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Molecular Epidemiology of Cholera Outbreaks during the Rainy Season in Mandalay, Myanmar

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Abstract. Cholera, caused by *Vibrio cholerae*, remains a global threat to public health. In Myanmar, the availability of published information on the occurrence of the disease is scarce. We report here that cholera incidence in Mandalay generally exhibited a single annual peak, with an annual average of 312 patients with severe dehydration over the past 5 years (since 2011) and was closely associated with the rainy season. We analyzed cholera outbreaks, characterized 67 isolates of *V. cholerae* serogroup O1 in 2015 from patients from Mandalay, and compared them with 22 *V. cholerae* O1 isolates (12 from Mandalay and 10 from Yangon) in 2014. The isolates carried the classical cholera toxin B subunit (*ctxB*), the toxin-coregulated pilus A (*tcpA*) of Haitian type, and repeat sequence transcriptional regulator (*rstR*) of El Tor type. Two molecular typing methods, pulsed-field gel electrophoresis and multiple-locus variable-number tandem repeat analysis (MLVA), differentiated the 89 isolates into seven pulsotypes and 15 MLVA profiles. Pulsotype Y15 and one MLVA profile (11, 7, 7, 16, 7) were predominantly found in the isolates from cholera outbreaks in Mandalay, 2015. Pulsotypes Y11, Y12, and Y15 with some MLVA profiles were detected in the isolates from two remote areas, Mandalay and Yangon, with temporal changes. These data suggested that cholera spread from the seaside to the inland area in Myanmar.

INTRODUCTION

Cholera is an acute enteric infection caused by *Vibrio cholerae*. *Vibrio cholerae* serogroups O1 and O139 are known to cause epidemic cholera.[1] Serogroup O1 is classified into two biotypes, classical and El Tor, which differ in phenotype as well as genotype.[2] Infection caused by this bacterium can cause profuse watery diarrhea with a tendency to severe dehydration. Severe fluid loss can lead to death within 1 day after the onset of symptoms.[1] Transmission occurs usually through the fecal-oral route of contaminated food or drinking water, especially in areas where safe water and sanitation facilities are inadequately available. Cholera remains an important public health issue in more than one-third of the countries of world, particularly in less-developed countries.[3]

A seasonal trend in the detection or isolation of *V. cholerae* O1 and prevalence of cholera in various areas has been observed worldwide.[4] Cholera dynamics in South India display regular seasonal cycles, which are related to climate factors such as rainfall.[5,6] Zambia and Zanzibar in Africa reported that increased temperature and rainfall signaled a coming surge in cholera cases.[7,8] A study based on cholera data that included all the World Health Organization reported cases from 1974 to 2005 in 140 countries described that cholera outbreaks showed seasonal patterns in higher absolute latitudes, whereas seasonal patterns did not persist near the equator.[4] However, there are several limitations in the study because of the incomplete record of all global cholera outbreaks.

Cholera has disseminated from the Indian subcontinent to other countries through pandemics. Nonetheless, availability of published information on the occurrence of cholera in Myanmar is scarce. Since the early 1990s, *V. cholerae* O1 strains, which possessed traits of both the classical and El Tor biotypes, have emerged.[2,9] Strains including Mozambique variants, altered El Tor variants, and Matlab variants exhibited a mixed genotype in the sequence of cholera toxin (CTX) phage regions such as cholera toxin B subunit (*ctxB*), repeat sequence transcriptional regulator (*rstR*), and other regions such as toxin-coregulated pilus A (*tcpA*).[9]

Pulsed-field gel electrophoresis (PFGE) is the most effective tool in epidemiological investigation of *V. cholerae* and is the gold standard of molecular typing. Multiple-locus variable-number tandem repeat analysis (MLVA) is a sequencing-based typing tool that targets five loci by examining short repeated DNA segments in the *V. cholerae* genome and provides a source of investigation of high genomic polymorphism among *V. cholerae* isolates.[10] PFGE and MLVA can provide comprehensive information on epidemiological relatedness among isolates from different sources or geographical regions.

In the present study, we described cholera outbreaks in Mandalay closely associated with the rainy season and conducted epidemiological investigations using PFGE and MLVA to identify the epidemiological linkage of *V. cholerae* O1 infection in Myanmar.

