypic and genetic characteristics. Of the 54 representative *V. cholerae* isolates tested, all proved phenotypically ET and were uniformly resistant towards sulphamithoxazole - trimethoprim (SXT) and furazolidone (FR), while resistance to tetracycline (TE) and erythromycin (E) showed temporal instability, varying between the years; and all isolates tested were susceptible towards gentamicin (CN) and ciprofloxacin (CIP). Year-wise data revealed E^R to be 33.3% during 2006 and 11% during 2011, while TE^R accounted for 33%, 78%, 0%, 100%, and 27% during 2006, 2007, 2008, 2009, and 2010, respectively; bu, all isolates tested were sensitive to TE during 2011. PCR assays confirmed all isolates to possess SXT element, but not *Intl*-integron-1. All of the tested isolates were *ctx*-positive, and possessed ET-specific markers such as $tcpA^{ET}$, $rstR^{ET}$ and *rtxC*, but had $ctxB^{CL}$ confirming all to be variant ET. DNA sequencing and analysis of *ctxB* gene allowed the detection of a point mutation at position 58 (C→A) that results in amino acid substitution from Histidine (H) to Asparagine (N) at position 20 (genotype 7) since 2008. Molecular fingerprinting determined by pulsed-field gel electrophoresis (PFGE) of genomic DNA and dendrogram revealed clonal nature of the strains, although minor divergence was observed for multi-drug resistance strains having TE^R marker. This study shows that multidrug resistant *V. cholerae* variant ET causing cholera in Dhaka now possess *ctxB* genotype 7 since 2008.

Key Words: Cholera, variant El Tor, antibiotic resistance, *ctxB* genotype, PFGE, clonal.

INTRODUCTION

Cholera is a life threatening form of dehydrating diarrheal disease caused by the toxigenic serogroup strains of *Vibrio cholerae*. Of more than 200 O-serogroups of *V. cholerae,* epidemics of cholera, until 1992, were caused by *V. cholerae* serogroup O1. *V. cholerae* O1 has two biological variants (designated biotypes), namely classical (CL) and El Tor (ET) that differs from each other in both phenotypic and genetic characteristics, including the type of cholera toxin (CT) that they harbor (Kaper *et al*., 1995; Dziejman *et al*., 2002, Olsvik *et al*., 1993). In addition, the two biotypes differ in the infection patterns of disease such as the CL biotype strains cause more severe disease while El Tor strains are more efficient in host-to-host transmission than that of classical strains (Woodward *et al*., 1972). The CL biotype is believed to have caused the first six pandemics, which occurred in the Indian subcontinent, and subsequently in other areas of the world between 1817 and 1923 (Politzer, 1959). *V. chol*erae O1 biotype El Tor, which was first reported in 1905 (Politzer, 1959), initiated the 7th cholera pandemic in the early 1960s by displacing the classical biotype (Kaper *et al*., 1995). In 1992, a *V. cholerae* non O1 serovar, designated *V. cholerae* O139 synonym Bengal, emerged as the cause of epidemic cholera in Bangladesh (Cholera Working Group 1993) and India (Ramamurthy, 1993). *V. cholerae* O139 Bengal emerged subsequently by displacing *V. chol*erae O1 El Tor and was considered a significant point in the history of cholera. *V. chol*erae O1 El Tor continues to be the major pathogen of cholera, although O139 Bengal continues to coexist by sharing niche with O1 ET in the estuarine ecosystem of Bay of Bengal (Alam *et al.*, 2006).

In spite of significant advancement in our understanding of diarrheal diseases including *V. cholerae* pathogenesis, endemic cholera kills many people in the Ganges delta of Bay of Bengal (Bangladesh and India), where cholera occurs forming two seasonal peaks, once during spring (March – May), and again in fall (September – November) (Alam *et al.*, 2006; Sack *et al.*, 2004). The treatment of cholera which includes a three-day course of effective antibiotic together with the appropriate oral or intravenous rehydration therapy can significantly shorten the duration of diarrhea (Sack *et al.*, 2004), disease severity and hospitalization (Lindenbaum *et al.*, 1967).

However, antibiotic therapy in recent years has faced difficulties as the rapid emergence of multiple antibiotic resistant strains of *V. cholerae* was reported from Africa, Asia, and America (Jesudason *et al.,* 1990, Maimone *et al.,* 1986,). During the past two decades, several cholera endemic countries including India and Bangladesh have reported *V. cholerae* serogroup O1 resistant to tetracycline (TE), ampicillin (AMP), kanamycin (K), sulphonamides, streptomycin (S), sulfomethoxazole-trimethoprim (SXT), norfloxacin (NOR), gentamicin (CN), furazolidone (FR), ciprofloxacin (CIP), and erythromycin (E) (Sack *et al.,* 2004, Faruque *et al.,* 2007, Jian *et al.*, 2011). The increasing trend of multi-drug resistance of *V. cholerae* associated with severe disease becoming a serious public health concern for the cholera endemic countries of Asia and Africa (Jain *et al.*, 2011; Kumar *et al.*, 2010; Quilici *et al.*, 2010).

