Myanmar Health Sciences Research Journal, Vol. 27, No. 3, 2015

Genotypic Characteristics of *Vibrio cholerae* Strains from Myanmar: Comparison between Past and Recent Isolates

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Atypical E1 Tor *Vibrio cholerae*, which possesses traits of both classical and E1 Tor biotypes, has replaced the seventh pandemic E1 Tor *V. cholerae* O1 in Asian and African countries. The origin and spread of these E1 Tor *V. cholerae* in Myanmar should be tracked by genomic analysis. The genotypic characteristic of recent (2012) and past (1982-1996) clinical *V. cholerae* O1 isolates from Yangon, Myanmar were investigated. *V. cholerae* isolates were confirmed by culture, biochemical identification, serotyping and polymerase chain reaction. Eight *V. cholerae* strains isolated during 1982-1996 and 34 strains isolated in 2012 were undergone genotypic analysis by Pulse Field Gel Electrophoresis Typing (PFGE) and Multilocus Variable Number Tamdem Repeat Analysis DNA sequencing. Recent 2012 isolates were atypical E1 Tor, which carried the classical cholera toxin B subunit gene (*ctxB*^{Cla}) and E1 Tor repressor gene (*rstR*^{E1}) and exhibited a total of 10 PFGE patterns. Among *V. cholerae* O1 strains isolated during 1982-1996, 4 pulsotypes were identified and they were different from 2012 PFGE patterns. Pulsotype Y1 and Y4 isolates carried *ctxB*^{El} and *rstR*^{E1} and they are related to seventh cholera pandemic E1 Tor strains. Pulsotype Y2 isolate was atypical E1 Tor which carried *ctxB*^{Cla} and *rstR*^{E1}. Remarkably, seventh cholera pandemic prototype E1 Tor was observed only in 1982 isolates and atypical E1 Tor with *ctxB*^{Cla} and *rstR*^{E1} was found to be existed in Yangon 30 years ago. This study provided basic genetic information on past and recent cholera strains in Myanmar.

Key words: Vibrio cholerae, Genotypic characteristics, Myanmar

INTRODUCTION

Cholera is a rapidly dehydrating acute enteric infection caused by the ingestion of toxigenic serogroup (01 and less commonly 0139) of *Vibrio cholerae* present in faecally contaminated water or food. It is characterized in its most severe form by a sudden onset of acute watery diarrhea that can lead to death by severe dehydration. Cholera has spread widely and affects at least 56 countries in the world at least. Cholera remains a global threat to public health. The global disease burden is estimated to be 3-5 million cases and 100,000-130,000 deaths per year. In 2009, a total of 221,226 cases, including 4,946 deaths, were reported to the World Health Organization from 45 countries.[1] The recent outbreaks of cholera were due to a new emerging form of *V. cholerae* O1, which possesses traits of both classical and El Tor biotypes. El Tor variants spread to several countries in

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Asia and Africa.[2-4] *Vibrio cholerae* harbours a virulence regulon consisting of genes involved in colonization, toxin production and bacterial survival within the host. In Bangladesh, all of the El Tor isolates of *V. cholerae* O1 obtained since 2001 produced classical cholera toxin. In Kolkata, India, El Tor variant strains carrying the hybrid CTX prophage, which carries the El Tor *rstR* (*rstR*^{El}) (*rstR*, CTX prophage repressor gene) and the classical *ctxB* (*ctxB*^{Cl}), have entirely replaced the El Tor type *ctxB* since 1995.[5] In northern Vietnam, the El Tor variant carrying this hybrid CTX prophage has been reported since late 2007.[6]

In Myanmar, cholera is a fifth priority disease in National Health Plan (2006-11). *V. cholerae* O1 El Tor outbreak was occurred in 1961 and spread throughout the country. In 1994, *V. cholerae* 139 outbreak occurred in some townships of Yangon Reagion and since then there was no more O139 detected and only *V. cholerae* O1 is circulating in Myanmar. According to the laboratory data of the National Health Laboratory, Yangon, there were 103 culture-confirmed cholera cases in 2011.[7]

With consideration of the increase in the global prevalence of cholera, the origin and spread of these El Tor variant strains of *V. cholerae* in Myanmar should be tracked by genomic analysis.