MATERIALS AND METHODS

Case definitions. A suspected cholera case was defined as a patient with clinical symptoms of severe dehydration from acute watery diarrhea, with or without vomiting. Suspected cholera cases were confirmed by laboratory isolation and identification of *V. cholerae* from diarrhea stool samples.

Bacterial isolates and phenotyping. The isolates were obtained from cholera patients admitted to hospitals in Mandalay in 2015 and were laboratory-confirmed as *V. cholerae* O1. Other *V. cholerae* O1 isolates isolated in Yangon and Mandalay in 2014 were included in the study for comparison. The isolates were confirmed as *V. cholerae* using standard culture methods and further identified using slide agglutination test with specific monoclonal antibodies (Denka Seiken, Tokyo, Japan) that identified the serogroups (O1 or O139) and serotypes (Ogawa or Inaba).[1] The study was approved by the Ethical

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TABLE 1
Number of cholera cases per month, rain days, and rain amount in Mandalay, 2015

Season	Cold	Summer			Rainy						Cold	
Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Suspected cases	42	20	28	27	101	112	382	721	78	35	43	28
Laboratory-confirmed cases	1	0	0	1	22 (2)*	6 (4)	121 (49)	151 (10)	9 (2)	6	9	5
Rain amount (mm)	19	2	2	14	276	26	96	116	134	263	23	2
Rain (days)	2	1	1	4	9	4	10	9	10	10	3	1

* Number in parenthesis indicates tested isolates in this study.

Committee of the Department of Medical Research, Ministry of Health, Myanmar (ref. No. 002316).

DNA preparation and polymerase chain reaction (PCR) amplification. DNA templates for PCR and MLVA were extracted with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) and quantified using a spectrophotometer. DNA samples were diluted to 10 ng/μL and used as templates for gene detection and MLVA. PCR for *ctxA*, *zot*, *ace*, *tcpA*, *ompU*, and *toxR* was performed to screen for toxigenic/pathogenic *V. cholerae*.[11] Mismatch amplification mutation PCR assay was used to detect sequence polymorphisms in *ctxB*.[12] In addition, PCR was performed for *rstR*[13] and *tcpA*.[14]

DNA fingerprinting. PFGE was performed as described previously.[15] Briefly, bacteria were grown on trypticase soy agar at 37°C for 18 hours, and the cells were suspended in 100 mM Tris- ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.0, and adjusted to an optical density $(OD)_{610}$ of 0.8. Agarose plugs were prepared by mixing 200 μL of the adjusted bacterial suspension with 200 μL of melted 1% SeaKem Gold Agarose (Lonza, Rockland, MD) and 10 μL of 20 mg/mL proteinase K. Bacteria in agar plugs were lysed in 5 mL lysis solution (50 mM Tris-EDTA, pH 8.0, 1% sarcosine, and 25 μL of the 20 mg/mL proteinase K) for 1 hour at 54°C with agitation. They were then washed twice with sterile distilled water and four times with Tris-EDTA buffer at 50°C. Plugs were equilibrated with NEBuffer 3.1 (New England Biolabs, Ipswich, MA) for 15 minutes and then with 40 U of *Not*I in the same buffer overnight at 37°C. After incubation, the plugs and standard lambda DNA ladder (Bio-Rad, Hercules, CA) were loaded into the wells of a 1% SeaKem Gold Agarose gel in 0.5X Tris borate EDTA buffer. Electrophoresis was performed with the CHEF-DRIII apparatus (Bio-Rad).

For MLVA, five variable number of tandem repeats loci (VC0147, VC0436-7, VC1650, VCA0171, and VCA0283) were amplified using specific primer sets.[16] The PCR products from each isolate were purified using the NucleoSpin Extract II kit (Macherey-Nagel), and sequenced using the ABI 3130xl automated sequencer (Applied Biosystems, Foster City, CA). Sequence data of the repeats present in the five loci of each isolate were counted manually. The PFGE and MLVA data were evaluated with BioNumerics software version 7.5 (Applied Maths).[15] Clustering analysis was performed using the unweighted pair group method with arithmetic averages by a categorical similarity coefficient.

Climate data. The rainfall data of the Mandalay region from 2011 to 2015 were provided by the Department of Meteorology and Hydrology (Upper Myanmar), Myanmar.

