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Dynamics of Cholera Epidemics in Haiti and Africa

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2 List of publications included in the thesis

Listed in order of appearance:

- 1 - **Moore, S**, N Thomson, A Mutreja, and R Piarroux. 2014. "[Widespread Epidemic Cholera Caused by a Restricted Subset of Vibrio Cholerae Clones.](#)" *Clinical Microbiology and Infection* 20 (5). European Society of Clinical Infectious Diseases: 373–79. doi:10.1111/1469-0691.12610.
- 2 - Baron, Sandrine, Jean Lesne, **Sandra Moore**, Emmanuel Rossignol, Stanislas Rebaudet, Pierre Gazin, Robert Barraïs, Roc Magloire, Jacques Boncy, and Renaud Piarroux. 2013. "[No Evidence of Significant Levels of Toxigenic V. Cholerae O1 in the Haitian Aquatic Environment During the 2012 Rainy Season.](#)" *PLOS Currents Outbreaks* Sept 13: 1–14. doi:10.1371/currents.outbreaks.7735b392bdc749baf5812d2096d331e.
- 3 - Rebaudet, Stanislas, Pierre Gazin, Robert Barraïs, **Sandra Moore**, Emmanuel Rossignol, Jean Gaudart, Jacques Boncy, Roc Magloire, and Renaud Piarroux. 2013. "[The Dry Season in Haiti: A Window of Opportunity to Eliminate Cholera.](#)" *PLOS Currents Outbreaks* June 10: 1–15. doi:10.1371/currents.outbreaks.2193a0ec4401d9526203af12e5024ddc.
- 4 - Miwanda, Berthe, **Sandra Moore**, Jean-jacques Muyembe, Georges Nguéfacktsague, Ickel Kakongo Kabangwa, Daniel Yassa Ndjakani, Ankur Mutreja, et al. 2015. "[Antimicrobial Drug Resistance of Vibrio Cholerae, Democratic Republic of the Congo.](#)" *Emerging Infectious Diseases* 21 (5): 847–51.
- 5 - **Moore, Sandra**, Berthe Miwanda, Adodo Yao Sadj, Hélène Thefenne, Fakhri Jeddi, Stanislas Rebaudet, Hilde de Boeck, et al. 2015. "[Relationship between Distinct African Cholera Epidemics Revealed via MLVA Haplotyping of 337 Vibrio Cholerae Isolates.](#)" *PLoS Neglected Tropical Diseases* 9 (6): e0003817. doi:10.1371/journal.pntd.0003817.
- 6 - Rebaudet, Stanislas, **Sandra Moore**, Anne-Cécile Normand, Lamine Koivogui, Eric Garnotel, Amara Jambai, and Renaud Piarroux. 2016. "[Direct Dried Stool Sampling on Filter Paper for Molecular Analyses of Cholera.](#)" *Am J Trop Med Hyg.* 95 (1): 251–52. doi:10.4269/ajtmh.16-0246a.
- 7 - **Moore S**, Dongdem AZ, Opare D, Cottavoz P, Fookes M, Sadj AY, Dzotsi E, Dogbe M, Jeddi F, Bidjada B, Piarroux M, Valentin OT, Glèlè CK, Rebaudet S, Sow AG, Constantin de Magny G, Koivogui L, Dunoyer J, Bellet F, Garnotel E, Thomson N and Piarroux R. [Dynamics of Cholera Epidemics in West Africa](#). Unpublished – submitted to *The Lancet*.

3 Abstract

Cholera is an acute diarrheal disease caused by consumption of water or food contaminated with toxigenic *Vibrio cholerae*. Over 200 serogroups of *V. cholerae* have been identified, although only O1 and O139 cause cholera epidemics. The bacteria associate with a variety of flora and fauna, and correlations have been observed between sea temperatures, rainfall and cholera outbreaks. Thus, according to the "cholera paradigm", the disease is contracted by exposure to environmental reservoirs of *V. cholerae*, with outbreaks driven directly by climatic factors. However, as recent findings argue against this dogma, we aimed to elucidate the dynamics of cholera outbreaks in three global foci: Haiti, Democratic Republic of the Congo (DRC) and West Africa. We combined spatiotemporal analysis of epidemics with genetic assessment of *V. cholerae* isolates. A literature review was also performed to examine the clonal nature of cholera epidemics, with the example of cholera in the Americas. In Haiti, we assessed whether outbreak re-emergence during the rainy season was due to toxigenic *V. cholerae* O1 strains that have settled into the aquatic environment. Instead, we found that the re-emergence of outbreaks was likely due to persisting outbreaks during the dry season that were insufficiently controlled, rather than an environmental reservoir of *V. cholerae* O1. In the DRC, analysis of *V. cholerae* isolates (1997-2012) revealed increasingly complex resistance to various antibiotics. Our study showed that the 2011/2012 epidemic that rapidly crossed the country was due to clonal expansion of a particular isolate genotype, which was already present in the DRC since 2009. We also found that the environmental isolates were largely unrelated to the epidemic isolates. In West Africa, our study revealed that Accra, Ghana was the hotspot of cholera in the entire region of West Africa, west of Nigeria. The Accra water network likely played a role in rapid diffusion of *V. cholerae* throughout the city. Cholera outbreaks spread from Accra into other countries in a wave-like fashion. Distinct outbreaks were linked via migration of at-risk populations, such as certain fishermen. In conclusion, our global reflection of cholera epidemics in these three distinct foci provides a coherent vision of the mechanisms of cholera emergence and diffusion. Our findings also raise serious doubts that a permanent reservoir of *V. cholerae* in the aquatic environment plays a significant role in cholera prevalence.

4 Résumé

Le cholera est une maladie diarrhéique aiguë due à la consommation d'eau ou d'aliments contaminés par des souches toxigéniques de *Vibrio cholerae*. Plus de 200 sérogroupes de *V. cholerae* ont été identifiés, mais seuls les sérogroupes O1 et O139 provoquent des épidémies. La bactérie peut être associée à une flore et une faune diverse et des corrélations ont été observées entre la température de la mer, la pluie et les épidémies de cholera. Ainsi, selon le "paradigme du choléra", la maladie est provoquée par une exposition à un réservoir environnemental de *V. cholerae* avec des épidémies directement modulées par des facteurs environnementaux. Cependant, comme divers arguments plaident contre ce dogme, nous avons voulu élucider les mécanismes de la dynamique des épidémies de cholera dans trois foyers situés en Haïti, en République Démocratique du Congo (RDC) et en Afrique de l'Ouest. Nous avons associé une analyse temporo-spatiale des épidémies à une étude génétique des isolats de *V. cholerae*. Une revue de la littérature a aussi été réalisée pour examiner la nature clonale des épidémies de choléra, en prenant l'exemple des Amériques. En Haïti, nous avons cherché à savoir si les épidémies actuelles étaient dues à des souches toxigéniques de *V. cholerae* O1 durablement implantées dans l'environnement aquatique. En fait, nous avons trouvé qu'en Haïti, les réémergences des épidémies de choléra durant la saison des pluies étaient plus probablement dues à des épidémies persistantes insuffisamment contrôlées plutôt qu'à la présence d'un réservoir environnemental de *V. cholerae* O1. En RDC, l'analyse d'isolats de *V. cholerae* isolées entre 1997 et 2012 a montré une complexification progressive de la résistance à divers antibiotiques. Notre étude a montré que l'épidémie de 2011/2012 qui a rapidement traversé la RDC était due à une expansion clonale d'un génotype particulier qui était déjà présent dans le pays depuis au moins 2009. Nous avons aussi trouvé que les isolats environnementaux étaient distincts des isolats épidémiques. En Afrique de l'Ouest, notre étude a révélé qu'Accra, la capitale du Ghana, était le principal foyer de choléra pour l'ensemble des pays d'Afrique de l'Ouest situés à l'Ouest du Nigeria. Le réseau d'eau d'Accra a probablement joué un rôle dans la propagation rapide de *V. cholerae* vers la majorité des quartiers de la ville. Les épidémies de choléra ont diffusé vers les autres pays sous la forme de vagues épidémiques et plusieurs épidémies ont

été liées à la migration de populations à risque comme certains pêcheurs. En conclusion, notre réflexion globale sur les épidémies de choléra dans ces trois foyers distincts nous donne une vision cohérente des mécanismes d'émergence et de diffusion du choléra. Nos résultats soulèvent des doutes sérieux sur le fait qu'un réservoir aquatique permanent de *V. cholerae* puisse jouer un rôle significatif dans la prévalence du choléra.

5 Introduction

5.1 Cholera overview

Cholera is an ancient disease, and its exact origins remain unknown. The first modern cholera pandemic was reported in 1817. Since this time, seven pandemics have circled the globe and cholera has eventually affected every continent (Echenberg 2011). Although the disease has since been virtually eradicated in many once heavily burdened nations, including Europe and the United States, cholera continues to cause anguish in many countries primarily in Southeast Asia, Sub-Saharan Africa and more recently Haiti (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

Cholera is an acute diarrheal disease caused by consumption of water or food contaminated with toxigenic forms of the Gram-negative bacterium *Vibrio cholerae* O1 (and less often O139) (J. B. Kaper, Morris, and Levine 1995). Cases often present with mild symptoms, although some patients experience profuse watery diarrhea and vomiting. Due to the short incubation period of the bacterium, however, severe cases who are left untreated experience a massive loss of bodily fluids, which can quickly provoke dehydration, hypovolemic shock and eventual death (J. B. Kaper, Morris, and Levine 1995; Sack et al. 2004a). The disease is easily treated with oral rehydration therapy, and antibiotics are required for critical cases (Centers for Disease Control and Prevention 2013). However, in settings where resources are limited, accessing (or administering) these simple treatments can be a major challenge.

The major risk factor associated with cholera is limited access to clean drinking water and proper sanitation. As a result, specific situations in which people live in such suboptimal sanitary conditions have also been considered related risk factors, such as severe overcrowding, peri-urban slums with limited basic health structures, temporary camps for refugees or internally displaced people, humanitarian or environmental crises and civil disruption (“Cholera Fact Sheet (number 107)” 2015). Thus, cholera outbreaks are also viewed as an indicator of hindered social development.

Currently, the scientific community remains divided on the life cycle of the bacterium and the mechanisms of cholera emergence and diffusion. In the early 1800s, cholera was believed to spread via miasma: pollution or “bad air”. It was not until the London cholera epidemic of 1854, when John Snow showed that residents of the overpopulated city contracted cholera by consuming well water contaminated with human sewage. That same year, Italian anatomist Filippo Pacini, isolated the bacterium when cholera struck Florence, although his results were largely ignored. It was not until 1884, that Robert Koch identified the pathogen as *Vibrio cholera* O1 during an outbreak in Egypt. The bacterium only causes disease in humans, which were considered to represent the main reservoir of the bacterium. At this time, it was believed that the disease spread from person-to-person via transitory vectors such as water and food contaminated with the toxigenic bacterium (Echenberg 2011).

However, when *V. cholerae* species were found in the Chesapeake Bay, it was suggested that the bacterium was a potential autochthonous inhabitant of brackish water and estuarine ecosystems (Morris 2003; Colwell, Kaper, and Joseph 1977). Over 200 serogroups of *V. cholerae* have since been identified in environmental samples, although only the serogroups O1 and O139 (a derivative of O1) cause cholera epidemics (Chun et al. 2009; Mutreja et al. 2011; Albert et al. 1993; Chatterjee and Chaudhuri 2003; Siddique et al. 1996; Shimada et al. 1994). The bacteria have been found associated with a wide range of flora and fauna, and copepods have been considered the most significant natural reservoir of *V. cholerae* strains (Vezzulli et al. 2010). Furthermore, certain correlations have been observed (especially in Bangladesh) between sea surface temperatures, copepod blooms, rainfall events and cholera outbreaks (Koelle 2009a; Huq et al. 2005; Constantin de Magny et al. 2008; A. Jutla et al. 2011a). The culmination of such observations ultimately led to the establishment of the “cholera paradigm”, which claims that cholera is contracted by exposure to environmental reservoirs of toxigenic *V. cholerae* and that outbreaks are driven directly by environmental factors (Colwell 1996).

It has been widely suggested that cholera is endemic in Southeast Asia, the majority of Sub-Saharan African countries and more recently Haiti, where the disease ebbs and flows supposedly in parallel with changes in ecological and climate conditions favorable to the growth of *V. cholerae* in the aquatic environment. Many scientific studies and some public health policies pivot on the assumption that aquatic reservoirs of *V. cholerae* are at the root of current cholera epidemics.

However, mounting evidence argues against this dogma. Whole-genome sequence-based phylogeny of a panel of seventh pandemic isolates has shown that the current pandemic is monophyletic and originated from a single clone, with a common ancestor dating to the 1950s. This study also showed that new epidemics paralleled major events of human migration. Environmental strains were also found to be largely unrelated to epidemic isolates (Mutreja et al. 2011). Furthermore, in contrast to the cholera paradigm, the majority of outbreaks in Sub-Saharan Africa between 2009 and 2011 occurred in non-coastal regions located over 100 km from coasts or estuaries (Rebaudet, Sudre, et al. 2013b; Rebaudet, Sudre, et al. 2013a). Finally, the existence of a perennial aquatic reservoir of toxigenic *V. cholerae* O1 has yet to be demonstrated, despite numerous surveys of environmental samples during which studies mostly identified non-toxigenic non-O1 strains. Overall, novel findings rather suggest that humans may serve as the permanent reservoir of the cholera-causing *V. cholerae*.

Cholera research and public health policy thus appear to be at a crossroads. Clearly, whether cholera outbreaks are triggered from an environmental or mobile human reservoir implies drastically different approaches to control the disease. Each of the seven cholera pandemics has radiated from Southeast Asia, where *V. cholerae* strains have continued to evolve and the dynamics of cholera appear to differ from those in other affected areas of the world. Therefore, to develop effective disease monitoring systems, efficiently control the spread of epidemics, and eventually prevent future cholera outbreaks, it is critical to understand the underlying mechanisms involved in both the emergence and diffusion of cholera in a region-specific manner.

5.2 *V. cholerae* classification

V. cholerae is scientifically classified as follows:

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Vibrionales

Family: Vibrionaceae

Genus: *Vibrio*

Species: *V. cholerae*

The *V. cholerae* species comprises over 200 serogroups, for which the nomenclature is based on O-antigen structure (Shimada, 1994). Only the serogroups O1 and O139 (a derivative of O1) cause cholera epidemics (Chun et al. 2009; Mutreja et al. 2011; Albert et al. 1993; Chatterjee and Chaudhuri 2003; Siddique et al. 1996); the latter is primarily restricted to Asia (Chun et al. 2009).

Pandemic *V. cholerae* O1 strains can be divided into two distinct biotypes: 'classical' and 'El Tor'. The classical biotype was responsible for at least the second and sixth pandemics (and is presumed to be the cause of all pandemics prior to the current seventh pandemic) (Kaper, Morris, and Levine 1995; Karaolis, Lan, and Reeves 1995; Devault et al. 2014). An independent strain lineage of *V. cholerae* O1 El Tor is responsible for the seventh pandemic, which started in Sulawesi in 1961 (Kaper, Morris, and Levine 1995; Safa, Nair, and Kong 2009).

Assessment of seventh pandemic strains has revealed the emergence of El Tor variants termed "atypical El Tor" and "hybrid", which are characterized by the rearrangement of classical and El Tor CTXΦ genes (Safa, Nair, and Kong 2009). Mutreja et al., have rather suggested to utilize the nomenclature wave I, II, and III referring to the successive evolution of this clonal population (Mutreja et al. 2011).

The O1 serogroup is further categorized into three serotypes: Ogawa, Inaba and the much rarer Hikojima (a variant of the Ogawa serotype). Ogawa and Inaba have been detected in both El Tor and classical strains (Stroeher et al. 1992). Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen (Sakazaki and Tamura 1971; Redmond, Korsch, and Jackson 1973), whereas Inaba strains express only the A and C antigens. Specific mutation of the *rfbT* gene causes conversion from Ogawa to Inaba (Stroeher et al. 1992).

5.3 Infection and clinical aspects of cholera

5.3.1 Infection

The disease is contracted by ingesting food or water contaminated with pathogenic forms of *V. cholerae* (Kaper, Morris, and Levine 1995). According to Cash et al., a minimum *V. cholerae* inoculum of 10^4 bacteria is required to develop symptoms of cholera (i.e., provoke diarrhea in healthy volunteers with neutralized gastric acid) (Cash et al. 1974). The bacterium has a short incubation period of two hours to five days (“Cholera Fact Sheet (number 107)” 2015). Once the bacterium is ingested, the bacteria colonize the small intestine, mediated by toxin coregulated pilus (TCP) (Herrington et al. 1988). Driven by the CTX, the bacteria produce and release the cholera toxin complex, which is comprised of one A subunit and five B subunits. The toxin then binds (via the B subunits) and is internalized by epithelial cells, where the A subunit indirectly induces constitutive cyclic AMP production (Gill and Kings 1975; D M Gill 1976b; Cassel and Pfeuffert 1978; D Michael Gill and Meren 1978). This causes secretion of water and electrolytes into the lumen of the small intestines and eventually provokes severe diarrhea and subsequent dehydration (Cassel and Pfeuffert 1978; Gill and Meren 1978; Kaper, Morris, and Levine 1995).