In the present study, the genotypic characteristics of recent (2012) and past (1982-1996) *V. cholerae* O1 isolates from Yangon were investigated. Observation of possible emergence of a new virulent variant strain will provide an effective control of cholera infections and outbreaks. Moreover, the molecular epidemiological data will provide insight knowledge of facilitating further studies to develop a safe vaccine against cholera.

MATERIALS AND METHODS

Study design and period

Cross-sectional, descriptive study was carried out during 2012-2014.

Study population

Suspected cholera cases attending hospitals in Yangon (Specialist Hospital, Waibargi, Insein General Hospital, North Okkalapa General Hospital, Thingangyun Sanpya Hospital, Yangon General Hospital and New Yangon General Hospital) in 2012 and *V. cholerae* isolates from Yangon during 1982-1996.

Laboratory procedure

Rectal swabs in Cary Blair Transport Media were collected from suspected cholera cases attending hospitals in Yangon and transported to DMR-LM. Isolation, identification, serotyping and PCR-based characterization were carried out at Bacteriology Research Division. Pulse Field Gel Electrophoresis Typing (PFGE) and genome sequencing were carried out at Thailand-Japan Research Collaboration Center on Emerging and Reemerging Infections, Nonthaburi, Thailand.

Isolation, identification, serotyping

Rectal swabs were placed in alkaline peptone water for 6 hours and then streaked on thiosulphate-citrate-bile salt-sucrose (TCBS) agar (Eiken Chemical, Japan) and incubated for 12-18 hrs at 37°C. Suspicious colonies in yellow, flat, shiny, and 2-3 mm in diameter on TCBS were tested for biochemical identification and slide aggluination with *Vibrio cholerae* specific antisera (Denka Seiken, Japan). Past *V. cholerae* isolates from stock cultures were inoculated onto TCBS after enrichment in alkaline nutrient agar for 6 hrs.

Drug susceptibility test

Drug susceptibility to 11 antibiotics (Becton Dickinson, Sparks, MD) is tested using a standard disc diffusion technique, according to the guidelines[8] of Clinical and Laboratory Standards Institute.

PCR-based characterization

The amplification of virulence-associated genes and/or biotype makers encoding the genome of the *V. cholerae* isolates is performed using LAMP method and hexaplex polymerase chain reaction (PCR).[9]

Pulsed-field gel electrophoresis (PFGE) typing

Intact agarose-embedded genomic DNA of the *V. cholerae* strains is digested with *No* I enzyme (New England Biolabs, MA, USA) and the fragments are separated in a contour-clamped homogeneous electric field apparatus (CHEF-DRIII; Bio-Rad, Hercules, CA, USA) according to the Pulsenet *V. cholerae* subtyping protocol.[10]

Multilocus variable-number tandem repeat analysis (MLVA) DNA sequencing

Five loci for MLVA are amplified using primers and PCR conditions as described in previous studies.[11] The purified PCR products were sequenced in both directions using a Big Dye Cycle Sequencing kit (Applied Biosystems) and sequencing was performed on an ABI 3770 automatic sequencer according to manufacturer's instructions.

PFGE and MLVA profiles

Not I PFGE profiles were compared digitally using Bionumerics 6.1 software (Applied Maths). Cluster analysis of Dice similarity indices based on the unweighted pair group method with arithmetic mean (UPGMA) was used to generate a dendrogram describing the relationships among PFGE profiles.

Ethical consideration

This study was approved by the Ethical Committee on Medical Research Involving Human Subjects, Department of Medical Research (Lower Myanmar).

RESULTS

Phenotypic characteristics of V. cholerae isolates

Of 20 *V. cholerae* stock culture isolates collected during 1982-1986, 8 *V. cholerae* O1 strains (serotypes: 4 Inaba and 4 Ogawa) were revived and preceded for molecular characterization. Of 312 rectal swab specimens collected during 2012, *V. cholerae* O1 were isolated from 72 cases (23.1%). The 34 out of 72 isolates of *V. cholerae* O1 were proceeded for molecular analysis. All isolates were *V. cholerae* O1, serotype Ogawa and tetracycline resistant.

Genotypic pattern of past (1982-1996) V. cholerae isolates

Of 8 *V. cholerae* O1 strains isolated during 1982 to 1996 in Yangon, a total of 4 pulsotypes were identified. The isolates exhibited pulsotypes Y1 and Y4 carrying *ctxB*^{El} and *rstR*^{El} and they are related to seventh cholera pandemic El Tor strains examined by the Multi-locus sequence typing.