RESULTS

In 2015, 1,617 suspected cholera cases were reported by the Public Health Laboratory in Mandalay. Of the total number of suspected cases, 331 (20.5%) were laboratory-confirmed cases (Table 1). The rainy season in Mandalay begins in May and extends to the end of October, generally with the heaviest rainfall in September/October. To obtain a correlation between cholera incidence and climatic factors, we compared the number of laboratory-confirmed cases each month with the rainfall. As shown in Figure 1, the number of confirmed cases rose sharply and coincided with the rainy season, except in 2012. Almost 90% of the confirmed cases occurred from May to October, and the number declined shortly afterward. No mortalities were reported throughout the year.

We selected and characterized 67 *V. cholerae* O1 isolates from May to September in 2015. All the isolates examined belonged to the serotype Ogawa, except for one isolate that belonged to the serotype Inaba. The use of a hexaplex PCR assay showed that all 67 isolates were toxigenic, possessing a set of virulence and regulatory genes, including *ctxA*, *zot*, *ace*, *tcpA*-El Tor biotype, *ompU*, and *toxR*. In addition, PCR genotyping and sequencing revealed that all isolates carried *ctxB*

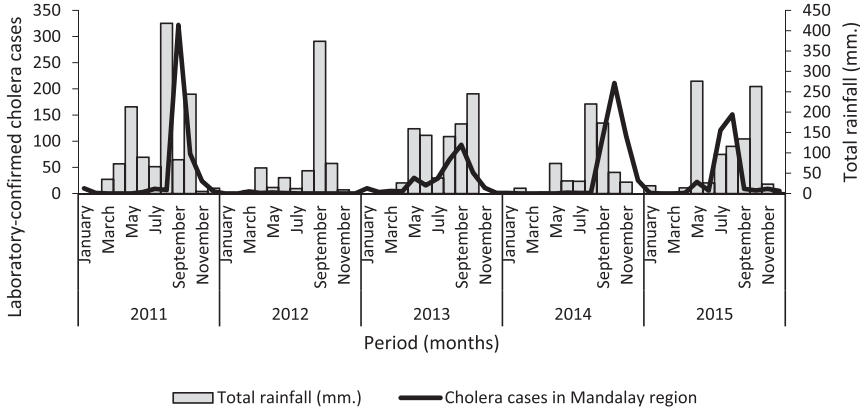

FIGURE 1. Number of cholera-confirmed cases and rainfall per month between January 2011 and December 2015 in Mandalay.

of the classical type (*ctxB*^{cla}), *rstR* of the El Tor biotype (*rstR*^{El}), and *tcpA* of the Haitian type (*tcpET*^{CIRS}) (Supplemental Figures 1–3).

PFGE fingerprints of *Not*I-digested genomic DNA differentiated the isolates into four pulsotypes, namely, Y12, Y15, Y16, and Y17; Y15 was the most frequent pulsotype, accounting for