5.3.2 Clinical symptoms, diagnosis and treatment

Although the infection is often mild or asymptomatic, some patients present with profuse watery diarrhoea and vomiting. Dehydration triggers cramps throughout the

body. The patient's skin becomes cold and coloration becomes black and blue (Kaper, Morris, and Levine 1995). However, due to the short incubation period of the bacterium, severe cases who are left untreated experience a massive loss of bodily fluids that can reach one liter per hour in adults, which can thereby quickly provoke dehydration, hypovolemic shock and eventual death. Cholera is laboratory diagnosed when culture samples test positive for the bacterium *V. cholerae* O1 or O139 (Sack et al. 2004a).

The disease is treatable with oral rehydration therapy, while antibiotics are necessary to care for critical cases who are moderately dehydrated and continue to pass a large volume of stool during rehydration treatment (Centers for Disease Control and Prevention 2013). Antibiotics assist in reducing the volume of stool released, the duration of diarrhea and the duration of positive bacterial cultures (Greenough III et al. 1964; Lindenbaum, Greenough, and Islam 1967; Rahaman et al. 1976). Various antibiotics have been proposed for cholera therapy, including erythromycin, ampicillin, tetracycline, ciprofloxacin and azithromycin (Roy et al. 1998; Kaushik et al. 2010; Khan et al. 1996; Saha et al. 2016). The choice of treatment primarily depends on the antibiotic susceptibility pattern of the responsible strains, which greatly varies from one locale to another (Materu et al. 1997).

5.3.3 Cholera risk factors

The major risk factor of cholera is limited access to both potable water, basic health structures and proper sanitation facilities, which is often the case for people living in conditions of severe overcrowding due to humanitarian or environmental crises ("Cholera Fact Sheet (number 107)" 2015). Consumption of raw or undercooked fish as also been occasionally linked with contracting the disease (CDCP 2014).

5.3.4 Prevention

The two WHO pre-qualified oral cholera vaccines are Dukoral and Shanchol. Dukoral is a monovalent inactivated vaccine based on formalin and heat-killed whole cells of *V. cholerae* O1 (classical and El Tor biotypes) and recombinant B-subunit of the cholera toxin. Dukoral triggers the production of antibacterial and antitoxin antibodies. The vaccine was developed in Sweden and first licensed in 1991; the current manufacturer is Crucell (the Netherlands). According to the manufacturer, primary immunization for individuals two years of age and older consists of two oral doses given > one week apart. Dukoral is stored at 2-8°C (Martin et al. 2014; WHO 2010). A review of vaccination campaigns from 1997 to 2014 show that delivery cost per fully immunized person ranged from \$0.53 USD to \$3.66 USD (Martin et al. 2014).

The initial trial conducted in Bangladesh to test Dukoral protective efficacy against El Tor and classical cholera combined revealed disparities in protection between children and the general population. In children (2-15 years) and women (over 16 years) protection was as follows: 85% (first 4–6 months after vaccination), 62% (after 1 year), 58% (after two years) and 18% (after three years). Protection among children aged 2–5 years was found to be 100% (4–6 months), 38% (after 1 year), 47% (after two years) and 0% (after three years) (Clemens et al. 1990).

Shanchol is a bivalent inactivated whole-cell vaccine based on serogroups O1 (classical and El Tor biotypes) and O139. The vaccine is based on a reformulated version of the non-WHO-qualified vaccine mORC-Vax (Vabiotech, Viet Nam). Shanchol was licensed in 2009 by the manufacturer Shantha Biotechnics Ltd in India. Shanchol was prequalified by the WHO in 2011. According to the manufacturer, Shanchol should be administered in two liquid doses, two weeks apart (for individuals one year of age and over). A booster dose is recommended after two years. Shanchol is also stored at 2-8°C, but unlike Dukoral, Shanchol does not require a buffer (Martin et al. 2014; WHO 2010). From 2011 to 2014, vaccine delivery costs ranged from \$1.13 USD to \$3.99 USD per fully immunized person (Martin et al. 2014). A randomized, placebo-controlled trial in India showed that Shanchol conferred 67% protective against cholera within

two years of vaccination, 66% at three years, and 65% at five years (Sur et al. 2009; Sur et al. 2011; Bhattacharya et al. 2013).

Many scientists and public health officials have however expressed concerns regarding oral cholera vaccines, due to cost, feasibility of carrying out large campaigns, community opinion of oral vaccines and the danger of diverting resources from other more effective interventions (Chaignat 2008). Therefore, vaccination programs should be designed based on thorough epidemiological studies and microbiological data to specifically and accurately target high-risk populations. Moreover, vaccination campaigns are most effective when launched in parallel with public awareness campaigns, water chlorination, and improvements in sanitation and access to clean water (WHO 2010).

It should be mentioned that the USA and Europe were rescued from the cholera burden following major public health and sanitation reform, in which improved piped water systems and sanitary facilities brought an end to the cholera plague without a single cholera vaccine. Access to clean water via improved water network systems and sanitary facilities therefore remains the most effective means of preventing cholera outbreaks (Fee and Brown 2005; Beau De Rochars et al. 2011; Rao 1992).

5.4 Ecology and life cycle of *V. cholerae*

Ever since *V. cholerae* species were found in the Chesapeake Bay, it was suggested that the bacterium was an autochthonous inhabitant of brackish water and estuarine ecosystems (Morris 2003). Certain forms of the bacterium are either found as free-swimming bacilli or associated with a wide range of flora and fauna, including cyanobacteria, phytoplankton, water hyacinths, free-living amoebae, copepods, blue crabs, marine bivalves as well as the intestines of certain fish, dolphins and aquatic birds (Vezzulli et al. 2010). Among these potential vectors, copepods are considered the most significant natural reservoir of *V. cholerae* strains (Vezzulli et al. 2010). Laboratory microcosm experiments have demonstrated that salinity levels of 15‰,

water temperatures ranging from 25°C to 30°C, and an alkaline pH of 8.5 are conducive to *V. cholerae* growth and attachment to copepods (Huq et al. 1984). These observations ultimately led to the establishment of the "cholera paradigm", which claims that cholera is contracted by exposure to environmental reservoirs of toxigenic *V. cholerae* and that outbreaks are driven directly by environmental factors (Colwell 1996).

According to this hypothesis, during stressful environmental conditions, *V. cholerae* strains transition into a viable but non-culturable (VBNC) state (Colwell et al. 1985). Strains then revert into a culturable and infectious form when water conditions become more suitable, including proper nutrients, salinity, pH and temperature (Colwell et al. 1985; Ravel et al. 1995).

Studies conducted in coastal Asia, the origin of all seven cholera pandemics, have revealed strong links between cholera, aquatic environments and climate. In the Bay of Bengal, the emergence of cholera outbreaks are associated with fluctuations in various seasonal events such as rainfall, river discharge, and increases in sea level and sea surface temperature (Koelle 2009a; Huq et al. 2005; Constantin de Magny et al. 2008). In this region, increased water temperatures driven by climatic events and subsequent plankton blooms have been shown to correlate with cholera incidence (Colwell 1996; Jutla et al. 2011a).

5.5 The seventh cholera pandemic in Haiti and Africa (Democratic Republic of the Congo and West Africa)

5.5.1 Cholera in Haiti

In 2010, a cholera epidemic struck Haiti for the first time since at least one hundred years (Jenson and Szabo 2011). From 2010 to the end of 2014, a total of 718,328 cases and 8,642 cholera-related deaths have been reported ("World Health Organization. Cholera, Number of Reported Cases (data by Country)" 2016). Initially, some experts suggested that the epidemic was triggered by climatic events, claiming that a rise in

the temperature and salinity of the Haitian waters induced the growth of *V. cholerae* presumably residing dormant in the environment (Knox 2010). Epidemiological data later demonstrated an exact spatio-temporal correlation between the arrival of United Nations Nepalese peacekeepers in Haiti and the first reported cholera cases in Meille, a small village 2 km south of Mirebalais (Piarroux et al. 2011). Microbiological analysis also demonstrated that the Haitian *V. cholerae* isolates were almost identical to isolates collected in Nepal a few weeks prior, thereby strongly suggesting that the Haitian strains were very recently imported from Nepal (Hendriksen, Price, and Schupp 2011; Frerichs et al. 2012b).

During the following years, cholera was disseminated throughout almost every region of Haiti, including the most remote rural areas. Case numbers markedly decreased during the dry seasons (Piarroux et al. 2011; Gaudart et al. 2013), but outbreaks reignited during rainy seasons with higher peaks after major climatic events, such as Hurricane Tomas in November 2010 and Hurricanes Isaac and Sandy in August and October 2012, respectively. As a result, cholera outbreaks continue to persist in the country to this day (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

5.5.2 Cholera in Africa

The seventh pandemic first reached Africa in 1970 with Guinean students traveling to Conakry, Guinea from Crimea, where an epidemic was ongoing. Once cholera arrived in Guinea, the disease spread throughout the continent, often following travel routes via sea and land (Echenberg 2011). Cholera incidence in Africa has increased since the beginning of the seventh pandemic (**Figure 1**). In contrast, the corresponding case fatality rate has gradually decreased from 9.1 (1991) to 1.79 (2014), in which deaths are often associated with remote rural areas and delayed treatment (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

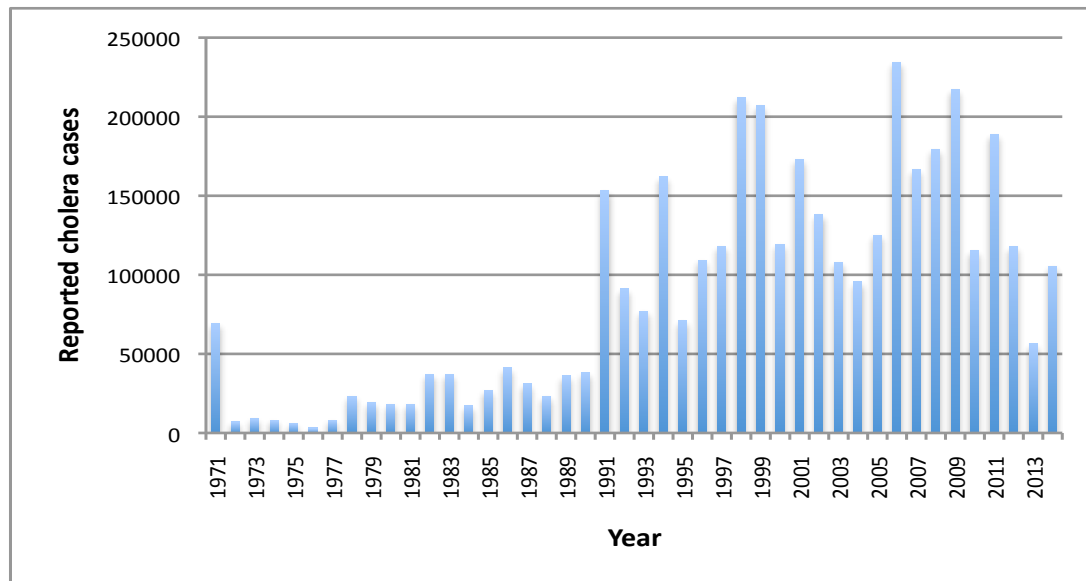


Figure 1. Annual cholera cases reported in Africa from 1971 to 2014. (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

Cholera outbreaks in Africa has often been linked with conflict, religious gatherings, and trade of slaves and African goods, such as sugar, rubber, ivory and gold. Large epidemics often occurred following war or major humanitarian conflicts. For example, the Rwandan Genocide in 1994 sparked a massive cholera epidemic in the African Great Lakes Region, in eastern Zaire (modern Democratic Republic of the Congo (DRC)), where unsanitary refugee camps in and around Goma led to a humanitarian catastrophe when over 50,000 people died, largely attributed to cholera (Echenberg 2011).

Current cholera cases reported from Africa have primarily clustered in the African Great Lakes Region of eastern DRC and West Africa, including the Lake Chad region (Nkoko et al. 2011; Oger and Sudre 2011) (**Figure 2**).

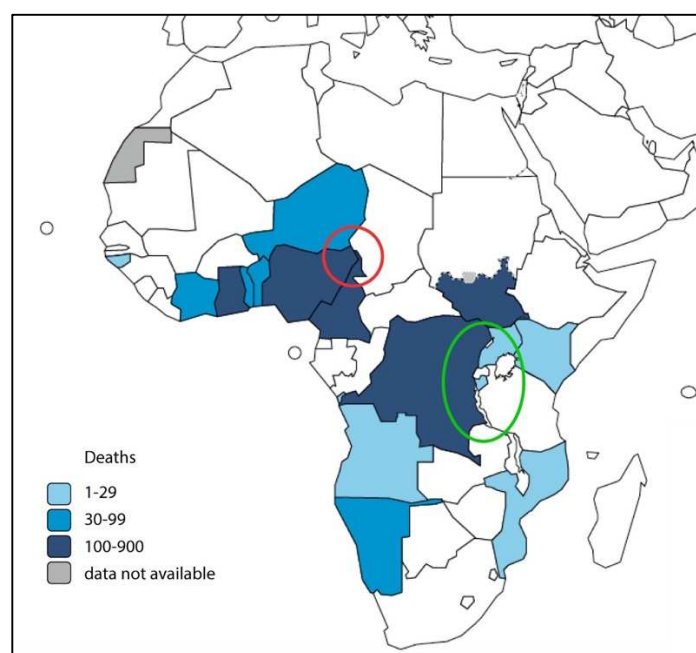


Figure 2. African countries affected by cholera in 2014 and corresponding cholera-related deaths. The Lake Chad Basin is indicated in the red circle, and the African Great Lakes Region is indicated in the green oval. Modified image extracted from the WHO - Weekly Epidemiological Records, 2014 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

5.5.2.1 *The Democratic Republic of the Congo*

In the African Great Lakes Region, the majority of cholera related cases (66%) and deaths (71%) reported between 2002 and 2008, occurred in the eastern DRC *provinces* of Katanga, South Kivu and North Kivu (Nkoko et al. 2011). Proximity to lakes has been shown to be a significant risk factor of cholera in the area (Bompangue et al. 2008; Bompangue et al. 2009); it has thus been hypothesized that the African Great Lakes Region may serve as a perennial aquatic reservoir of *V. cholerae* (Shapiro et al. 1999).

In contrast eastern *provinces*, the western DRC *provinces*, including the capital and largest city, Kinshasa, have only been intermittently affected (Nkoko et al. 2011; Bompangue et al. 2012). However, in 2011, an epidemic spread violently along the Congo River, reaching Kinshasa in only 130 days. This epidemic spilled over into 2012

and cause major outbreaks in Kinshasa for the first time in ten years (Bompangue et al. 2012).

5.5.2.2 West Africa

Ever since the seventh pandemic first reached Africa in 1970, cholera cases have been reported every year in West Africa. In 2014, the WHO reported a total of 30,475 cholera cases in the 11 countries along the Atlantic Coast from Benin to Mauritania (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Furthermore, cholera incidence has increased in recent years in certain countries, such as Ghana. According to the WHO, the majority of countries in West Africa are considered cholera-endemic; although the underlying mechanism behind cholera emergence and diffusion remain unclear.

6 Research Question

Following the establishment of the “cholera paradigm”, many current studies and public health policies to combat cholera epidemics pivot on the assumption that the causative agent is omnipresent in the aquatic ecosystem and that outbreaks are triggered by climatic conditions that favor proliferation of *V. cholerae* (Colwell 1996). These reports deducing that climate change represented a greater threat to humans than proper sanitation have also turned the focus away from the provision of clean water and adequate sewage disposal. However, recent evidence contradicts this dogma and rather suggests that human populations represent the principal reservoir of the toxigenic *V. cholerae* O1 bacterium and that drinking water (or food sources) play a role in cholera epidemics as a transient vector.

Using whole-genome sequencing-based phylogeny, Mutreja *et al.* have recently shown that the seventh pandemic has spread from the Bay of Bengal in at least three independent waves with a common ancestor in the 1950s. Their findings suggest that

cholera epidemics spread in parallel with population movement. Furthermore, they showed that the strains responsible for cholera epidemics exhibit a specific genome, disparate from the genetically diverse *V. cholerae* strains resident of the environment. Most, if not all, studied environmental *V. cholerae* strains lacked the cholera toxin and have never been shown to cause large epidemics (Mutreja et al. 2011).

The 2010 cholera epidemic in Haiti was caused by the imported of *V. cholerae* O1 from Nepal. As cases continue to be reported over five years later with seasonal exacerbations during the rainy seasons, many experts claim that the cholera-causing *V. cholerae* O1 strains have now settled in the Haitian coastal waters and that current outbreaks in Haiti are directly caused by exposure to this environmental reservoir (Alam et al. 2014). However, a perennial aquatic reservoir of toxigenic *V. cholerae* O1 in Haiti has yet to be identified. Surveys of environmental samples have mostly identified non-toxigenic non-O1 strains. A few studies have managed to isolate O1, although with on-going cholera cases nearby, patient derived contamination cannot be excluded (Rebaudet and Piarroux 2015).