Vibrio cholerae O1 biotype El Tor (ET), cause of current 7th pandemic, has recently been replaced in Asia and Africa by an altered ET possessing cholera toxin (CTX) of the classical (CL) biotype. Over the past few years, ET causing Asiatic cholera has shown remarkable changes in its phenotypic and genetic characteristics (Nair *et al.,* 2002). Recent molecular analysis of ET strains causing acute watery diarrhea in Bangladesh shows them to be hybrid because they possess phenotypic and genotypic traits of the CL biotype against an ET background (Nair *et al.*, 2002). Subsequent retrospective studies showed that all of the O1 ET strains isolated in Bangladesh since 2001 were hybrids of both CL and ET biotypes, while those isolated before 2001 contained all the ET attributes of the 7th pandemic *V. cholerae* O1 (Nair *et al.,* 2006). *V. cholerae* hybrid ET continues to be routinely isolated from clinical cholera cases in Asia and Africa (; Safa *et al.,* 2008) and has been reported to be a new pandemic pathogen capable of causing more severe disease (Siddique *et al.,* 2009) and spreading globally (Chin *et al.*, 2011). A recent study in India reported that new cholera toxin variant of *V. cholerae* O1 of El Tor biotype having an amino acid substitution at position 20, caused a large cholera outbreak in Orissa, Eastern India (Kumar *et al.*, 2009). To better understand the phenotypic and molecular genetic traits of contemporary *V. cholerae* causing epidemic cholera in Bangladesh, 54 representative *V. cholerae* O1 strains isolated from endemic cholera between 2006 and 2011 in Dhaka were critically characterized and data presented in this study.

MATERIAL AND METHODS

Bacterial strains

In this study, a collection of 54 randomly selected strains isolated as part of 2% surveillance from cholera patients who were seeking treatment to the hospital of International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) in between 2006 and 2011. Rectal swabs were collected from suspected cholera patients and transported to the laboratory within 3 hours using Cary Blair transport media. The isolation of *V. cholerae* starts with the enrichment of rectal swab in alkaline peptone water (APW) (pH-8.4) at 37°C for 4-6 h followed by culturing on selective media as described previously (Alam *et al*., 2007). *V. cholerae* was identified and confirmed by using standard cultural, biochemical and molecular methods (Alam *et al*., 2007).

Serogrouping

The serogroups of *V. cholerae* strains that were identified using biochemical and molecular methods were confirmed serologically by a slide agglutination test using specific polyvalent antisera for *V. cholerae* O1 and O139, followed by a monoclonal antibody that is specific for serotypes (Alam *et al*., 2007).

Biotyping

Biotyping involved a number of phenotypic tests: chicken erythrocyte agglutination (CCA), sensitivity to polymyxin B, and Mukerjee CL phage IV and Mukerjee ET phage V tests (Kaper *et al*., 1995). To complement the biotype characterization by phenotypic traits, PCR assays were carried out using previously described procedures that were targeted to detect *tcpA* (CL and ET) (Alam *et al*., 2010), the type of the *rstR* gene encoding the phage transcriptional regulator (Kimsey *et al*., 1998) and *rtxC* gene of RTX (repeat in toxin) (Chow *et al*., 2003).

Genomic DNA preparation

Genomic DNA extraction was carried out following previously described methods (Nusrin *et al*., 2009)

Confirmation of serogrouping by PCR assay

All strains that were primarily identified as *V. cholerae* were reconfirmed using a *V. cholerae* species-specific *ompW* PCR. The serogroups of these strains were reconfirmed using polyvalent O1 and monovalent Inaba and Ogawa anisera and by multiplex PCR targeted to identify genes encoding O1 (*wbe*) and O139 (*wbf*) specific O biosynthetic genes and the cholera toxin (CT) gene (*ctxA*) (Hoshino *et al.*, 1998).

Antimicrobial susceptibility

Bacterial susceptibility to different antimicrobial agents was determined by the NCCLS agar disk diffusion method (NCCL 2008). The *V. cholerae* strains were tested for antibiotic sensitivity against six commercially available antibiotic impregnated discs (Oxoid international) with Erythromycin (E) (15μg), Tetracycline (TE) (30μg), Gentamicin (CN) (10μg), Ciprofloxacin (CIP) (5μg), Sulphamithoxazole-trimethoprim (SXT) (30μg) and Furazolidone (FR) (100μg).