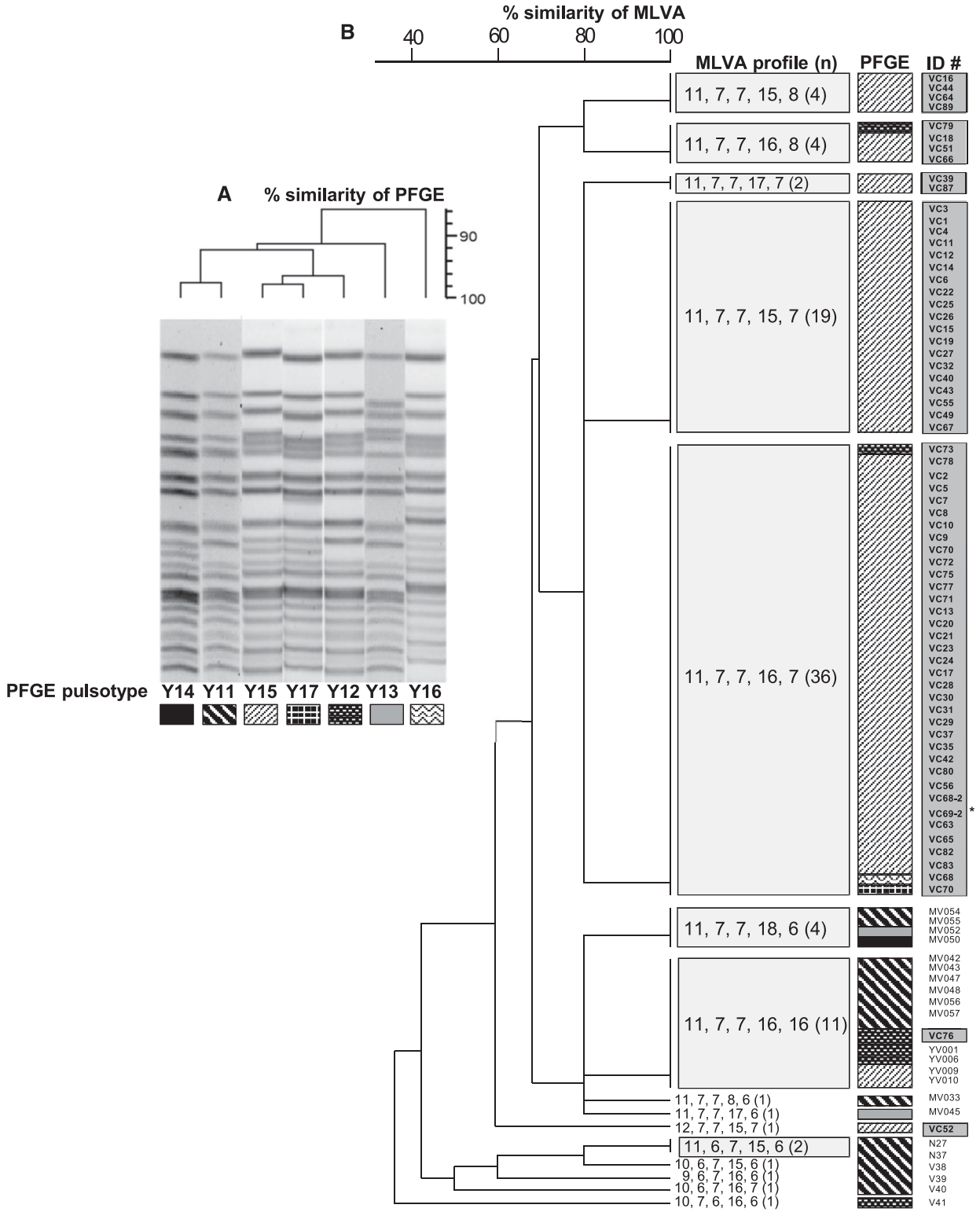

FIGURE 2. Clustering dendrogram of 89 *Vibrio cholerae* O1 isolates. Dendrogram shows the relationship between (**A**) seven pulsed-field gel electrophoresis (PFGE) and (**B**) 15 multiple-locus variable-number tandem repeat analysis (MLVA) profiles for the *V. cholerae* O1 isolates. The initial of ID# indicates the isolation place and period; VC, Mandalay from May to September 2015 (shown in dark gray boxes); MV, Mandalay in November 2014; YV, Yangon in December 2014; N and V, Yangon in June 2014. Asterisk (*) indicates serotype Inaba.

TABLE 2
Molecular profiles based on pulsotyping and MLVA of *Vibrio cholerae* O1 isolates, Mandalay and Yangon, 2014–2015

Isolation period	No. of cholera-confirmed cases	No. of isolates tested	Pulsotype (no. of isolates)	MLVA (no. of isolates)
Mandalay 2015				
May	22	2	Y12 (2)	11,7,7,16,7 (1); 11,7,7,16,8 (1)
June	6	4	Y15 (3)	11,7,7,16,7 (2); 11,7,7,15,7 (1)
			Y12 (1)	11,7,7,16,6 (1)
July	121	49	Y15 (48)	11,7,7,16,7 (26); 11,7,7,15,7 (17); 11,7,7,15,8 (2)
				11,7,7,16,8 (2); 12,7,7,15,7 (1)
			Y17 (1)	11,7,7,16,7 (1)
August	151	10	Y15 (9)	11,7,7,16,7 (6); 11,7,7,16,8 (1); 11,7,7,15,7 (1)
				11,7,7,15,8 (1)
			Y16 (1)	11,7,7,16,7 (1)
September	9	2	Y15 (2)	11,7,7,17,7 (1); 11,7,7,15,8 (1)
Mandalay 2014				
November	110	12	Y11 (9)	11,7,7,16,6 (6); 11,7,7,18,6 (2); 11,7,7,8,6 (1)
			Y13 (2)	11,7,7,17,6 (1); 11,7,7,18,6 (1)
			Y14 (1)	11,7,7,18,6 (1)
Yangon 2014				
June	–	6	Y11 (5)	11,6,7,15,6 (2); 10,6,7,15,6 (1); 9,6,7,16,6 (1); 10,6,7,16,7 (1)
			Y12 (1)	10,6,7,16,6 (1)
December	–	4	Y12 (2)	11,7,7,16,6 (2)
			Y15 (2)	11,7,7,16,6 (2)