In Africa, the dynamics of cholera epidemics fail to correlate with the concept that outbreaks are triggered by climatic conditions and an environmental reservoir of *V. cholerae*. The majority of cholera cases in Africa have been unassociated with the coastal environment (Rebaudet, Sudre, et al. 2013a). Indeed, the largest recorded epidemic in Africa struck the landlocked country of Zimbabwe in 2008-2009, which was responsible for 128,208 cases and 5,634 deaths during the two-year period and occurred independent of any coastal waters, lakes or rivers (WHO 2009; "World Health Organization. Cholera, Number of Reported Cases (data by Country)" 2016). Although a correlation has been periodically observed between rainfall events and cholera incidence, epidemics have also arisen during the dry season. Indeed, outbreaks have been reported in Mali during periods of drought and famine (Tauxe et al. 1988). Dry season outbreaks appear to emerge rather due to overcrowding and subsequent contamination of the last available water source or famine-triggered weakened immunity of the populations (Tauxe et al. 1988). Likewise, a permanent reservoir of *V. cholerae* in the aquatic environment has yet to be described in Africa.

Thus, recent data question the validity of the “cholera paradigm”, at least in the context of cholera epidemics in Haiti and Sub-Saharan Africa. Whether cholera outbreaks are due to an environmental or human reservoir would require markedly different public health strategies to monitor and control the disease.

7 Aims and objectives

The aim of this thesis was to gain a clear understanding of the mechanisms involved in both the emergence and diffusion of cholera epidemics.

To address this question, we first conducted a literature review assessing the clonal nature of cholera epidemics. Integrated studies of cholera epidemics in Haiti, the DRC and West Africa were then performed to determine whether cholera epidemics in these regions arise due to a permanent environmental reservoir of *V. cholerae* or rather strains that circulate among human populations. We present the results via our publications corresponding to each topic.

In **Chapter 8**, we present the article **Widespread epidemic cholera caused by a restricted subset of *Vibrio cholerae* clones**. With this review, we examined the clonal nature of cholera epidemics, with the example of the recent history of cholera in the Americas. Epidemiological data and genome sequence-based analysis of *V. cholerae* isolates demonstrated that the cholera epidemics of the 1990s in South America were triggered by the importation of a pathogenic *V. cholerae* strain that gradually spread throughout the region until local outbreaks ceased in 2001. Latin America remained almost unaffected by the disease until a new toxigenic *V. cholerae* clone was imported into Haiti in 2010.

We then assess the dynamics of the Haitian cholera epidemic in **Chapter 9**. In the article **No Evidence of Significant Levels of Toxigenic *V. cholerae* O1 in the Haitian Aquatic Environment During the 2012 Rainy Season**, we aimed to determine whether toxigenic *V. cholerae* O1 strains have settled into the local aquatic environment. With our collaborators, we examined a series of environmental water samples collected from several areas in the West and Artibonite Departments, in areas heavily affected by cholera outbreaks. We found that the re-emergence of cholera outbreaks in Haiti during the rainy seasons was likely due to persisting outbreaks that were insufficiently controlled during the dry periods, which is addressed in a subsequent report: **The dry**

season in Haiti: a window of opportunity to eliminate cholera. In this study, we investigated cholera dynamics and control strategies during the dry season.

In **Chapter 10**, we analyze the dynamics of cholera epidemics in the DRC, with a focus on both clinical and environmental *V. cholerae* isolates. As the phylogeny study conducted by Mutreja *et al.* lacked strains from Africa, we examined a comprehensive panel of *V. cholerae* isolates from the DRC and other African countries. We assessed the characteristics of strain fluctuation in the DRC over an extended period of time. First, together with our collaborators at the University of Kinshasa, we focused on the patterns of antibiotic resistance in the article **Antimicrobial Drug Resistance of *Vibrio cholerae*, Democratic Republic of the Congo**. In this study we analyzed 1,093 *V. cholerae* isolates collected from 1997 to 2012. In a subsequent analysis, we applied MLVA (Multi-Locus Variable Number Tandem Repeat Analysis) typing of 337 *V. cholerae* isolates from recent cholera epidemics in the DRC, Zambia, Guinea and Togo. In the publication **Relationship between Distinct African Cholera Epidemics Revealed via MLVA Haplotyping of 337 *Vibrio cholerae* Isolates**, we aimed to investigate the relationship between outbreaks in the DRC and West Africa. MLVA is a highly discriminatory technique that allows for the differentiation between various cholera strains isolated during an epidemic. Using specialized algorithms, MLVA data can further be applied to infer genetic relatedness and therefore provide insight into the mechanism by which strains evolve and the bacterial diffusion pattern over the course of a single epidemic (Danin-poleg *et al.* 2007; Olsen *et al.* 2009).

The underlying mechanisms of cholera epidemics in West Africa is examined in **Chapter 11**. First we investigated the link between the epidemics that almost simultaneously struck Guinea and Sierra Leone in 2012. Field investigation reports had strongly suggested that the epidemic in Guinea was due to importation of a *V. cholerae* clone imported from Sierra Leone. In the Letter to the Editor titled **Direct Dried Stool Sampling on Filter Paper for Molecular Analyses of Cholera**, we analyzed the genetic relationship between *V. cholerae* isolates from Guinea and Sierra Leone using MLVA to determine the origin of these epidemics. Next, we extend our investigation of cholera epidemics throughout West Africa, as described in the article

Dynamics of cholera epidemics in West Africa. In this study, we performed an extensive spatiotemporal assessment of outbreaks spanning from Benin to Mauritania between 2009 and 2015. We also carried out a series of field investigations in seven West African countries to assess social and environmental factors that played a role in cholera transmission. This study was complemented with MLVA-based typing and whole-genome sequencing of isolates responsible for the majority of recent outbreaks in the region from 2009 to 2015.

Chapters 12 concludes the thesis by discussing the overall findings and public health implications of this global reflection of current cholera epidemics.

8 Widespread epidemic cholera due to a restricted subset of *Vibrio cholerae* clones

Cholera is an acute diarrheal infection caused by the bacterium *Vibrio cholerae* (*V. cholerae*), which provokes disease exclusively in humans. Since 1817, seven cholera pandemics have plagued mankind. Although the infection is often mild or asymptomatic, some patients present with profuse watery diarrhea and vomiting (Kaper, Morris, and Levine 1995). If left untreated, severe cases exhibit a massive loss of bodily fluids that can quickly provoke dehydration, hypovolemic shock and death (Sack et al. 2004a). The clinical symptoms of the disease are directly associated with the major virulence factors, cholera toxin (CTX) (Kaper, Morris, and Levine 1995) and toxin coregulated pilus (TCP), the latter being essential for bacterial colonization of the intestine (Herrington et al. 1988). Upon colonization of the small intestine, the bacterium produces and releases the cholera toxin, a protein complex comprised of one A subunit and five B subunits (Gill 1976a). Once the toxin binds epithelial cells, a portion of the A subunit is internalized (Gill and Kings 1975; Kaper, Morris, and Levine 1995) and subsequently induces constitutive cyclic AMP (cAMP) production (Cassel and Pfeuffert 1978; Gill and Meren 1978). Increased cAMP causes excessive secretion of water and electrolytes into the lumen of the small intestines and, in acute cases, eventually provokes the severe diarrhea recognized as cholera (Cassel and Pfeuffert 1978; Gill and Meren 1978; Kaper, Morris, and Levine 1995).

A diverse spectrum of the *V. cholerae* species flourish in the aquatic environment. The bacterium is found in brackish and estuarial waters either as planktonic bacilli or associated with a wide range of flora and fauna (Vezzulli et al. 2010). Elevated water temperatures, copepod blooms and rainfall have been suggested to correlate with elevated concentrations of *V. cholerae* in the environment (Huq et al. 1984; Jutla et al. 2011a; Huq et al. 2005; Koelle et al. 2005). Conversely, *V. cholerae* levels are controlled in the ecosystem by bacteriophages and predation by bacterivorous protozoa (Brüssow et al. 2004; S. Faruque 2013; Seda 2007). A study in Bangladesh has shown that increased concentrations of certain bacteriophages targeting toxigenic *V. cholerae* coincided with a decreased concentration of the corresponding *V. cholerae* serogroup in environmental water samples (Rodo et al. 2002). As this

phenomenon was also associated with a reduced number of locally reported cholera cases, it has been suggested that bacteriophages may influence the course of cholera outbreaks (Jensen et al. 2006) and the emergence of new *V. cholerae* clones.

Cholera has been extensively portrayed as a prototypical waterborne disease. Some studies, primarily conducted in the Bay of Bengal, have revealed links between cholera outbreaks and flux in climate and aquatic conditions (Huq et al. 2005; Koelle et al. 2005; Rodo et al. 2002). These observations have led many experts in the field to conclude that cholera epidemics are triggered by exposure to local reservoirs of *V. cholerae* (Sack et al. 2004b), driven directly by environmental factors that promote bacterial growth in water bodies (Colwell 1996). However, the mechanisms of cholera outbreaks around the Bay of Bengal are poorly understood perhaps due to the lack of large-scale spatial and temporal studies conducted in the area. In fact, in contrast with Sub-Saharan African countries, Bangladesh and India are reluctant to report suspected cholera cases. Furthermore, there is little evidence that blooms of toxigenic strains occur in aquatic environments prior to epidemics; therefore, toxigenic *V. cholerae* present in water sources may be derived from patients with diarrhea. Seasonal cholera epidemics in the Bay of Bengal may also be associated with other factors such as rainfall and drought, which can promote the contamination of drinking water with patient-derived toxigenic *V. cholerae*. Recent reports examining the evolution of a variety of *V. cholerae* strains responsible for the current pandemic have provided novel insight into cholera epidemiology. In the current report, we show that mounting epidemiological findings as well as genome sequence analysis of clinical isolates indicate that cholera epidemics are largely unassociated with most of the *V. cholerae* strains found in aquatic ecosystems. Indeed, current evidence demonstrates that only a specific subset of *V. cholerae* ‘types’, which have become increasingly specialized in inter-human transmission, have triggered epidemics in the current cholera pandemic. Moreover, the current pandemic can be largely attributed to *V. cholerae* isolates derived from a single ancestral clone that has spread globally in successive waves.

Surveys of *V. cholerae* isolates derived from clinical and environmental samples have shown the species to be highly diverse, comprising more than 200 serogroups

(Chatterjee and Chaudhuri 2003; Kaper, Morris, and Levine 1995). However, only the serogroups O1 and O139, a derivative of serogroup O1 that was first identified in 1992, cause cholera epidemics (Chun et al. 2009; Mutreja et al. 2011; Albert et al. 1993; Chatterjee and Chaudhuri 2003). Although the O1 serogroup is widespread throughout affected regions, the O139 serogroup is almost exclusively restricted to Asia (Chun et al. 2009).

Pandemic *V. cholerae* O1 strains can be divided into two distinct biotypes: 'classical' and 'El Tor'. The classical biotype was responsible for at least the sixth cholera pandemic and reputedly more (Kaper, Morris, and Levine 1995; Karaolis, Lan, and Reeves 1995). Indeed, a recent study has applied targeted high-throughput sequencing to reconstruct the genome of a *V. cholerae* isolate recovered from the intestine of a victim of the 1849 Philadelphia cholera outbreak (i.e., the second pandemic). According to these results, this O1 isolate displayed 95 to 97% genetic similarity with the classical O395 *V. cholerae* genome (Devault et al. 2014). Meanwhile, the El Tor biotype has been described as the causative agent of the seventh pandemic, which emerged in 1961 in the Sulawesi Archipelago (Kaper, Morris, and Levine 1995; Safa, Nair, and Kong 2009). Recent high definition genomic evidence (see below) as well as isolated historical records all indicate the Bay of Bengal as a major hub linking the spread of cholera around the globe during the 19th and early 20th centuries. To understand the underlying phylogeny of the lineage responsible for the current pandemic, Mutreja et al. have performed a genome-wide high-resolution marker analysis by defining single nucleotide polymorphisms (SNPs) in the core genome of 123 seventh pandemic strains, using a pre-seventh pandemic strain (M66) as an outgroup to root the constructed phylogenetic tree. Genomic elements including SNPs in probable genomic islands were excluded, as these regions are mobile and therefore exhibit distinct phylogenies to that of their bacterial host. As recombination can also distort the ability to draw an accurate phylogeny, these SNPs were also excluded from the final phylogenetic analysis. This work on the core genome sequence of *V. cholerae* O1 El Tor unequivocally demonstrated that the current pandemic is monophyletic and originated from a single clone, very distinct from the classical strains of the previous pandemic. Phylogenetic analysis of the seventh pandemic strains also shows that *V.*

cholerae has continually evolved in Southeast Asia and spread around the globe in at least three independent but overlapping waves. The three waves share a common ancestor dating back to the 1950s (Mutreja et al. 2011).

The evolution of toxigenic *V. cholerae* has been characterized by the acquisition, loss and rearrangement of specific mobile genetic elements that play a major role in provoking cholera epidemics (Mutreja et al. 2011). Strains responsible for the sixth pandemic harbored certain mobile elements specific to the classical biotype: the *tcpA* gene, the cholera toxin core genes (i.e., *ctxA* and *ctxB*) and RS elements (*rstR*, *rstA* and *rstB*), which play a role in the replication and chromosomal integration of CTX Φ , the active lysogenic bacteriophage that carries the cholera toxin genes themselves (Waldor et al. 1997; Safa, Nair, and Kong 2009). In contrast, the initial wave I El Tor strains harbored a genetically distinct set of these core mobile elements with an extra gene, *rstC*, which is an anti-repressor and enhances the production of cholera toxin (Davis et al. 2002). Interestingly, El Tor wave I isolates seem to have largely disappeared from most regions of the world with the large majority of the current epidemics instead being attributed to isolates falling within the observed wave II or III global expansions of *V. cholerae* O1 El Tor. Each subsequent wave of the current pandemic has been distinguished by the emergence of O1 El Tor variant strains, first identified in 1991, which are marked by a rearrangement of a repertoire of a few classical and El Tor CTX Φ genes (Nair et al. 2002; Ansaruzzaman et al. 2004; Nair et al. 2006; Safa, Nair, and Kong 2009; Mutreja et al. 2011). The seventh pandemic waves II and III are also characterized by the acquisition of the SXT/R391 family of integrative and conjugative elements that encode resistance to several antibiotics, which has undoubtedly played a role in epidemic spread (Mutreja et al. 2011).

These genetic variations also coincide with contrasts in pathogenic potential among *V. cholerae* subspecies. The classical biotype of the sixth pandemic was associated with more severe clinical manifestations (Sack et al. 2004b; J. B. Kaper, Morris, and Levine 1995), while the early El Tor strains are more frequently associated with asymptomatic infections, fewer fatalities and more efficient transmission than classical strains (Sack et al. 2004b). However, the variant El Tor strains harboring classical gene elements

have been suggested to be associated with more severe clinical symptoms than conventional El Tor strains (Nair et al. 2006).

In sharp contrast to the epidemic-provoking strains, non-O1/non-O139 *V. cholerae* strains, usually associated with the environment, lack the complex array of CTXΦ genetic elements required to produce the cholera toxin and are therefore rarely toxigenic (Faruque and Mekalanos 2012). Although non-O1/non-O139 strains have been sporadically linked to a few cases of mild diarrhoea (Onifade et al. 2011), such strains have never been implicated as the source of a cholera epidemic.

The recent history of cholera in the Americas provides insight into cholera outbreak onset, spread and disappearance following the importation of a pathogenic *V. cholerae* strain. In the 1990s, Latin America experienced the first cholera epidemic since 1895, when an outbreak struck Peru in 1991 and subsequently spread throughout most of South America and Mexico (Seas et al. 2000; “World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). During the following decade, Latin America was then continuously hit by successive outbreaks. The origin of the cholera epidemic was initially suggested to be due to *V. cholerae* species that had settled in the coastal environment following importation and then proliferated following El Niño-Southern Oscillation (ENSO) events (i.e., oscillations in sea surface temperatures that have been linked to interannual variations in cholera outbreaks in Bangladesh) before provoking epidemics (Seas et al. 2000).