The 5 isolates isolated in 1982, 1986, and 1996, exhibited pulsotype Y3 which were corresponding to atypical El Tor strains carrying *ctxB*^{Cla} and *rstR*^{Cla}. The remaining pulsotype Y2 was atypical El Tor which carried *ctxB*^{Cla} and *rstR*^{El} (Table 1).

Table 1. Genotypic patttern of past (1982-1996) *V. cholerae* isolates (n=8)

No.	Year	Serotype	*ctxB* gene pattern	*rstR* gene pattern	PFGE pattern
1	1982	Inaba	El	El	Y1
2	1982	Inaba	Cl	El	Y2
3	1982	Ogawa	Cl	Cl	Y3
4	1982	Inaba	El	El	Y4
5	1982	Inaba	Cl	Cl	Y3
6	1966	Ogawa	Cl	Cl	Y3
7	1986	Ogawa	Cl	Cl	Y3
8	1996	Ogawa	Cl	Cl	Y3

EL=ELTOR, CL=Classical

Genotypic pattern of recent (2012)V. cholerae isolates

All 34 *V. cholerae* O1 Ogawa carried *tcpA* (encoding the structural subunit of the toxin-coregulated pilus) and *rstR* (repressor gene in CTX phage) of El Tor biotype. All sequences toxin B subunit gene were classical type (*ctxB*^{Cla}). All recent Myanmar isolates were atypical El Tor, which carried *ctxB*^{Cla} and *rstR*^{El} and exhibited a total of PFGE 10 patterns (Y5 to Y14). Y10 pulsotype was predominantly found in 50% isolates (17/34). Multilocus variable-number tandem-repeat analysis of Y10 isolates revealed that five MLVA types were shown and they were closely related (Table 2 & Fig. 1).

Table 2. Genotypic pattern of recent (2012) *V. cholerae* O1 isolates (n=34)

No.	Date	Age	Sex	PFGE pattern	MLVA type
1	10-Feb	18	M	Y14	-[†]
2	13-Feb	24	M	Y5	-
3	13-Mar	1	M	Y6	-
4	15-Mar	12	M	Y8	-
5	16-Mar	18	F	Y9	-
6	19-Mar	68	M	Y11	-
7	20-Mar	24	F	Y10	M1[‡]
8	20-Mar	7	M	Y11	-
9	23-Mar	56	M	Y10	M1
10	26-Mar	54	M	Y8	-
11	29-Mar	62	M	Y8	-
12	29-Mar	63	F	Y11	-
13	2-Apr	18	M	Y7	-
14	2-Apr	50	F	Y10	M5
15	2-Apr	20	M	Y12	-
16	2-Apr	28	F	Y10	M1
17	2-Apr	31	F	Y10	M1
18	2-Apr	22	F	Y11	-
19	4-Apr	48	F	Y10	M1
29	4-Apr	17	F	Y11	-
21	23-Apr	35	M	Y10	M3
22	2-May	10	F	Y10	M1
23	7-May	4	M	Y10	M2
24	14-May	2	M	Y10	M2
25	15-May	2	M	Y10	M1
26	5-Jun	5	F	Y10	M1
27	5-Jun	2	F	Y10	M1
28	6-Jun	2	F	Y10	M2
29	6-Jun	4	M	Y10	M2
30	14-Jun	24	F	Y10	M1
31	28-Jun	1	F	Y12	-
32	4-Jul	1	M	Y10	M2
33	30-Jul	5	F	Y11	-
34	6-Aug	56	F	Y11	-

MLVA types, M1 (11, 6, 5, 17, 17), M2 (11, 6, 5, 17, 18), M3 (10, 6, 5, 17, 17), M4 (11, 6, 5, 12, 17), and M5 (11, 6, 5, 16, 17)

The numbers of repeats were counted and listed sequentially for the five VNTR loci (VC0147, VC0436-7, VC1650, VC0171, and VCA0283) to generate an isolate pattern.