MLVA = multiple-locus variable-number tandem repeat analysis.

92.5% (62 of 67) of the isolates (Figure 2A and Table 2). In addition, MLVA, based on the variation in the number of repeats at the five loci, yielded seven different types (Table 2). Thirty-six isolates (53.7%) exhibited an MLVA profile (11, 7, 7, 16, 7), and the remaining isolates also showed closely related profiles (Figure 2B).

The next 22 isolates of *V. cholerae* O1 from Mandalay and Yangon collected in 2014 were characterized to examine temporal trends. The 2014 isolates were all found to be toxigenic *V. cholerae* O1, biotype El Tor, serotype Ogawa, and carried genes *ctxB*^{cla}, *rstR*^{El}, and *tcpET*^{CIRS}. The Mandalay isolates (75%, 9 of 12) from November 2014 belonged to pulsotype Y11, which was identical to that of the Yangon isolates (83%, five of six) from June 2014. However, the pulsotype Y11 disappeared in 2015. In contrast, the pulsotypes of the four Yangon isolates from December 2014 (Y12 or Y15)

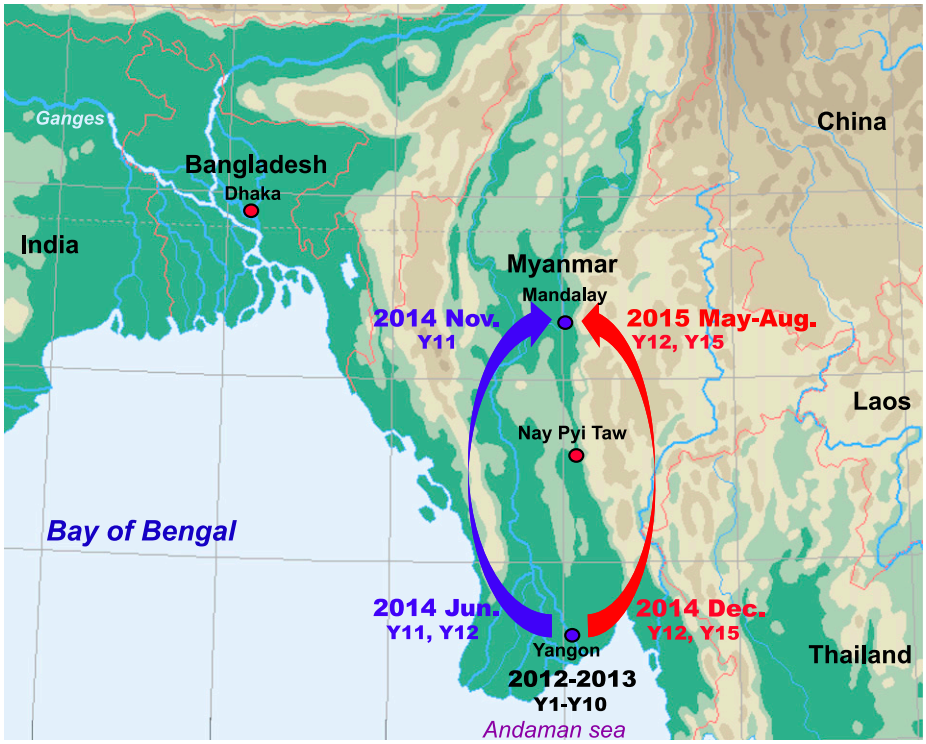

FIGURE 3. Cholera transmission within Myanmar inferred from this study. The arrows denote the transmission from Yangon to Mandalay. Cholera in Mandalay was related to the incidences in Yangon. Pulsotypes Y11 and Y12 were first found in Yangon in June 2014 and then found in Mandalay in November 2014. Pulsotypes Y12 and Y15 were found in Yangon in December 2014, and Y15 was dominant in an outbreak in Mandalay, 2015. This figure appears in color at www.ajtmh.org.

were identical to those isolated in Mandalay from May to September 2015.