However, a recent assessment of isolates responsible for the South American outbreaks of the 1990s shows that the isolates causing disease form a distinct genetic lineage, denoted WASA 1, with a recent common ancestor branching within wave I of the *V. cholerae* O1 El Tor phylogenetic tree (**Figure 3**). Currently, the most basal isolate represented within the WASA 1 sequenced lineage is an isolate collected in 1989 from the Portuguese-speaking West African country of Angola (Mutreja et al. 2011). This is evidence that these strains were all derived from a single clone sharing a common origin and raises the possibility that the disease was imported by an individual, which argues against the hypothesis implying that the epidemic was triggered by the

proliferation of multiclonal *V. cholerae* populations in the environment. Further addressing the hypothesis of environmental influence on cholera emergence, a recent study has indicated that the rains provoked by the 1991 ENSO event arrived in Peru much later than the initial outbreak, and therefore could not have triggered cholera emergence in Peru (Ramírez, Grady, and Glantz 2013). Regardless of the origin of cholera in Latin America, the succession of ensuing epidemics led many experts to presume that once *V. cholerae* was introduced, it would settle in the coastal environment and cause epidemic re-emergence for many years. However, after a resurgence of outbreaks in 1998, cholera unexpectedly receded from Latin American following the final outbreak report of 2001 (i.e., 494 cases in Peru) (Centers for Disease Control and Prevention 2002). Although it is possible that a few sporadic cases may have arisen, no epidemic was reported in America for nearly a decade until a new clone was imported into Haiti in 2010 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

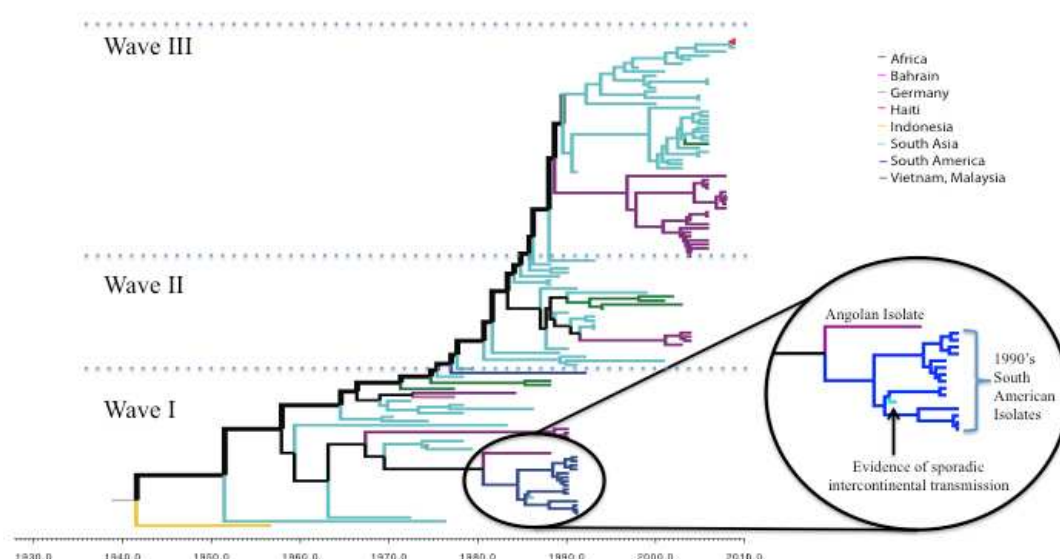


Figure 3. A maximum-likelihood phylogenetic tree of the *Vibrio cholerae* seventh pandemic lineage based on whole core genome single-nucleotide polymorphism (SNP) variations. The pre-seventh pandemic phylogeny-based core genome SNP analysis of 123 seventh pandemic *V. cholerae* strains, excluding probable recombination events, is shown. The pre-seventh pandemic strain M66 served as an outgroup to root the tree. The branch colors correspond to the indicated region of strain isolation. The branches representing the three major waves are indicated. The wave I branch responsible for the 1990s South American epidemic is enlarged in the circle, and the related Angolan isolate is indicated. A case of sporadic intercontinental transmission in wave I is also designated. The Haitian strain is indicated in red. The dates shown are the median estimates for the indicated nodes, derived from the results of the BEAST analysis.

The unexpected disappearance of cholera epidemics in Latin America for almost a decade may be explained by a 2004 study assessing the *in situ* elimination rate of *V. cholerae* O1 El Tor in brackish water collected from Mecocan lagoon and the athalassohaline lake of Alchichica in Mexico. The study findings demonstrated that fluorescently labelled *V. cholerae* O1 were eliminated from water samples derived from the brackish lagoon and athalassohaline lake waters at an average rate of 32% and 63% per day, respectively (Perez, Macek, and Galvan 2004). Thus, it seems that the clone responsible for the epidemics of the 1990s in the Americas exhibited a strong propensity to spread among human populations, but limited capacity to gain a foothold in the aquatic environment, at least at significant concentrations.

In 2010, cholera was re-introduced into Latin America with the Haitian epidemic (Centers for Disease Control and Prevention 2011). The outbreak commenced with the massive contamination of the Artibonite River, the ensuing infection of the population of the Artibonite Delta and eventual dissemination throughout almost the entire country. Although it was initially suggested that the epidemic was triggered by the proliferation of local *V. cholerae* reservoirs due to geophysical and climatic events, epidemiological and microbiological analyses have unequivocally demonstrated that the epidemic *V. cholerae* strain had been very recently imported from Nepal (Piarroux et al. 2011; Hendriksen, Price, and Schupp 2011; Frerichs et al. 2012a; Chin et al. 2011; Cravioto et al. 2011). The Haitian epidemic strain is a variant *Vibrio cholerae* O1,

serotype Ogawa, biotype El Tor harboring the classical ctxB-7 allele (Talkington et al. 2011; Centers for Disease Control and Prevention 2011), which clusters with Nepalese isolates in the third wave of the seventh pandemic (Frerichs et al. 2012a; Hendriksen, Price, and Schupp 2011).

Although the disease had previously never in recorded history been reported in the country (Jenson and Szabo 2011), the Haitian epidemic represents the largest national epidemic of the seventh pandemic (Barzilay et al. 2013b; “World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Cholera has persisted in Haiti for more than 3 years, with seasonal exacerbation of the epidemic during the rainy periods (Gaudart et al. 2013). The disease has subsequently spread throughout Latin America, with outbreaks reported in Venezuela (Centers for Disease Control and Prevention 2012), the Dominican Republic (Centers for Disease Control and Prevention 2010; Sealfon et al. 2012), Mexico (Aleccia; Knox 2013; “Cholera in Mexico – Update, Disease Outbreak News” 2013) and Cuba (“Cholera in Mexico – Update, Disease Outbreak News” 2013; Mascarello et al. 2013); each of these epidemics have been epidemiologically and/or molecularly linked to the Haitian strain. The rapid identification of these epidemics also validated the efficient outbreak detection system in Latin America despite the 10-year absence of epidemic cholera, which has been questioned to explain the lack of cholera reports following the outbreaks of the 1990s in the region.

As cholera has persisted in Haiti, it has been suggested that the Haitian/Nepalese epidemic strain has since settled and proliferates in the aquatic environment and that it may exchange DNA fragments with environmental *V. cholerae* strains (Hasan et al. 2012). However, an extensive sequence analysis of Haitian clinical isolates and environmental *V. cholerae* strains has clearly demonstrated that, at least in Haiti, the epidemic strains exhibit no evidence of acquired genetic elements via horizontal gene transfer with environmental strains (Katz et al. 2013a). The same study further demonstrated that the epidemic strain was poorly transformable (Katz et al. 2013a). Furthermore, a study assessing coastal waters conducive to *V. cholerae* growth during the warmest period of the rainy season was unable to detect toxigenic *V. cholerae* O1

isolates via PCR, although non-O1/non-O139 *V. cholerae* strains were isolated at each of the stations tested (Baron et al. 2013). Other studies have either failed to detect the epidemic *V. cholerae* strain in the Haitian environment (Hasan et al. 2012) or identified only a couple epidemic strains in freshwater canals during the peak of the epidemic, when transient patient-derived contamination of local water systems was unavoidable (Hill et al. 2011). Overall, the cumulative data suggest that the *V. cholerae* O1 El Tor clone imported from Nepal has failed to settle into the Haitian waters at least at levels that pose a risk to local populations, but instead circulates among the population through sporadic but persisting outbreaks, which are exacerbated by latrine overflow during rainy seasons (Rebaudet et al. 2013).

The demonstration of cholera in South America supports the concept of the clonal nature of the disease at an intercontinental scale. Similar phenomena have been observed in Africa, although data concerning the entire African continent is currently limited. A recent study has revealed that the 2012 Guinean cholera epidemic was triggered by a single *V. cholerae* clone that was imported by a fisherman travelling from Sierra Leone (Bellet 2012). Furthermore, whole-genome DNA sequence analysis of Kenyan *V. cholerae* O1 El Tor strains isolated between 2005 and 2010 has shown that the isolates are clonally related to other El Tor *V. cholerae* isolates found throughout affected regions of the globe, falling into two distinct clades of the third wave of the seventh pandemic (Kiiru et al. 2013).

Current epidemic strains exhibit enhanced capacity for inter-human transmission, which appears to be in part due to the exchange of key mobile elements required for cholera toxin production and the more recent acquisition of antibiotic resistance elements. The *Vibrio* seventh pandemic islands I (VSP-I) and II (VSP-II) are hallmark features specific to the seventh pandemic El Tor O1 lineage (Dziejman et al. 2001), and at least VSP I has been shown to encode factors, such as di-nucleotide cyclase (DncV), that enhance the intestinal colonization and likely transmission of *V. cholerae* (O'Shea et al. 2004; Davies et al. 2013). The catalytic activity of DncV is required to downregulate chemotaxis, which has been shown to significantly enhance *V. cholerae* intestinal colonization of the intestine and subsequently promote hyperinfectivity

(Davies et al. 2013; Butler et al. 2006). Altogether, the acquisition of both VSP-I-encoded factors and SXT-encoded drug resistance factors may explain the improved fitness of the variant strains, as these elements enhance human intestinal colonization and transmission and not environmental fitness. However, the horizontal gene transfer mechanism of such elements among bacteria and whether this occurs in the environment or host, remain poorly understood.

In conclusion, the gamut of epidemiological data supplemented with recent microbiological analyses of epidemic *V. cholerae* strains based on whole-genome sequencing approaches have provided further insight into the dynamics of cholera epidemics and called into question some of the basic assumptions concerning epidemic cholera. Contrary to the contemporary understanding of cholera epidemiology suggesting that epidemics originate from aquatic environmental reservoirs of a spectrum *V. cholerae* species, the current data rather show that a specific subset of *V. cholerae* clones have emerged from this diverse population to provoke cholera epidemics, which are often spread via inter-human transmission. Present-day epidemics are caused by variant *V. cholerae* El Tor strains harboring a varying repertoire of CTX Φ genes, which have gradually replaced former classical and wave I El Tor strains. Indeed, whole-genome sequence analysis of a series of temporal and spatial representative strains has revealed that the seventh pandemic is monophyletic and all epidemic strains are derived from a single ancestral clone. Finally, the current understanding of cholera dynamics suggests that the disease can be eliminated, as observed in South America, by robustly controlling the diffusion of epidemic clones. Although the emergence of new toxigenic *V. cholerae* clones is possible, the culmination of studies shows that such an event is very rare.

9 Cholera in Haiti

Are current cholera outbreaks in Haiti due to the proliferation of toxigenic *V. cholerae* strains in the local aquatic environment? It has indeed been suggested that following the initial importation of the *V. cholerae* O1 El Tor strain from Nepal in 2010, that these cholera-causing strains have since settled into the coastal waters. In this chapter, we present our study examining a series of environmental water samples from several heavily affected areas in the West and Artibonite Departments to ascertain whether toxigenic *V. cholerae* could be detected in Haitian waters at concentrations representing a significant risk to the local population. As the title suggests, we found no evidence of a perennial reservoir of cholera-causing *V. cholerae* O1 in the local Haitian waters. We then present our report of cholera dynamics and control strategies during the dry season in Haiti, which reveals that cholera re-emergence during the rainy season in the country likely stems from persisting cholera foci that are inadequately controlled during the dry season.

9.1 No evidence of significant levels of toxigenic *V. cholerae* O1 in the Haitian aquatic environment during the 2012 rainy season

9.1.1 Introduction

On October 21, 2010, Haiti was struck by cholera for the first time in over a century (Jenson and Szabo 2011). The Haitian epidemic represents the largest national cholera epidemic of the seventh pandemic with 604,634 cases and 7,436 deaths reported from October 2010 to October 2012 (Barzilay et al. 2013a).

Epidemiological data has demonstrated an exact spatiotemporal correlation between the first reported cholera cases in Meille, a small village 2 km south of Mirebalais, and the arrival of UN Nepalese peacekeepers in Haiti (Piarroux et al. 2011). According to Frerichs et al. (2012), the Nepalese soldiers were exposed to a cholera epidemic in Nepal in late September just before embarking for Haiti, where they were primarily stationed in a camp near Mirebalais, situated on the banks of the Meille River (Frerichs et al. 2012b). The initial cases were biologically confirmed as *Vibrio cholerae* O1, serotype Ogawa, biotype El Tor (Centers for Disease Control and Prevention (CDC) 2010). Genetic analysis has demonstrated that the Haitian cholera isolates were almost identical to isolates collected in Nepal a few weeks prior, which displayed only one- or two-base pair differences throughout the entire genome, thereby strongly suggesting that the Haitian cholera strains were very recently imported from Nepal (Hendriksen, Price, and Schupp 2011).

During the first 2 years of the epidemic, cholera was disseminated throughout almost every region of Haiti, including the most remote rural areas. Meanwhile, outbreaks seemed to be aggravated by major climatic events, such as Hurricane Tomas in November 2010 and the hot and rainy seasons of 2011 and 2012. Moreover, it appears that cholera particularly affected certain coastal areas, such as the Artibonite Delta following the floods provoked by Hurricane Tomas and the low altitude wards of Port-au-Prince during the 2011 rainy season. In contrast, a major reduction in the number

of cases was observed during the dry seasons (Gaudart et al. 2013; Barzilay et al. 2013a), when cholera transmission retracts in a few rural locations and urban quarters (Rebaudet et al. 2013). The persistence of cholera in the country for more than two years associated with a seasonal exacerbation of the epidemic during the rainy seasons, especially in coastal areas, has prompted us to examine the level of toxigenic *V. cholerae* contamination in the Haitian aquatic environment.

Indeed, in the Bay of Bengal, it has been shown that the environment can play a role in the durable establishment of cholera. Studies in the 1980s and 1990s have demonstrated that *V. cholerae* species can grow in various aquatic ecosystems, such as fresh waters, brackish waters and estuaries (Colwell 2004). In such environments, *V. cholerae* species associates with phytoplankton and zooplankton in a pH- and salinity-dependent manner (Colwell 2004). Increases in water temperature and subsequent plankton blooms have been shown to correlate with the fluctuation in cholera cases in the Bay of Bengal (Jutla et al. 2011b; Koelle 2009b).

To investigate whether the aquatic environment presents a major risk of cholera transmission to local populations, we conducted a microbial assessment of toxigenic *V. cholerae* O1 levels on a panel of water samples isolated from several areas in the West and Artibonite departments. These areas were selected because they were heavily affected by cholera during either the floods that followed hurricane Tomas in 2010 (Artibonite Department) or the 2011 rainy season (Port-au-Prince and Gonaïves areas). Most of the sample sites were coastal areas considered to be favorable environments for *V. cholerae* growth; although, other inland sites (Cul-de-Sac Plain) suitable for *V. cholerae* proliferation were also examined.

9.1.2 Materials and Methods

9.1.2.1 *Study period*

To search for toxigenic *V. cholerae* present in the Haitian environment, the study was performed during the warmest period of the rainy season (**Figure 4**). Aquatic samples were collected between July 3 and July 10, 2012, a period characterized by high surface water temperatures. During this period, approximately 168 suspected cholera cases were reported per day in the departments of West (including the Port-au-Prince metropolitan area) and Artibonite (**Figure 4**).

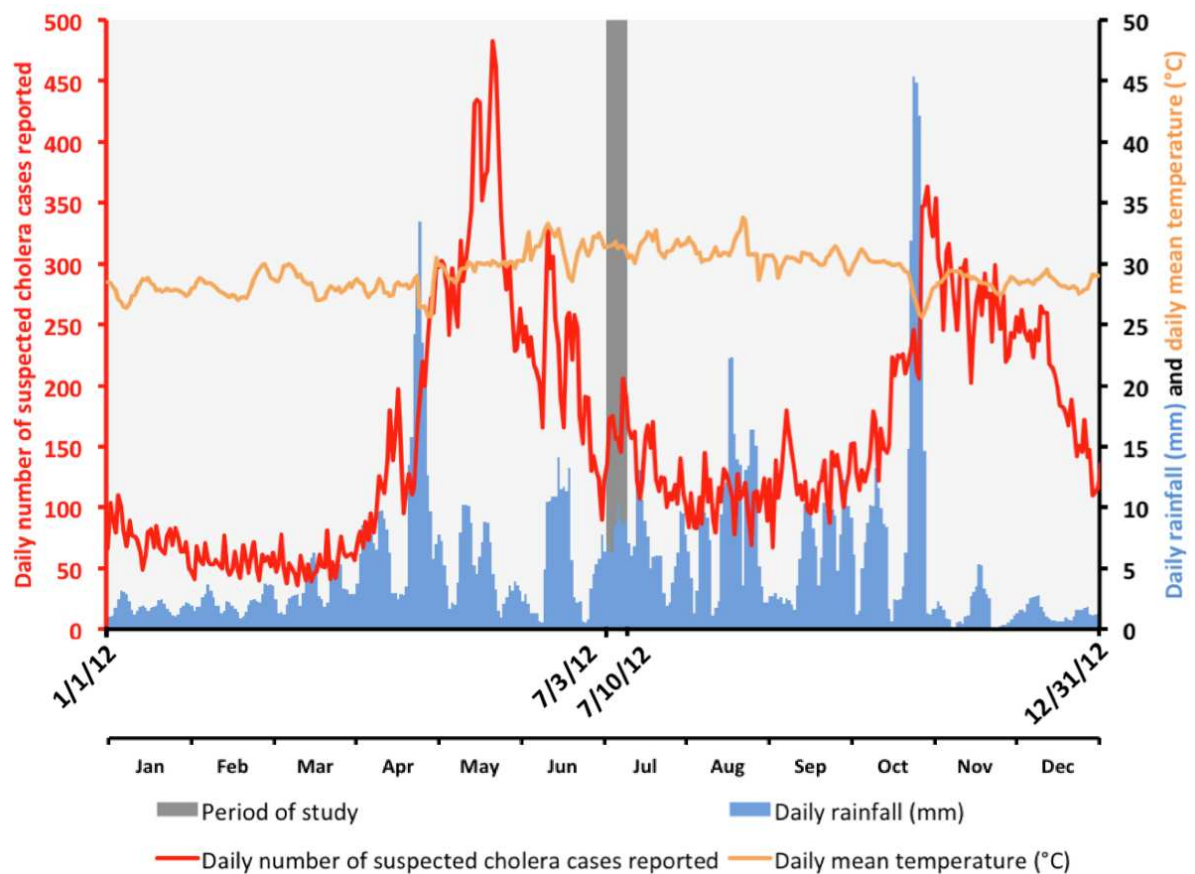


Figure 4. Evolution of the daily suspected cholera cases in the departments of West (including Port-au-Prince conurbation) and Artibonite, daily accumulated rainfall in the area and the daily mean temperature in Port-au-Prince in 2012. Time point of the sampling period (July 3 to 10 2012). Accumulated rainfall data were obtained from satellite estimates (TMPA-RT 3B42RT derived) averaged on the position 18.25N-19.75N / 74.25W-71.75W and available at http://disc2.nascom.nasa.gov/Giovanni/tovas/realtime.3B42RT_daily.2.shtml (accessed April 29, 2013).