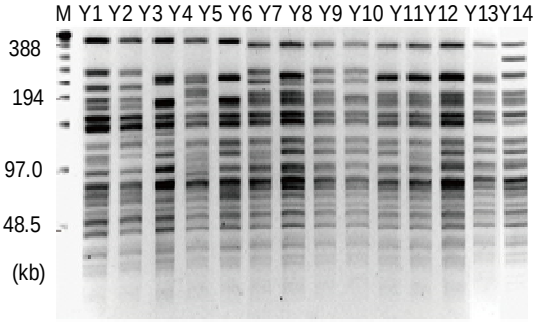

Fig. 1. Y1-Y14 PFGE pattern of *V. cholerae* isolates showing multiple bands

DISCUSSIONS

In Myanmar, morbidity rate of severe diarrhea (assumed to include cholera cases but not laboratory confirmed) is an estimated 2.6-3.5/100,000 population and mortality rate is 0.04-0.1/100,000.[12]

Yangon is the largest city and the total population of the city is 4.5 million (2014 Census). Cholera outbreaks caused by El Tor biotype were recorded in 1961 in Yangon, although it is unknown to be related with the seventh cholera pandemic wave which began in Indonesia, 1961. *V. cholerae* O139 Bengal was initially appeared in Bangladesh and India 1992,[13] In 1994, several outbreaks caused by O139 cholera pathogen were firstly recognized in some townships of Yangon.[14] In recent two decades, atypical El Tor *V. cholerae*, which possesses traits of both classical and El Tor biotypes, has replaced the seventh pandemic prototypic El Tor *V. cholerae* O1 in Asian and African counties.[15]

All 2012 isolates were serotype Ogawa, tetracycline resistant, and carried *tcpA* (encoding the structural subunit of the toxin-coregulated pilus) and *rstR* (repressor gene in CTX phage) of El Tor biotype,[16] while all sequences of cholera toxin B subunit gene were classical type ($ctxB^{Cla}$). There is no O139 Bengal isolate, O1 isolates carrying Haitian variant cholera toxin gene[17] and MS6 strain which we previously found as a new genetic line in Thai Myanmar border area.[18] Recent 2012 *V. cholerae* isolates from Yangon were of atypical El Tor, which carried $ctxB^{Cla}$ and $rstR^{El}$ and exhibited a total of 10 patterns of Pulsed Field Gel Electrophoresis (PFGE).

Between February and April, 2012, the majority of patients was adults and 9 variation of PFGE patterns were observed. Whereas, since May to August, most patients infected with *V. cholerae* O1 were children less than 5 years old and almost exhibited pulsotype Y10. This pulsotype was predominantly found in 50% of isolates. For further distinguish, we apply all isolates of Y10 for multilocus variable-number tandem-repeat analysis and

revealed that five MLVA types were shown and they were closely related. Sporadic cholera among children since May probably attributed to limited sources of infection during rainy season in Yangon.

V. cholerae O1 strains isolated during 1982 to 1996 in Yangon were analyzed. A total of 4 pulsotypes were identified and they were different from 2012 PFGE patterns. The isolates exhibited pulsotypes Y1 and Y4 carried $ctxB^{El}$ and $rstR^{El}$ and they are related to seventh cholera pandemic El Tor strains examined by the Multi-locus sequence typing. The 5 isolates isolated in 1982, 1986, and 1996, exhibited pulsotype Y3 which were corresponding to atypical El Tor strains carrying $ctxB^{Cla}$ and $rstR^{Cla}$. The remaining pulsotype Y2 was atypical El Tor which carried $ctxB^{Cla}$ and $rstR^{El}$. Nevertheless, their pulsotypes in past and present were differentiable.

The largest bands of PFGE pattern (ca. 400 kb) in Y1-Y4 of past isolates and in Y5 could be due to the insertion of bacteriophage K139 (35 kb) (referenced MJ1236 complete genome, accession no. NC_012667/ NC_012668) in the large chromosome confirming by PCR and Southern hybridization (using probes against the genes encoding phage replication (VCD_002162) and capsid (VCD_002167) proteins).

Taken together, in 1982, Yangon would had been attacked by several waves of cholera transmission, at least three cholera organisms which carried different CTX phages. Remarkably, seventh cholera pandemic prototype El Tor were observed only in 1982 isolates. In 1986-1996, *V. cholerae* O1 were only atypical El Tor with $ctxB^{Cla}$ and $rstR^{Cla}$. Notably, atypical El Tor with $ctxB^{Cla}$ and $rstR^{El}$ existed in Yangon 30 years ago. Nevertheless, this type of atypical El Tor has replaced in 2012. Variation of PFGE in adult patients and on the contrary, limited PFGE pattern of less 5 aged, could have led to understand the transmission routes in Yangon. Continuous and systematic surveillance on cholera in Yangon would contribute especially to prevention and control.

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