PCR assay for the detection of SXT and class1 integron

All antibiotic resistant *V. cholerae* O1 strains were examined for the presence of SXT element and class1 integron by PCR. The detection of SXT and *intI1* were performed using primers and procedures described previously (Thungapathra *et al.*, 2002).

MAMA-PCR for determination of *ctxB* gene type

The mismatch amplification mutation assay (MAMA) was recently developed to detect the sequence polymorphism between the CL and ET *ctxB* genes ($ctxB^{CL}$ and $ctxB^{ET}$, respectively) by focusing on nucleotide position 203 of the *ctxB* gene (Morita *et al.*, 2008). MAMA-PCR was performed to test for the presence of the *ctxB* genes specific for the CL and ET biotypes. A conserved forward primer (Fw-con, 5'-ACTATCTTCAGCATATGCACATGG-3') and two allele-specific polymorphism detection primers, Rv-cla (5'-CCTGGTACTTCTACTTGAAACG-3') and Rv-elt (5'-CCTGGTACTTCTACTTGAAACA-3'), were used. PCR conditions were as follows: after initial denaturation at 96°C for 2 min, 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 10 s, extension at 72°C for 30 s, and a final extension at 72°C for 2 min.

The resulting *V. cholerae* O1 isolates, O395 CL and N16961 ET, were used as standard reference strains.

Nucleotide sequence analysis of *ctxB* gene

To determine the nucleotide sequence of the *ctxB* subunit of CT, PCR amplification of *ctxB* genes of 14 representative strains of *V. cholerae* O1 were performed in a 25-mL reaction mixture in an automated Peltier thermal cycler (PTC-200, M. J. Research). PCR primers and conditions were as described previously (Olsvik *et al.*, 1993). PCR products were purified with a Microcon centrifugal filter device (Millipore Corporation, Bedford, MA) and sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 310 automated sequencer (Applied Biosystems). The nucleotide sequence data generated using different *V. cholerae* O1strains were submitted to GenBank with accession numbers.

PFGE

The whole agarose-embedded genomic DNA from *V. cholerae* was prepared. Pulsed-field gel electrophoresis (PFGE) was carried out with a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad), according to procedures described elsewhere (Alam *et al.*, 2010). The conditions used for separation were as follows: 2 to 10 s for 13 h, followed by 20 to 25 s for 6 h. An electrical field of 6 V/cm was applied at an included field angle of 120°. Genomic DNAs of the test strains were digested by the *NotI* restriction enzyme (Gibco-BRL, Gaithersburg, MD), and *Salmonella enterica* serovar Braenderup was digested by *XbaI*, with the fragments being used as molecular size markers. The restriction fragments were separated in 1% pulsed-field-certified agarose in 0.5X TBE (Tris-borate-EDTA) buffer. In the postelectrophoresis gel treatment step, the gel was stained and destained. The DNA was visualized using a UV transilluminator, and images were digitized via a one-dimensional gel documentation system (Bio-Rad).

Image analysis

The fingerprint pattern in the gel was analyzed using a computer software package, Bionumeric (Applied Maths, Belgium). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity using Dice similarity coefficient and unweighted-pair group method using average linkages (UPGMA) clustering methods, as recommended by the manufacturer; these were graphically represented as dendrograms.

RESULTS

Microbiological and serological tests

All tested strains (n=54) produced characteristic colonies typical of *V. cholerae* when they were grown on selective medium taurocholate tellurite gelatin agar (TTGA). The characteristic colonies gave biochemical reactions typical of *V. cholerae*, and all strains reacted to polyvalent antibody specific for *V. cholerae* serogroup O1 followed by positive agglutination with monovalent Ogawa antisera suggesting that all belonged to *V. cholerae* O1 of Ogawa serotype (Table 1).

Amplification of primers specific for *V. cholerae* serogroup O1 and *ctxA* by PCR assay

All tested strains (n=54) amplified the primers for *V. cholerae* species-specific gene *ompW*, and O-antigen biosynthetic gene *wbeO1*, but failed to amplify the primers specific for *wbfO139* gene. In addition, all the strains serologically identified to be O1 amplified the primers for the cholera toxin gene *ctxA*, confirming that all strains harbor toxigenic CTX pro-phage in the genome of tested *V. cholerae* O1 strains (Table 1).

Phenotypic and related genetic characteristics

The result of phenotypic and related genetic characteristics of the *V. cholerae* serogroup O1 strains are presented in Table 1. All of the *V. cholerae* O1 strains were primarily identified as El Tor biotype based on the presence of specific phenotypic characteristics such as, positive agglutination reaction with chicken erythrocytes (CCA), sensitivity to ET-specific phage V, whereas those were resistant to both polymyxin B (50U) and CL specific phage IV. All