Mean daily temperatures observed at Port-au-Prince airport were obtained from the following:

<http://gis.ncdc.noaa.gov/map/viewer/#app=cdo&cfg=cdo&theme=temp&layers=1&node=gis> (accessed April 29, 2013).

9.1.2.2 Sampling sites

Thirty-six stations were sampled from four distinct areas (**Table 1, Figure 5**). The sampling sites were selected based on field observations, focusing on the nature of

the water bodies (surface water or wastewater), excluding well water and domestic tanks. The urban sampling areas included Port-au-Prince (seven stations) in the West department, and Pont-Sondé (one station), L'Estère (one station) and Gonaïves (four stations) in the department of Artibonite. Lakes and ponds unconnected to coastal waters were also tested, as their salinity levels are compatible with *V. cholerae* proliferation. These sites were located at lakes (Trou Caïman and Etang Saumâtre in the Cul-de-Sac plain, three stations) and a large pond located southwest of Saint Marc (Etang Bois-Neuf, one station). Rural areas of the Artibonite Plain were represented by 17 stations at both the river and diversion canals. Among these 17 stations, nine were frequently accessed for toilet or laundry activity. The two remaining stations were salt marshes located near Gonaïves.

| Sampling stations | | | | Water characteristics | | | |
|--------------------------|----------------|----|--|---------------------------|------------------|-----|---------------------------------------|
| Department | Commune | ID | Location | Salinity (‰) ¹ | Temperature (°C) | pH | Fecal contamination (E. coli /100 mL) |
| West (Metropolitan area) | Port-au-Prince | 2 | Martissant (street wastewater) | ND | ND | ND | ND |
| | | 32 | Mariani (wastewater in the river) | 0.21 | 38.5 | 6.9 | 106,000 |
| | Carrefour | 33 | Mariani (river shore) | 0.20 | 38.5 | 7.0 | 35,000 |
| | | 34 | Mariani (river shore) | 0.21 | 38.5 | 7.1 | 46,000 |
| | | 35 | Mariani (macrophyte lagoon) | 25.02 | 38.5 | 7.5 | 12,400 |
| | | 36 | Mariani (microphyte lagoon) | 10.72 | 38.5 | 6.7 | 50,000 |
| | | 37 | Mariani (river output into the sea) | 0.31 | 38.8 | 6.5 | 1,000 |
| West | Thomazeau | 3 | Trou Caïman lake | 1.27 | 29.8 | 7.9 | ND |
| | | 4 | Etang Saumâtre lake (shore) | 4.90 | 28.6 | 7.8 | ND |
| | | 5 | Etang Saumâtre lake (far from the shore) | 5.62 | 27.7 | 7.5 | ND |
| Artibonite | Gonaïves | 19 | Small canal of wastewater | 2.09 | ND | ND | 91,000 |
| | | 20 | Small canal output into the sea | 28.49 | 33.9 | 7.4 | 13,700 |
| | | 21 | Seashore near the small canal output | 28.49 | 32.7 | 7.5 | 1,100 |
| | | 22 | Salt marsh: canal | 32.17 | 36.1 | 7.7 | <100 |
| | | 23 | Salt marsh: basin | 29.54 | 33.9 | 8.0 | <100 |
| | | 24 | Large canal of waste water | 1.40 | 33.8 | 7.5 | 36,000 |
| | Saint Marc | 18 | Etang Bois-Neuf | 12.41 | 32.0 | 8.4 | 2,000 |
| | | 6 | Pont-Sondé (river Artibonite) | 0.14 | 28.8 | 7.3 | 4,800 |
| | Grande Saline | 17 | Main canal 1 | 0.15 | 32.5 | 7.5 | 2,800 |
| | | 16 | Main canal 2 | 0.15 | 32.4 | 7.4 | 3,600 |
| | | 7 | Drouin - main canal 3 (point-of-use) | 0.15 | 30.2 | 7.3 | 2,100 |
| | | 9 | Artibonite river estuary 1 | 0.14 | 30.2 | 7.4 | 2,100 |
| | | 10 | Artibonite river estuary 2 | 0.15 | 30.5 | 7.3 | 3,100 |
| | | 8 | Artibonite river estuary 3 (pontoon) | 0.15 | 31.1 | 7.3 | 2,900 |
| | | 13 | Artibonite river estuary 4 | 0.14 | ND | 7.4 | 2,000 |
| | | 11 | Artibonite river estuary 5 | 0.20 | ND | ND | 1,200 |
| | | 12 | Artibonite river estuary 6 | 4.87 | 30.0 | 7.2 | 800 |
| | | 14 | Basin 1 | 0.75 | 36.1 | 7.1 | 1,000 |
| | | 15 | Basin 2 | 0.27 | 34.8 | 7.9 | <100 |
| | L'Estère | 25 | L'Estère (river) | 0.15 | 32.5 | 7.6 | 300 |
| | | 26 | L'Estère (small canal) | 0.15 | 31.9 | 7.4 | 400 |
| | | 27 | L'Estère (large canal) | 0.14 | 32.2 | 7.4 | 300 |
| | | 28 | L'Estère (roadside) | 0.37 | 31.9 | 6.5 | 4,700 |
| | Desdunes | 29 | Route de Desdunes (small canal) | 0.26 | ND | ND | 200 |
| | | 30 | Route de Desdunes (large canal) | 0.52 | 34.1 | 7.0 | 300 |
| | | 31 | Route de Desdunes (rice field) | 0.30 | 33.5 | 8.0 | <100 |

Table 1. Characteristics of the sampling stations (see Figure 5 for localization). ¹ fresh water, salinity <0.5‰; brackish water, 0.5-16‰; saline water, ≥16‰; ND, no data.

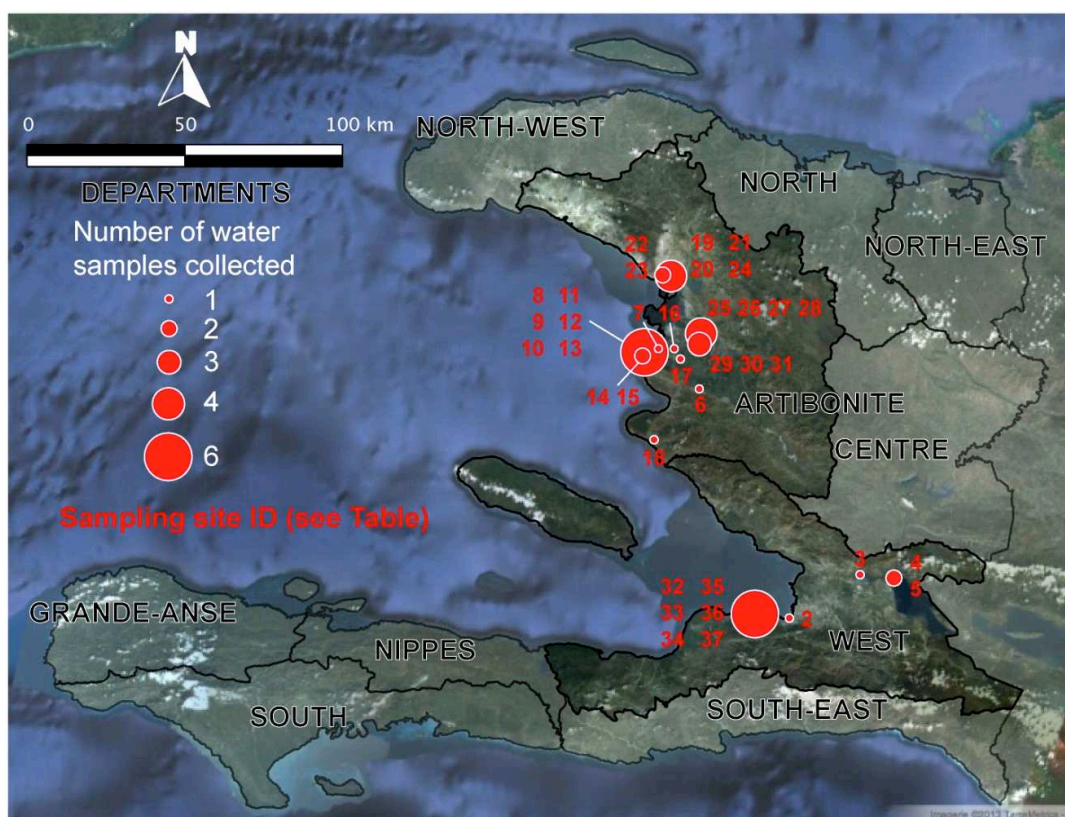


Figure 5. Localization of the sampling stations in the West and Artibonite departments. (See Table 1 for the corresponding characteristics).

9.1.2.3 Sample collection and processing

Grab water samples were collected 20 cm below the surface with sterilized narrow-mouth plastic bottles. Sample collection was performed by boat at Etang Saumâtre Lake, Etang Bois-Neuf Pond and the Artibonite estuary. The other samples were collected from the shore with a telescopic pole. Water samples were transported in a cooler (containing frozen packs at the bottom for minimum contact with the bottles) to the Haitian National Laboratory of Public Health (LNSP), and the analysis was performed within 6 to 24 hours of collection.

Surface water temperature and pH levels were measured at the sampling sites using a field pH meter (Hanna HI-98127, Grosseron, Nantes, France); conductivity was

assessed at the laboratory with a field conductometer (Hanna HI-99301, Grosseron, Nantes, France). Fecal contamination was determined using Petrifilm™ Select E. coli (Département Microbiologie Laboratoires 3M Santé, Cergy, France), which was incubated overnight at 42°C.

9.1.2.4 *Enrichment and selective cultures*

The analyzed serial volumes for each water sample were selected based on the type of water and the expected abundance of *V. cholerae*. At six stations, only a 1-L sample volume was analyzed. At 10 other stations, a 1-L sample volume was analyzed in association with smaller volume samples (100 mL, 10 mL and 1 mL). For the remaining 20 stations, the range of sample volumes analyzed only included 100 mL and smaller sample volumes (e.g., 10 mL, 1 mL and sometimes even smaller sample sizes).

Sample volumes of 1 L and 100 mL were filtered (Diaphragm pump N035.3 AN.18 KNF Neuberger, Village-Neuf, France) successively with glass microfiber filters GF/D (grade D, 2.7 µm; Whatman, Maidstone, UK), glass microfiber filters GF/C (grade C, 1.2 µm; Whatman, Maidstone, UK) and 0.45 µm cellulose ester membranes (Millipore, Watford, UK). The filters were sequentially used for the filtration of water samples. The various filter sizes guaranteed the isolation of both fixed-form and free-living bacteria. The filters were placed in 250 mL of sterile Alkaline Saline Peptone Water (ASPW; composition for 1 liter: 10 g peptone, 20 g NaCl and 5 g yeast extract; post-autoclave pH: 8.6 ± 0.2). Sample volumes of 10 mL were incorporated in 100 mL of ASPW, and sample volumes of 1 mL to 0.001 mL were incorporated in 10 mL of ASPW.

The enrichment cultures were incubated from 16 to 24 hours at $41 \pm 1^\circ\text{C}$ (Muic 1990) and subsequently cultured on selective TCBS (Thiosulfate Citrate Bile Sucrose) agar (Difco, provided by Bio-Rad, Marne la Coquette, France) to isolate *V. cholerae* colonies.

The screening procedure was based on phenotypic traits. Up to 20 sucrose-fermenting colonies were transferred with sterile toothpicks onto nutrient agar without NaCl (NA₀ – Difco, provided by Bio-Rad, Marne la Coquette, France) to test for growth at 37°C

and then submitted for an oxidase test (Bactident oxidase strips, Merck, Darmstadt, Germany). All sucrose-fermenting isolates that were able to grow on NA₀ agar and tested oxidase-positive were considered to be presumptive isolates of *V. cholerae* (Baron, Chevalier, and Lesne 2007).

9.1.2.5 Isolate serotyping

Presumptive *V. cholerae* isolates were examined to determine whether they were members of the O1 serogroup via slide agglutination using a polyclonal antibody specific for the O1 surface antigen (Bio-Rad, Marne la Coquette, France). A saline solution was used as a control to identify self-agglutinating isolates.

9.1.2.6 Molecular identification

At the LNSP laboratory, DNA extraction of the enrichment cultures was performed automatically after adding 10 µL proteinase K to 200 µL of each enrichment broth using Boom technology (TANBead viral auto kit, Taiwan) with the robot Medipro super pure system-32 (Taiwan). All 107 DNA extracts obtained from 32 sampling sites (stations 6 to 37), both pure and 10-fold dilutions, were assessed via two multiplex PCR (Nandi et al. 2000; Hoshino et al. 1998). The detection of *V. cholerae* species was performed via PCR targeting a gene encoding an outer membrane protein (*ompW*) (Nandi et al. 2000) (**Table 2**). Two different cholera toxin gene (*ctxA*)-specific PCR assays were used to detect the cholera toxin (Nandi et al. 2000; Hoshino et al. 1998) (**Table 2**). The gene coding for the O1 and O139 surface antigens (*rfb*) was assessed via PCR using O1- and O139-specific primers (Hoshino et al. 1998) (**Table 2**). The PCR assays were conducted using a G-Storm thermal cycler (Gene Technologies Ltd, Braintree, UK) with the cycling conditions described in **Table 2**.

| Primer ID | Sequence | Amplicon size (bp) | Target | PCR conditions ¹ | Reference |
|------------------|--------------------------------|--------------------|---|-----------------------------|-----------------------|
| O139F2 (forward) | 5'-AGCCTCTTTATTACGGGTGG-3' | 449 | rfb gene: O139-specific region | 1 | (Hoshino et al. 1998) |
| O139R2 (reverse) | 5'-GTCAAACCCGATCGTAAAGG-3' | | | | |
| O1F2-1 (forward) | 5'-GTTTCACTGAACAGATGGG-3' | 308 | rfb gene: O1-specific region | 1 | (Hoshino et al. 1998) |
| O1R2-2 (reverse) | 5'-GGTCATCTGTAAGTACAAC-3' | | | | |
| VCT1 (forward) | 5'-ACAGAGTGAG TACTTTGACC-3' | 192 | ctxA gene: A subunit of the cholera toxin | 1 | (Hoshino et al. 1998) |
| VCT2 (reverse) | 5'-ATACCATCCATATATTTGGGAG-3' | | | | |
| ompW (forward) | 5'-CACCAAGAAGGTGACTTTATTGTG-3' | 588 | ompW gene: Outer Membrane Protein | 2 | (Nandi et al. 2000) |
| ompW (reverse) | 5'-GAACTTATAACCAACCCGCG-3' | | | | |
| ctxA (forward) | 5'-CTCAGACGGGATTTGTTAGGCACG-3' | 301 | ctxA gene: A subunit of the cholera toxin | 2 | (Nandi et al. 2000) |
| ctxA (reverse) | 5'-TCTATCTCTGTAGCCCCTATTACG-3' | | | | |

Table 2. Primer sequences and multiplex PCR assay conditions. PCR conditions¹: initial denaturation (5 min at 94°C); 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (1 min at 72°C); final extension (7 min at 72°C). PCR conditions²: initial denaturation (5 min at 94°C); 30 cycles of denaturation (30 sec at 94°C), annealing (30 sec at 64°C) and extension (30 sec at 72°C); no final extension.