DISCUSSION

This study revealed that the cholera outbreaks in Mandalay during the rainy season in 2015 were caused by the El Tor variant of *V. cholerae* O1 genetically identical or very similar (isolates showed ≥ 86% and ≥ 60% similarity by PFGE and MLVA, respectively). These data suggested that a clonal *V. cholerae* O1 exhibiting a single PFGE and MLVA type, spread in Mandalay during the rainy season in 2015. Although the number of isolates was limited, incidences of cholera in Mandalay were found to be related to those in Yangon. Pulsotypes Y11–Y17 reported in this study were not previously observed in Yangon from 2012 to 2013.[17] However, the MLVA profiles of pulsotypes Y11, Y12, and Y15 showed a linkage in the cholera incidences between the two sites from 2014 to 2015, implying that the pathogen had originated in Yangon and disseminated to Mandalay within a span of 6 months. Interestingly, Mandalay and Yangon are located 600 km apart, and Yangon is located in the southern part of the country, at a distance of 40 km from the Gulf of Martaban in the Andaman Sea (Figure 3).

The infection patterns of the cholera outbreaks across the most affected areas showed a single annual peak,[18] whereas those in the Bengal Delta region showed biannual peaks. Spring outbreaks usually occur in coastal areas, whereas fall outbreaks dominate inland regions.[19,20] The propagation of cholera outbreaks in the region from the coastal to the inland areas occurs through the premonsoon transmission cycle, whereas that from spring to fall takes place through the postmonsoon transmission cycle. The two cycles are distinctly different, with the former and latter being influenced by coastal and terrestrial hydroclimatic processes, respectively.[18,21,22] We observed that the distribution of cholera in Mandalay was closely associated with the rainy season. Mandalay has experienced at least four cholera outbreaks over the past 5 years (Figure 1). During the period 2011–2015, cholera incidence generally exhibited a single annual peak with an annual average of 312 cholera cases, whereas a small peak in cholera cases was observed in 2013 and 2015 at the first peak of rainfall. However, most cholera cases were found to occur during or after the initial second peak of rainfall, possibly because time was needed for the water sources to get contaminated before the initiation of outbreaks.[23] In addition, rainstorm and flood cause poor availability of safe water and destruction of sanitation infrastructures. The overpopulation after the monsoon floods may also facilitate the spatial transmission of cholera.[18,22] On the other hand, cholera cases in 2012 were unrelated to rainfall, because only eight laboratory-confirmed cholera cases were detected despite the heavy rainfall recorded. Further investigation to identify risk factors associated with cholera by epidemiological study, before and during the rainy season, would be crucial for countermeasure of cholera in Mandalay.

A genome-wide single-nucleotide polymorphism (SNP) analysis of seventh pandemic isolates performed by Mutreja et al.[24] revealed that the ongoing seventh pandemic was monophyletic and has spread around the world in at least three independent waves. All Mandalay isolates were categorized as Wave 3 isolates of the seventh cholera pandemic based on the CTX type estimated from the genotypic data and were similar to CIRS101, which was an atypical El Tor strain from Bangladesh harboring *tcpET*^{CIRS} (*tcpA* sequence of CIRS101 has single SNP at nucleotide position 266 [A→G], which differs from typical El Tor strains),[25] *ctxB*^{cla}, *rstR*^{El}, and other similar variants found in Nigeria, Pakistan, Afghanistan, South Africa, Sri Lanka, Vietnam, India, Bangladesh, and Thailand.[15,24–29] *Vibrio cholerae* O1, that exhibited other CTX types related to Wave 1 or Wave 2 isolates, was not found in Myanmar, at least since 2012. These data also support that *V. cholerae* O1 is circulating in multiple countries in Asia and beyond.

For the prevention of cholera in Mandalay, Myanmar in the future, improvement of sanitation conditions, sufficient supply of clean drinking water, and adequate knowledge about the mechanism of cholera dissemination from the seaside to inland area are essential.

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