9.1.3 Results

9.1.3.1 Surface water characteristics and fecal contamination

The disparity in pH levels between the different stations was low (**Table 1**), with a mean pH of 7.4 (standard deviation: 0.4, minimum: 6.5 and maximum: 8.4). The Etang Bois-Neuf site displayed the highest water pH levels (8.4), which is a pH level known to be favorable for *V. cholerae* growth. The in situ water temperatures ranged between 27.7°C (Etang Saumâtre) and 38.8°C (Mariani) (mean: 33.3°C; standard deviation: 3.3°C). Salinity levels varied greatly between the sampling zones. Therefore,

the sampling sites were categorized into three groups based on this characteristic: (1) 20 freshwater stations: salinity levels inferior to 0.5‰; (2) 11 brackish water stations (including three oligohaline wastewaters): salinity levels between 0.5‰ and 16‰; and (3) five saline water stations: salinity levels between 16‰ and 40‰.

The levels of fecal contamination varied greatly between samples (**Table 1**). Accordingly, the risk of contracting intestinal infections was low in the rice field and salt marshes (less than 100 Colony Forming Units (CFU) per 100 mL), high at the Artibonite plain stations located a pronounced distance from housing (101 to 1000 CFU per 100 mL), and very high near housing settlements (more than 1000 CFU per 100 mL). In wastewaters, fecal contamination levels ranged from 10,000 to 100,000 CFU per 100 mL.

*9.1.3.2 Absence of toxigenic *Vibrio cholerae* O1*

From a total of 141 enrichment cultures derived from the water samples collected at the 36 sampling sites, 411 presumed isolates of *V. cholerae* were isolated. The distribution of the isolates by sampling site is provided in **Table 3**. Nine sampling sites failed to yield any *V. cholerae* isolates. Five of these sites (sites 20, 21, 22 and 23 at Gonaïves and site 35 at Port-au-Prince) were saline waters. The other four stations (sites 8, 11, 12 and 13), which were fresh waters, were located at the mouth of Artibonite estuary.

| Station ID | Culture and identification | | Specific PCRs | | | |
|------------|--|--|---|---------------------------------------|---|--------------------------------|
| | Culturable <i>V. cholerae</i> ¹ (limit volume ²) | No. O1 / No. tested <i>V. cholerae</i> strains | <i>V. cholerae</i> : ompW gene (limit volume ²) | <i>V. cholerae</i> O1: rfb gene | <i>V. cholerae</i> O139: rfb gene | cholera toxin: ctxA gene |
| 2 | pos (0.1 mL) | 0/1 | ND | ND | ND | ND |
| 32 | pos (100 mL) | 0/1 | pos (10 mL) | neg | neg | neg |
| 33 | pos (0.1 mL) | 0/17 | pos (1 mL) | neg | neg | neg |
| 34 | pos (0.1 mL) | 0/11 | pos (0.001 mL) | neg | neg | neg |
| 35 | neg (100 mL) | NI | pos (0.1 mL) | neg | neg | neg |
| 36 | pos (100 mL) | NT | pos (0.01 mL) | neg | neg | neg |
| 37 | pos (10 mL) | 0/8 | pos (10 mL) | neg | neg | neg |
| 3 | pos (0.1 mL) | 0/40 | ND | ND | ND | ND |
| 4 | pos (10 mL) | 0/25 | ND | ND | ND | ND |
| 5 | pos (10 mL) | 0/8 | ND | ND | ND | ND |
| 19 | pos (0.01 mL) | 0/18 | pos (0.01 mL) | neg | neg | neg |
| 20 | neg (100 mL) | NI | pos (0.01 mL) | neg | neg | neg |
| 21 | neg (100 mL) | NI | pos (0.01 mL) | neg | neg | neg |
| 22 | neg (100 mL) | NI | pos (10 mL) | neg | neg | neg |
| 23 | neg (100 mL) | NI | pos (100 mL) | neg | neg | neg |
| 24 | pos (0.01 mL) | 0/15 | pos (0.01 mL) | neg | neg | neg |
| 18 | pos (0.1 mL) | 0/34 | pos (0.1 mL) | neg | neg | neg |
| 6 | pos (0.1 mL) | 0/30 | pos (1 mL) | neg | neg | neg |
| 17 | pos (100 mL) | 0/16 | pos (100 mL) | neg | neg | neg |
| 16 | pos (0.1 mL) | 0/27 | pos (1000 mL) | neg | neg | neg |
| 7 | pos (100 mL) | 0/12 | pos (100 mL) | neg | neg | neg |
| 9 | pos (1000 mL) | 0/2 | pos (1000 mL) | neg | neg | neg |
| 10 | pos (1000 mL) | 0/8 | pos (1000 mL) | neg | neg | neg |
| 8 | neg (1000 mL) | NI | pos (1000 mL) | neg | neg | neg |
| 13 | neg (1000 mL) | NI | pos (1000 mL) | neg | neg | neg |
| 11 | neg (1000 mL) | NI | pos (1000 mL) | neg | neg | neg |
| 12 | neg (1000 mL) | NI | pos (1000 mL) | neg | neg | neg |
| 14 | pos (1 mL) | 0/24 | pos (1 mL) | neg | neg | neg |
| 15 | pos (0.1 mL) | 0/33 | pos (1 mL) | neg | neg | neg |
| 25 | pos (10 mL) | 0/6 | pos (10 mL) | neg | neg | neg |
| 26 | pos (10 mL) | 0/3 | pos (10 mL) | neg | neg | neg |
| 27 | pos (10 mL) | 1 [*] /9 | pos (10 mL) | neg | neg | neg |
| 28 | pos (1 mL) | 0/1 | pos (1 mL) | neg | neg | neg |
| 29 | pos (0.01 mL) | 0/11 | pos (1 mL) | neg | neg | neg |
| 30 | pos (1 mL) | 0/22 | pos (1 mL) | neg | neg | neg |
| 31 | pos (10 mL) | 0/8 | pos (10 mL) | pos | neg | neg |
| Total | 27 pos | 1 [*] /390 | 32 pos | 1 | 0 | 0 |

Table 3. Results of *Vibrio cholerae* cultures, identifications and PCR assays. ¹Characteristic appearance on TCBS (sucrose-fermentation), translucent colony growth on 0% NaCl nutrient agar (Difco), and positive oxidase reaction. ²Limit volume is the smallest with positive culture or the biggest with negative culture. * Late agglutination. NI: no isolate; NT: no isolate tested; ND: no DNA extraction.

The O1-agglutination test was performed on 390 presumed *V. cholerae* isolates, of which 56 strains were positive for auto-agglutination. Only a single isolate, which was isolated from sampling site 27 (a large canal south of L'Estère), displayed a late positive reaction with polyvalent anti-O1 serum, without auto-agglutination.

The *V. cholerae* PCR assays were found to be more sensitive than the culture assay, as positive results were obtained from all of the 32 tested stations (a total of 77 enrichments out of 107 yielded a positive *V. cholerae*-specific PCR on *ompW* gene). However, only one sample derived from station 31 in the rice field near Desdunes yielded a positive result for *V. cholerae* O1-specific PCR (*rfb* gene). The *ctxA*-specific PCR was performed on all water enrichments, including from station 27 (where an isolate displayed a late positive reaction with polyvalent anti-O1 serum) and from station 31 (positive O1 PCR). However, the cholera toxin was never detected with either pair of *ctxA*-specific primers, even from 100 mL or 1 mL water samples. Therefore, despite the high number of *V. cholerae* isolates obtained, we could not demonstrate the presence of toxigenic *V. cholerae* among all the samples collected.

9.1.3.3 Abundance of *Vibrio cholerae* in surface waters and wastewaters

Table 3 provides the smallest sample volume displaying the presence of *V. cholerae*, or in case of non-detection of *V. cholerae*, the largest volume analyzed for each station. The results of *V. cholerae* detection via culture or PCR are consistent with an abundance ranging from 1 to 10⁶ bacteria per liter depending on the type of water.

9.1.4 Discussion

The aim of this study was to ascertain whether toxigenic *V. cholerae* could be detected in Haitian aquatic systems at concentrations representing a significant risk to the local population. Our purpose was therefore not to prove the absence of toxigenic *V. cholerae* in the environment, a goal that would require other methods and sampling

strategies. Aquatic samples characterized by a wide range of salinity levels were collected during a warm period, at both the time point and locations one would expect to find a high abundance of *V. cholerae* in the environment. Sampling sites were located in Haitian areas profoundly affected by cholera, and most of locales presented medium to high fecal contamination levels, thereby presenting aquatic conditions appropriate for the study of contamination by the epidemic *V. cholerae* clone.

To enhance culturable *V. cholerae* detection, environmental water samples were enriched. Sample enrichment facilitates the detection of *V. cholerae* (including culturable toxigenic *V. cholerae*) regardless of the bacterial form (i.e., free-living bacteria or bacteria attached to phytoplankton, zooplankton and copepods). Various dilutions of the samples were enriched to maximize the chances of isolating and identifying *V. cholerae* clones, and every enrichment culture was analyzed.

Moreover, as it has been reported that cholera bacteria are also found in a viable but non-culturable state in the environment (Xu et al. 1982; Gauthier 2000), we also performed several PCR assays on each enrichment culture to detect toxigenic *V. cholerae* bacteria that may remain non-culturable. The application of both bacterial culture and PCR techniques on enrichment samples has been proposed by other groups (Mendes-Marques et al. 2013; Hill et al. 2011). This two-sided approach has proven to be effective as every station presented positive results by either one method or the other with respect to the presence of *V. cholerae*. However, the two approaches may yield conflicting results for technical reasons; a gene associated with a specific phenotype may be detected via PCR, while it may remain unexpressed or absent in isolated colonies. The inverse situation is also possible, as we analyzed diverse aliquots. It is probably for these reasons that we observed two discrepancies between selective culture and PCR regarding the detection of *V. cholerae* O1 with samples 27 and 31. Finally, to improve our chances of detecting toxigenic *V. cholerae*, we used two distinct PCR assays targeting the *ctxA* gene and systematically tested both pure and 10-fold dilutions of the DNA extracts.

Nevertheless, despite all the precautions that were taken, we found no evidence of the presence of toxigenic *V. cholerae* O1 in any of the samples collected. The absence of the *ctxA* gene not only highlights the absence of *V. cholerae* CT + bacterium, but it also suggests the absence of phages carrying the gene in water samples. These findings seem to contrast with those described by Hill et al. (2011) who isolated two culturable toxigenic *V. cholerae* O1 strains from two 30-L water samples among 14 samples (Hill et al. 2011). However, these two studies were not carried out in the same context. Hill et al. searched for toxigenic *V. cholerae* during the first epidemic wave in October-November 2010, when attack rates of cholera exceeded 2,000 new cases per day, with an epicenter around the Artibonite coast (Piarroux et al. 2011; Gaudart et al. 2013). At that time, many more infected individuals were likely to contaminate the environment with toxigenic *V. cholerae* via open-air defecation compared with the July 2012 period.

In contrast with the absence of toxigenic *V. cholerae* O1, numerous non-toxigenic non-O1 *V. cholerae* isolates were isolated in our study, even in very small sample volumes. Our results indicate that non-toxigenic *V. cholerae* are well established in freshwater and brackish Haitian aquatic environments. This is not surprising, as it has been demonstrated that the *V. cholerae* species can be isolated from many aquatic ecosystems throughout the world, including cholera-free areas, whether in freshwater (Bockemühl et al. 1986; Uchiyama 1998), brackish water (Barbieri et al. 1999; Jiang and Fu 2001; Louis et al. 2003) seawater (Wu et al. 1996; De Silva, Mantilla, and Agudelo 1997; Maugeri et al. 2004) or even wastewater (Lesne et al. 1991).

Our study shows that in July 2012 the bacterial levels of the imported toxigenic clone were far below the levels required for direct transmission to local human populations, despite the massive biomass disseminated in 2010-2011 by more than half a million cholera patients in a country where open-air defecation (WHO and UNICEF 2012) and the washing of clothes in rivers are widely practiced. The true level of exposure required to contract cholera is difficult to precisely assess. In a study performed in rural Bangladesh, Spira et al. (1980) have shown that people infected during the course of the study were unlikely to have ingested more than 10^5 viable organisms per

day (Spira et al. 1980), whereas a study by Cash et al. (1974) has established 10^4 as the minimum inoculum required to provoke diarrhea in healthy volunteers with neutralized gastric acid (Cash et al. 1974). In our study, the lack of toxigenic *V. cholerae* O1 detection using PCR assays on enrichment cultures was well established for 31 stations with 100-mL or 1-L sample volumes. This strongly suggests that all water samples analyzed contained less than 10 toxigenic *V. cholerae* bacteria per liter, a level 1000-fold below the dose that has been shown by Cash et al. to provoke diarrhea in healthy adults with neutralized gastric acid (Cash et al. 1974). Notably, as well water and domestic tanks were excluded from our sampling design, our findings do not preclude the possibility of higher levels of toxigenic *V. cholerae* O1 in peri-domestic water bodies following recent contamination by infected individuals.

Non-toxigenic *V. cholerae*, such as those identified in the Haitian aquatic environment in this study, may provoke gastroenteritis or sporadic cholera-like diarrhea in humans; however, these strains have never been implicated in large-scale cholera epidemics (Faruque and Mekalanos 2008). Only *V. cholerae* serogroup O1, both ‘classical’ and ‘El Tor’ biotypes, and the derivative serogroup O139 are known to cause cholera epidemics (Faruque and Mekalanos 2008). The relationship between all cholera isolates implicated in the seventh pandemic has recently been elucidated by Mutreja et al. (2011) in a study based on whole-genome sequencing of 154 *V. cholerae* strains collected from all over the world (Mutreja et al. 2011). By analyzing high-resolution markers (genome-wide single nucleotide polymorphisms), they showed that all strains isolated from various outbreaks during the seventh pandemic have a single common ancestor that emerged during the 1950s. Over time, the clones diversified. Most strains disappeared within a few years, while the remaining strains gave way to new pandemic waves spread by human activity. As we demonstrate that the imported epidemic *V. cholerae* strain has failed to settle in high levels in the aquatic environment of Haiti, our results are in total accordance with the Mutreja et al. findings. If the epidemic strains disseminated by humans could gain a foothold in the environment for an extended duration and eventually proliferate to levels compatible with epidemic reactivation via environment-to-human contamination, phylogenetic assessment of the 154 *V. cholerae* isolates analyzed by Mutreja et al. would not have

revealed the diversification and extinction phases that characterized the distinct pandemic waves since the emergence of the seventh pandemic (Mutreja et al. 2011).

According to Faruque and Mekalanos, the precursors of the pandemic clones probably displayed traits that are lacking in environmentally adapted *V. cholerae*, regardless of the serogroup (Faruque and Mekalanos 2008). As specified by these authors, the evolution of environmental strains into typical pathogenic strains would require more widespread gene transfer events than that shown to occur with known phages (Faruque and Mekalanos 2008). Inversely, the transmission of a toxigenic *V. cholerae* O1 strain could be dependent on the amplification-via-disease lifestyle, and the inability of the bacteria to re-establish in the environment might be due to the requirement of human host-dependent replication and transmission. Importantly, a recent study has found that the Haitian *V. cholerae* strain has failed to acquire any genes via horizontal gene transfer from the population of non-toxigenic *V. cholerae* bacteria residing in the local aquatic environment, thereby suggesting that environmental strains have probably played no role in the evolution of the outbreak strain (Katz et al. 2013b). In fact, our findings suggest that despite its massive dissemination, the toxigenic strain imported into Haiti may no longer be present in the environment at levels required for transmission to humans.

In conclusion, these findings provide hope that cholera could be eliminated from Hispaniola with the recovery of the last patient. Such an objective seems all the more realistic as the elimination of epidemic-causing *V. cholerae* strains has already been observed in Latin American countries such as Peru and Mexico (Harvez and Ávila 2013). Mexican coasts present many aquatic environments conducive to *V. cholerae* proliferation, and many rural populations still suffer from limited access to potable water and suitable health care (WHO and UNICEF 2012). As over 43,000 cases were reported in Mexico with a higher incidence in coastal states from 1991 to 1996, Mexico was predicted to become a cholera-endemic region (Borroto and Martinez-Piedra 2000). However, this pessimistic prediction failed to materialize, and the annual cholera incidence throughout the entire country dwindled down to 5 cases by the year 2000 (World Health Organization 2001) and 1 case the following year (World

Health Organization 2002). Strikingly, the disease has not been observed in this supposedly endemic country since 2001, and has obviously been extinguished in Mexico. Based on the current observations, the same outcome also seems plausible for Haiti, where cholera outbreaks during the rainy season appear to reemerge from persistent transmission foci insufficiently tackled during the dry periods rather than the commonly suspected aquatic reservoir of toxigenic bacteria.

9.2 The Dry Season in Haiti: a Window of Opportunity to Eliminate Cholera

9.2.1 Introduction

In October 2010, the importation of a toxigenic *Vibrio cholerae* strain in Haiti (Chin et al. 2011; Hendriksen, Price, and Schupp 2011; Frerichs et al. 2012a) led to a massive epidemic that rapidly spread throughout all 140 Haitian communes (Gaudart et al. 2013; Barzilay et al. 2013b). By the end of March 2013, the Public Health and Population Ministry (MSPP) had reported 652,730 cases, corresponding to an attack rate of 6.4%. With 8,060 reported deaths, the global case fatality rate was 1.2%. This historic epidemic, which occurred in a small but densely populated country (10.2 million inhabitants in 27,750 km²), accounted for over half of cholera cases and over one-third of cholera-associated deaths reported globally between 2010 and 2011 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). The disease also spread throughout the rest of Hispaniola island, and by late 2012, the Dominican Republic had recorded 29,433 cases and 422 deaths associated with cholera (Pan American Health Organization 2013).

Since the beginning of the epidemic, cholera incidence in Haiti has been characterized by alternating peak and lull phases, which were partly associated with the fluctuating dry, rainy and cyclonic seasons (Chin et al. 2011) (**Figure 6**).

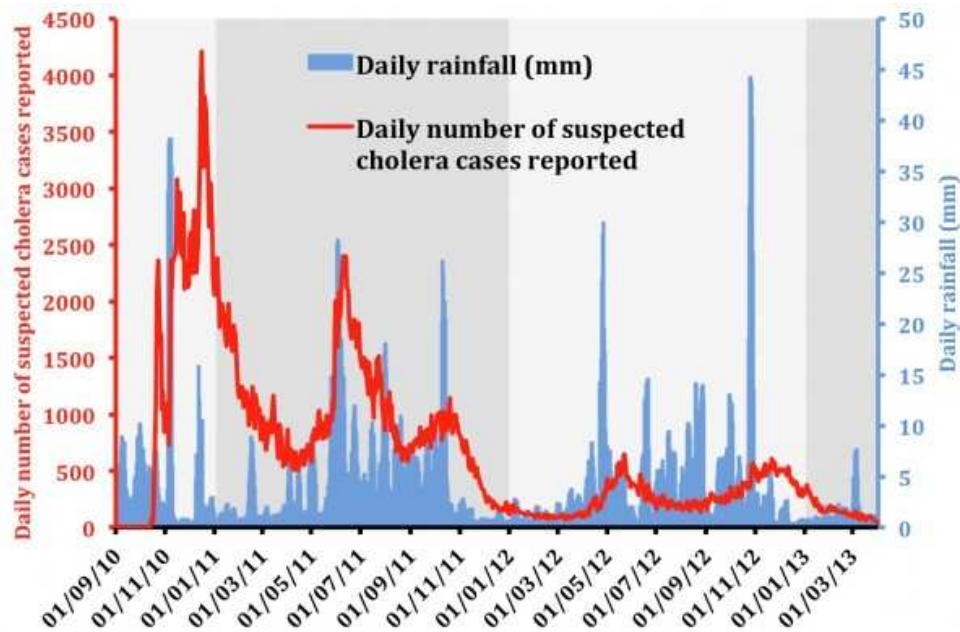


Figure 6. Evolution of the daily suspected cholera cases and rainfall between September 2010 and March 2013. Accumulated rainfall data were obtained from satellite estimates (TMPA-RT 3B42RT derived), averaged on the position 18.25N-19.75N / 74.25W-71.75W, and available at: http://disc2.nascom.nasa.gov/Giovanni/tovas/realtime.3B42RT_daily.2.shtml.

Haiti experienced the first marked decrease in cholera transmission during the dry season in early 2012 (67 cases/day reported in March). At that time, cholera had almost completely disappeared from the Southern Peninsula as well as the North-East and North-West departments according to the Haitian National Cholera Surveillance System (Ministère de la Santé Publique et de la Population 2013). In contrast, residual transmission was recorded in a few dozen communes located in the North (DSN), Artibonite (DSA) and Centre (DSC) departments as well as the conurbation of Port-au-Prince (West department). During the rainy season of April-May 2012, the reappearance of the epidemic was first reported in these geographic areas, before spreading to the areas where transmission seemed to have stopped during the dry season. By the end of 2012, 903 additional cholera-associated deaths had been reported.

However, the evolution of cholera epidemics is always difficult to predict, and there is no undisputable study demonstrating that cholera will settle in Haiti for decades to

come. Despite poor access to improved drinking water sources (69% of the population in 2010) and very poor use of improved sanitation facilities (17% of the population in 2010) (WHO and UNICEF 2012), the country and the rest of the island of Hispaniola had been spared from cholera for at least a century and probably since the beginning of recorded history (Jenson and Szabo 2011). Moreover, a Franco-Haitian study conducted in 2012 (Baron et al. 2013) has shown that the O1 strain responsible for the outbreak was overwhelmed by local non-O1 *V. cholerae* strains in a large set of Haitian aquatic environments. None of the rare O1 strains isolated in this study proved to be toxigenic. Thus, there is currently no evidence of a perennial and significant environmental reservoir for toxigenic *V. cholerae* in Haiti.

Therefore, we hypothesize that some areas of lingering cholera transmission during the dry season play an important role in the persistence of epidemics from one rainy season to the next and that the struggle against cholera in Haiti has failed to take advantage of the dry season opportunity. In this study, we aimed to describe both the dynamics of cholera during the dry season and the fight against the disease by targeting the remaining foci.

9.2.2 Methods

At the request of the MSPP, a field study was conducted from February 19 to March 29, 2013 to both establish an inventory of the remaining cholera transmission foci during the dry season and assess the prevention actions carried out by Haitian and international organizations. The four components of the survey consisted of the following aspects: (1) identify affected communes by analyzing the information available in the national database; (2) identify transmission foci based on the basic data recorded by cholera treatment health structures; (3) conduct a field study of the principal foci to confirm the presence of cholera, assess factors associated with transmission and examine the actions taken to control the epidemic since the beginning of the current dry season (in collaboration with representatives of the National Department of Drinking Water and Sanitation (DINEPA)); and (4) perform

interventions at the household level involving public awareness campaigns and the distribution of chlorine tablets.

Cholera-associated morbidity and mortality data at the commune level were provided by the Haitian Directorate of Epidemiology Laboratory and Research (DELR), which gathers, validates and analyzes anonymous data that are prospectively collected in the field by epidemiological surveillance officers. According to the WHO standard definition (Global Task Force on Cholera Control 2010), a probable cholera case is defined as a patient aged five years or older who develops acute watery diarrhea, with or without vomiting, located in an area where there is a cholera epidemic. In Haiti, all acute watery diarrhea cases are reported as suspected cholera, but the surveillance system separately records cases <5 and ≥5 years-old. Bacteriological confirmation of cases is routinely performed at the National Laboratory of Public Health (LNSP) using standard methods (Bopp, Ries, and Wells 1999).

The identification of concentrations of active cholera transmission was carried out by analyzing February and March 2013 cholera national databases. We assessed any commune presenting a bacteriological-confirmed cholera case OR a suspected cholera death (associated with concomitant reported cholera cases) OR more than 1 suspected cholera case per day (excluding patients less than five years of age to enhance the case definition specificity). We then visited the health facilities of as many at-risk communes as possible to identify the exact origin of cholera patients by interviewing the medical staff and reviewing the case registers of the last one to three months.

Field investigations were subsequently performed in identified suspected cholera foci to search for possible factors linked to cholera transmission. People and local health actors were also interviewed regarding the actions undertaken to stop the spread of cholera since the beginning of the dry season in December 2012. In Port-au-Prince, data on patient origin was provided by the staff of Médecins sans Frontières – Holland, who also described the actions performed to limit cholera transmission. Due to the time limitation, not all identified at-risk communes and transmission foci were

investigated. However, medical staff declaring the suspected cholera cases and/or local epidemiologists were interviewed by phone.

Maps of cholera morbidity, mortality and prevention interventions were generated using Quantum-GIS® v1.8.0 (Open Source Geospatial Foundation Project, Beaverton, OR, USA).

9.2.3 Results

From December 1, 2012 to March 31, 2013, 21,695 suspected cholera cases (≥ 5 years of age) and 238 related deaths were reported by the national surveillance system. However, most cases occurred in December and January (16,700 cases and 208 deaths) and a strong decrease in cholera incidence was noted in February and March with only 4,995 recorded cases and 30 deaths. In parallel, the percentage of positive samples cultured at the LNSP dropped from 68% in December and January to 41% in February and March (**Table 4**).

| Department | December 2012 | | January 2013 | | February 2013 | | March 2013 | | Entire study period | | |
|------------|---------------|-------------|--------------|-------------|---------------|-------------|-------------|--------------|---------------------|--------------|-------|
| | pos | neg | pos | neg | pos | neg | pos | neg | pos | neg | Total |
| West | 85 (77%) | 25 (23%) | 50 (57%) | 38 (43%) | 38 (39%) | 59 (61%) | 32 (51%) | 31 (49%) | 205 (57%) | 153 (43%) | 358 |
| Artibonite | 44 (70%) | 19 (30%) | 31 (70%) | 13 (30%) | 23 (56%) | 18 (44%) | 7 (26%) | 20 (74%) | 105 (60%) | 70 (40%) | 175 |
| South-East | 25 (66%) | 13 (34%) | 12 (80%) | 3 (20%) | 6 (38%) | 10 (63%) | 4 (36%) | 7 (64%) | 47 (59%) | 33 (41%) | 80 |
| Centre | 15 (75%) | 5 (25%) | 0 (0%) | 8 (100%) | 0 (NA) | 0 (NA) | 0 (0%) | 15 (100%) | 15 (35%) | 28 (65%) | 43 |
| North | 2 (100%) | 0 (0%) | 0 (0%) | 2 (100%) | 0 (0%) | 4 (100%) | 6 (86%) | 1 (14%) | 8 (53%) | 7 (47%) | 15 |
| Total | 171 (73%) | 62 (27%) | 93 (59%) | 64 (41%) | 67 (42%) | 91 (58%) | 49 (40%) | 74 (60%) | 380 (57%) | 291 (43%) | 671 |

Table 4. Bacteriological confirmation of cholera at the LNSP. Evolution of the monthly number of positive and negative samples (% of total) from December 2012 to March 2013. LNSP, National Laboratory of Public Health, Port-au-Prince pos; positive culture; neg, negative culture.

As shown in **Figure 7**, cholera morbidity and mortality distribution also exhibited a high degree of spatial heterogeneity, particularly at the end of the study period, when cholera seemed to persist almost exclusively in the three departments of DSN, DSC and DSA. Notably, only a few communes were significantly affected, even at these sites. In the South Peninsula and Port-au-Prince, the few sporadic cases were associated with a very low fatality rate and were probably non-cholera-associated diarrhea.

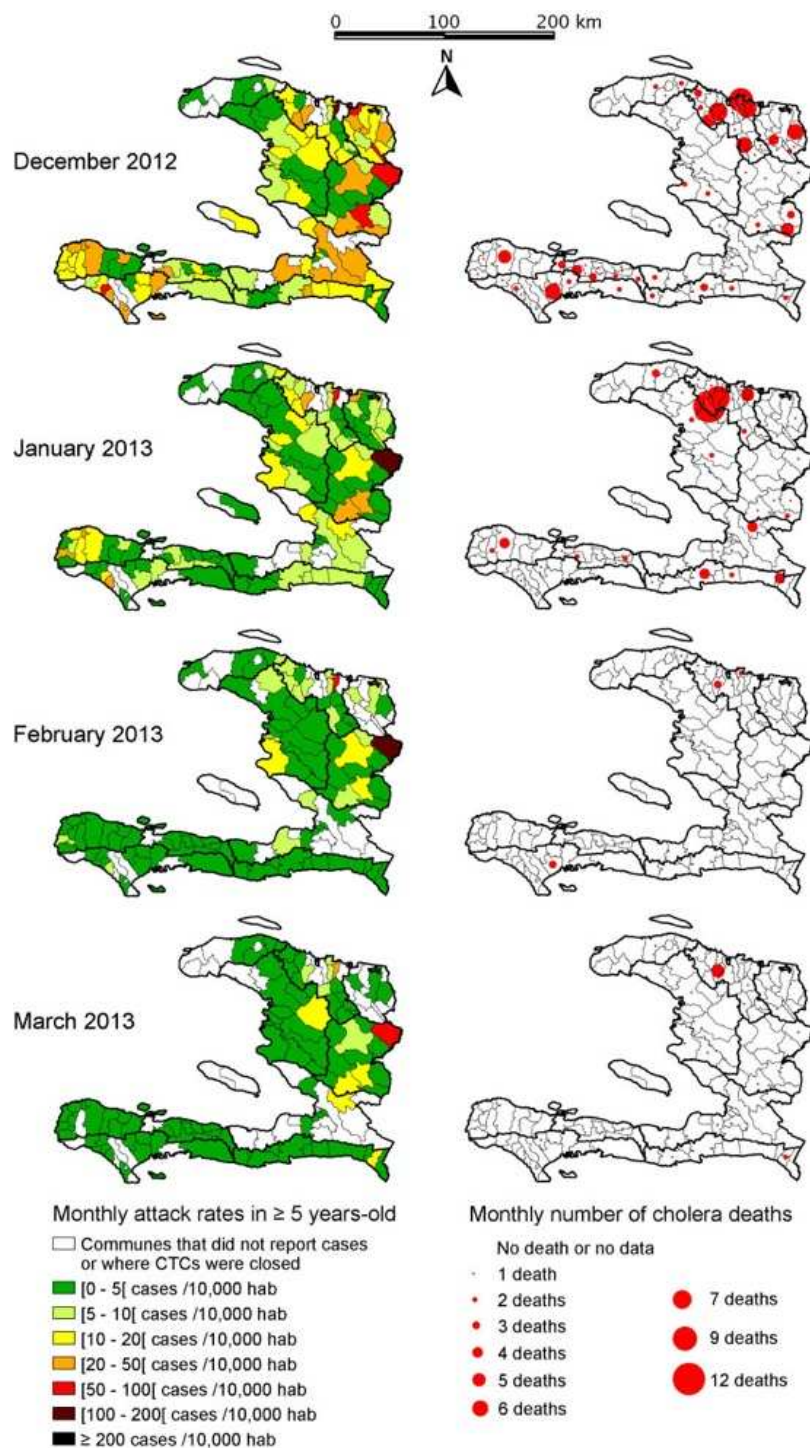


Figure 7. Monthly cholera attack rates and number of cholera-associated deaths in patients ≥ 5 years of age during the dry season 2012-2013.

Twenty-four provincial communes with likely active cholera transmission were identified (**Table 5, Figure 8**). All but eight communes were localized in the Artibonite (DSA), Centre (DSC) and North (DSN) departments. These 24 communes accounted for

69% of the total cases (≥ 5 years of age) recorded throughout the country, with a monthly attack rate of 6.0 cases/10,000 inhabitants. A total of 26 deaths was reported in these communes, and the cholera case fatality rate was 0.8%.

| Department | Commune | Estimated population in 2012 ² | <5 ³ No of cases ³ | ≥ 5 ³ No of cases ⁴ | ≥ 5 ³ Attack rate ⁵ | ≥ 5 ³ Cholera deaths | Bacteriological confirmation | Date of last confirmation |
|--|---------------------|---|---|---|---|---|------------------------------|---------------------------|
| Artibonite | Gonaïves | 340,155 | 70 | 198 | 5.8 | 1 | | 13/12/12 |
| Artibonite | Gros Morne | 148,627 | 13 | 141 | 9.5 | 0 | Yes | 14/02/13 |
| Artibonite | Saint-Marc | 254,543 | 60 | 515 | 20.2 | 1 | Yes | 05/03/13 |
| Artibonite | Saint-Michel | 143,679 | 81 | 198 | 13.8 | 0 | | |
| Artibonite | Verrettes | 138,242 | 44 | 110 | 8.0 | 1 | | |
| Centre | Cerca-la-Source | 5,397 | 97 | 113 | 209.4 | 1 | | |
| Centre | Hinche | 115,381 | 160 | 223 | 19.3 | 0 | | |
| Centre | Lascahobas | 43,790 | 60 | 147 | 33.6 | 0 | | 07/12/12 |
| Centre | Mirebalais | 93,319 | 98 | 251 | 26.9 | 1 | | 21/12/13 |
| North | Borgne | 63,885 | 32 | 55 | 8.6 | 1 | | |
| North | Cap-Haitien | 261,952 | 43 | 359 | 13.7 | 3 | Yes | 22/03/13 |
| North | Limbe | 81,431 | 28 | 146 | 17.9 | 8 | | |
| North | Pilate | 51,597 | 7 | 66 | 12.8 | 0 | | |
| North | Plaisance | 66,426 | 13 | 50 | 7.5 | 1 | | |
| North | Quartier Morin | 26,117 | 87 | 247 | 94.6 | 0 | Yes | 11/03/13 |
| North | Saint Raphael | 51,316 | 7 | 44 | 8.6 | 1 | | |
| North-West | Port de paix | 194,719 | 13 | 65 | 3.3 | 1 | | |
| West-West | Saint Louis du Nord | 111,070 | 11 | 60 | 5.4 | 1 | | |
| West | Cabaret | 65,148 | 95 | 65 | 10.0 | 0 | | |
| West | Leogane | 190,746 | 0 | 147 | 7.7 | 0 | | |
| West | Thomazeau | 50,558 | 5 | 74 | 14.6 | 0 | | |
| South | Les Cayes | 144,813 | 3 | 56 | 3.9 | 3 | | |
| South-East | Jacmel | 178,756 | 2 | 40 | 2.2 | 0 | Yes | 25/02/13 |
| South-East | Thiotte | 33,341 | 5 | 54 | 16.2 | 2 | | |
| Total of the 24 selected provincial communes | | 2,855,008 | 1,034 | 3,424 | 12.0 | 26 | | |
| Port-au-Prince conurbation (7 communes) ¹ | | 2,599,952 | 231 | 738 | 2.8 | 1 | Yes | 01/03/13 |
| Rest of Haiti (109 communes) | | 4,913,113 | 301 | 833 | 1.7 | 3 | No | |
| Total Haiti (140 communes) | | 10,368,073 | 1,566 | 4,995 | 4.8 | 30 | | |

Table 5. Communes with likely active cholera transmission in February and March 2013 (See Figure 8 for localizations). ¹ Port-au-Prince conurbation = communes of Port-au-Prince, Carrefour, Delmas, Petionville, Cite-Soleil, Tabarre and Kenskoff; ² calculated from 2003 Census and provided by Haitian Ministry of the Interior and Territorial Collectivities; ³ <5 years of age and ≥ 5 years of age; ⁴ cases suspected by acute watery diarrhea; ⁵ attack rates presented in cases /10,000 inhabitants.

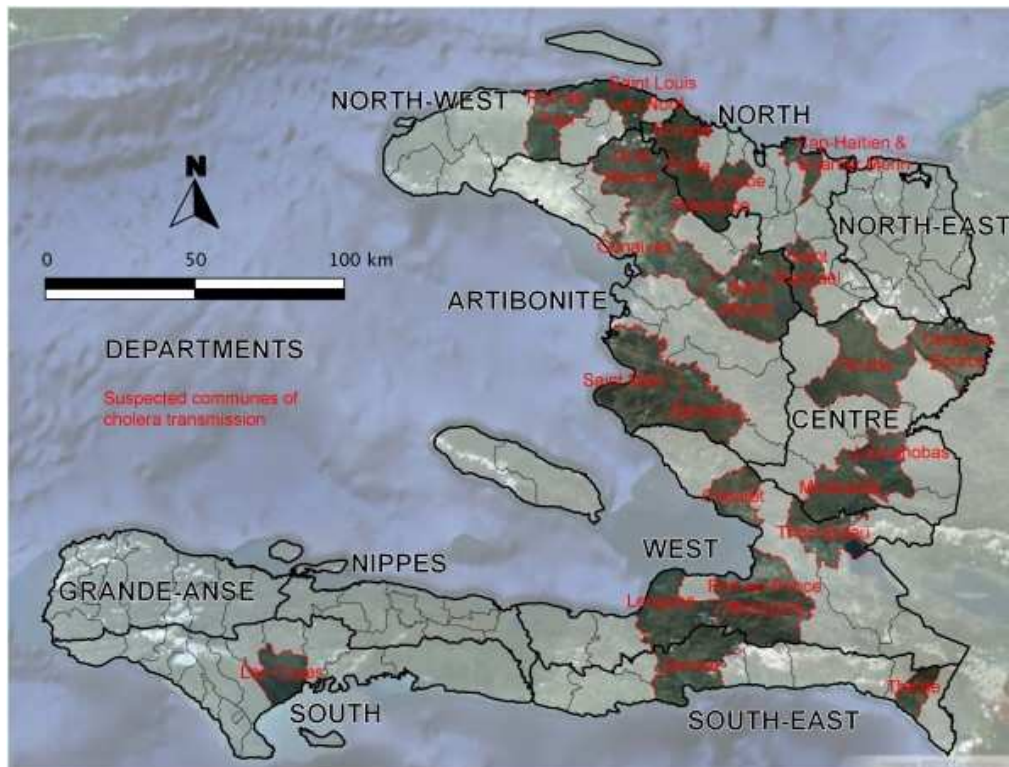


Figure 8. Communes likely affected by active cholera transmission in February and March 2013 (See Table 5 for characteristics). Several communes, such as Jacmel, Leogane and Plaisance, were only affected in February. Thiottle was exclusively affected in March.

A few dozen confirmed cases were reported in Port-au-Prince conurbation with only one suspected cholera-associated death (**Table 4** and **Table 5**). However, transmission remained sporadic in the capital, thereby yielding a low attack rate (1.4 cases/month/10,000 inhabitants). Moreover, the low case fatality rate (0.1%) demonstrates that most of these cases of acute watery diarrhea were probably not due to cholera infection.

In the 109 remaining communes, the attack rate (0.8 cases/month/10,000 inhabitants) was even lower than that observed in Port-au-Prince. Only three deaths were recorded (one in Camp Perrin, one in Grande-Rivière-du-Nord and one in Bainet), and cholera was not biologically confirmed for any of these cases. Therefore, in the absence of other suspected cholera cases at the same period, we considered it unlikely these cases were associated with a local transmission of cholera.

Investigations were carried out in 12 of the 24 communes with suspected active cholera transmission. These communes were located in DSN (Cap-Haïtien, Quartier-Morin, Port-Margot and Borgne), DSC (Hinche, Mirebalais and Cerca-la-Source) and DSA (Saint-Marc, Gonaïves, Saint-Michel-de-l'Attalaye, Ennery and Gros Morne). Based on the field investigations, we identified 49 areas where clusters of cases had recently been reported. Taken together, these 49 areas accounted for 56% of cases reported in these 12 communes during the analysis period.

In DSN, the principal cholera residual focus was located in the city of Cap-Haïtien. Some cases were biologically confirmed by the LNSP. Note that patients from Cap-Haïtien were also treated in the nearby commune of Quartier Morin, which remained almost free of cholera. In Cap-Haïtien, cases were concentrated in several neighborhoods without proper access to clean drinking water. The water supply network had been out of order for 25 years throughout most of the city, and the population used water treated via reverse osmosis, which does not have the anti-bacterial properties of chlorinated water. The field assessment highlighted the presence of numerous boreholes and traditional shallow wells widely contaminated with fecal matter due to their proximity to unprotected latrines. The collection of feces in plastic bags thrown onto the roof was said to be a common practice. Markets with no access to clean water and very poor food preservation practices were also likely sites of contamination.

In DSC, the commune of Cerca-la-Source reported the highest attack rates (126.0 and 83.4 cases/month/10,000 inhabitants in February and March, respectively). Only one suspected cholera-related death was reported (**Figure 7**). The field assessment showed that patients treated at the Cholera Treatment Center (CTC) were primarily children. As all samples delivered to LNSP for bacteriological confirmation were negative for *V. cholerae* and 90% (38/42) of the cholera rapid tests performed during the first half of March were negative, we noted a low level of cholera in this commune.

In DSA, the most active remaining foci were located in the town of Saint-Marc and several of its neighborhoods. Even though a private company sold chlorinated water,

most of the population still relies on manually operated boreholes without home water treatment. In certain fishermen quarters, the general practice of defecating along the beach renders the contamination of open storage buckets of water highly likely. Cholera outbreaks occurred in several rural localities near Saint-Marc, where people rely exclusively on unprotected natural water sources. In addition to Saint-Marc, some cases were regularly reported in Gonaïves, where the municipal water network had been severely damaged by the hurricanes, as well as Saint-Michel-de-l'Attalaye. In the latter commune, most recent cases originated from the urban and peri-urban areas. Two water networks supplied this town with water and standposts. However, water was not treated for several weeks because of a chlorine shortage. In-house chlorination practices were almost never observed in those quarters, which were also largely deprived of latrines.

In all areas affected by cholera, community health activities appeared insufficient and inappropriately targeted since the beginning of the dry season 2012-2013 (**Figure 9**). Twenty-three of the 49 identified foci had not been previously investigated since December 2012. Thirteen had been investigated, especially in Gonaïves and Saint-Michel-de-l'Attalaye, but no intervention aimed to prevent transmission in the community had been initiated by MSPP, DINEPA or international partners. Only 13 foci among those visited had thus benefited from at least one intervention since early December 2012. At three of these 13 foci, prevention activities had been limited to awareness campaigns, which was sometimes due to the absence of available chlorination products (such as that found in Borgne, DSC). At least one distribution of water treatment products +/- soaps associated with an awareness campaign had nevertheless been organized in the 10 remaining foci, often with the assistance of NGOs such as Action-Contre-la-Faim (e.g., Saint-Michel-de-l'Attalaye in December) or Partners in Health (e.g., the Goyaviers section of Saint-Marc in December and January). A few distributions had been organized by the MSPP (e.g., Ennery in December and the rural Grand-Boucan portion of Mirebalais in January), while only one distribution event was launched by DINEPA in Borgne in December. Unfortunately, the majority of these distributions failed to target the principal transmission foci of the corresponding communes, which were primarily located in towns or the close outskirts, and instead

exclusively focused on rural communities. Yet, some of these well-performed rural prevention events, such as that carried out in Gros Morne (DSA), proved effective with the subsequent local disappearance of cholera.

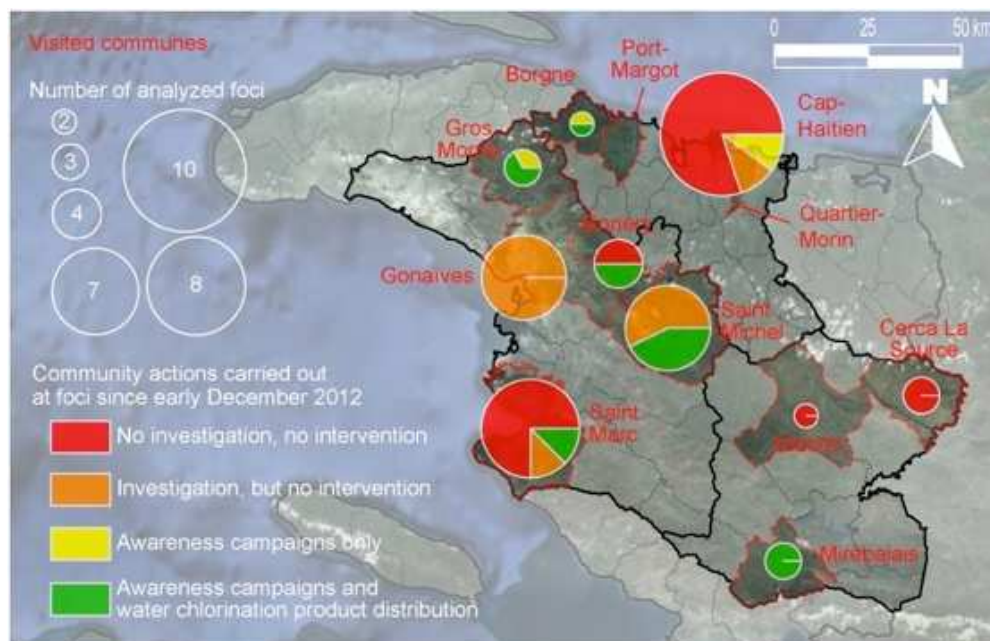


Figure 9. Residual transmission foci identified in the 12 visited communes and community actions carried out since early December 2012.

In Port-au-Prince conurbation, the low incidence and death rate associated with cholera may be due to the community actions implemented by NGOs. In particular, during the 2013 dry season, Médecins sans Frontières – Holland staff continued identifying the areas associated with clusters of cases, where they organized awareness campaigns, water treatment product distribution and free bucket chlorination stations.

9.2.4 Discussion

Thirty months after cholera onset, the disease is still present in Haiti. The disease attack rate has varied considerably since the beginning of the epidemic, with peaks during the rainy season and relative lulls during the dry season in 2011 and 2012 (Barzilay et al. 2013b). Our results show that the lull was even more pronounced in

February and March 2013. In particular, of the 140 Haitian communes, 109 showed no sign of cholera transmission during early 2013 for more than two months. Indeed, in these 109 communes, there has been no confirmed case, significant group of cases, or death in the context of grouped diarrheic patients. Even if small cholera outbreaks may have remained unnoticed by the surveillance system in resource-deprived areas, it is unlikely, in the current context of Haiti, that an outbreak with subsequent cholera deaths would go completely unnoticed for two consecutive months. Only sporadic suspected cholera patients were reported, with an average of less than five cases per commune per month, which represents less than one case per 10,000 inhabitants per month. It is thus likely that, in these 109 communes, almost all of isolated and unconfirmed cases were associated with afflictions other than cholera. Note that, even if these cases of diarrhea were not due to cholera, the background noise of one monthly case of acute watery diarrhea per 10,000 inhabitants would yield 1,000 cases per month or 12,000 cases per year for the entire country of Haiti, which could portray the false impression of persistent endemic cholera. It is therefore of upmost importance to perform a microbiological confirmation of cases as attack rates decrease.

In February and March 2013, the majority of suspected cases were concentrated in a small number of urban and rural foci, almost all of which were located in the northern half and often in inland locales. Haitian estuaries did not seem to be particularly affected. Even in these residual foci, an overestimation of cases is possible. A pooled case fatality rate of only 0.8% in the 24 communes with potential cholera transmission appears indeed implausible, considering persisting difficulties at numerous CTCs (Doctors Without Borders 2013). A marked proportion of these cases of acute watery diarrhea, especially in children under five years of age, were probably not due to cholera. For instance, in the commune of Cerca-la-Source (DSC), biological tests performed on various inpatient samples were negative.

Nevertheless, cholera did not completely disappear, as active foci with laboratory-confirmed cases remained, such as that observed in the urban community of Cap-Haïtien. At this site, the vast majority of reported cases (87%) corresponded to

patients over five years of age, which represents a percentage consistent with an ongoing cholera outbreak. Finally, the metropolitan area of Port-au-Prince displayed a markedly lower attack rate (1.4 cases per 10,000 inhabitants per month) than the 24 municipalities that were likely affected. Moreover, the extremely low lethality rate (0.1%) indicates that the majority of cases were probably associated with other diarrheal diseases. However, even in Port-au-Prince, a few laboratory-confirmed cases were reported at least until the beginning of March.

Currently, no evidence of persistence of toxigenic *V. cholerae* in the Haitian environment at a significant level has been reported. In fact, an environmental study performed during the warm and rainy season 2012 failed to detect toxigenic *V. cholerae* via both culture and polymerase chain reaction analyses, even in estuaries (Baron et al. 2013). This does not allow to completely exclude the presence of a few toxigenic *V. cholerae* bacteria in the aquatic environment; however, considering that a minimum inoculum is required to provoke cholera (Cash et al. 1974), it is unlikely that undetectable concentrations of culturable or viable-but-non-culturable toxigenic *V. cholerae* in Haitian surface waters may greatly influence the dynamic of the current epidemic. Conversely, the remaining cases and small outbreaks that were still ongoing at the end of the dry season will thus likely play a leading role in the re-emergence of cholera during the rainy season. The fight against the infectious agent must therefore target this persisting human reservoir. As incidence of cholera decreases during the dry season, it is all the more important to enhance the fight against cholera transmission. Unfortunately, our field investigations show that low attack rates were often interpreted as evidence of “residual disease”. The few reported cases and deaths were considered as “acceptable”, and the investigation of small outbreaks and the hunt for the last remaining cases were therefore neglected. Such an attitude does not appear relevant if, as stated in the governmental strategic plan to fight cholera, the aim of the struggle is to eliminate cholera (République d’Haïti, Ministère de la Santé Publique et de la Population, and Direction Nationale de l’Eau Potable et de l’Assainissement 2012). In 2012, the lull of the dry season had been followed by new epidemic waves responsible for 903 additional deaths (Ministère de la Santé Publique et de la Population 2013). Such a number of deaths is too high to not consider cholera

as a priority. This is all the more important now that international funding is currently lacking, which has resulted in a deterioration in the quality of care at treatment centers as recently noted in an MSF press release (Doctors Without Borders 2013) and a halt in community prevention activities.

To enhance the effectiveness of the fight during the dry season, interventions should be targeted on active foci, which must be detected as early as possible and immediately investigated. Suspected cases should be confirmed via microbiological testing, as the risk of inaccurate cholera diagnosis is important especially when the number of reported cases diminishes. Actions should primarily focus on access to clean water via the establishment or rapid repair of distribution networks when possible and the free distribution of treatment products in the other cases. These actions are all the more effective when competent technicians apply practical solutions adapted to the local context in populations still plagued by recent cholera cases.

Vaccination should only be a supplementary element in the strategy to eliminate the disease. Indeed, a meta-analysis conducted in 2011 by the Cochrane collaboration showed that the effectiveness of current vaccines was only 52% in the first year and 62% during the second year (Sinclair et al. 2011). This overall rate of protection is lower in children less than five years of age (38% versus 66% for 5 years and older). As long-term effectiveness of the vaccine has not been demonstrated, WHO recommends re-vaccination after two years (WHO 2010). Given the lack of demonstrated efficacy in young children, vaccination should therefore be reserved for individuals over five years of age. Vaccination programs should always be accompanied with public awareness campaigns and actions to improve access to safe water and sanitation. In the current context of vaccine shortages, massive untargeted vaccination throughout the country remains unrealistic. Instead, vaccination sites should be determined in real time based on epidemiological observations and microbiological confirmation. Inadequately targeted campaigns would have a high cost and yield poor results in impeding cholera transmission. Finally, there is a need to ensure that the financial and manpower resources required for vaccination

campaigns do not impair those necessary to conduct preventive actions based on the promotion of enhancing hygienic practices and supplying safe water.

9.2.5 Conclusion

As observed at the beginning of 2013, it is evident that cholera continues to affect Haiti. Epidemiological data and studies on environmental strains have shown that toxigenic *V. cholerae* O1 persists only in the human reservoir, without a significant presence in the aquatic environment. As seen in 2011 and 2012, there is a great risk that cholera will re-emerge during the upcoming rainy season. The current situation should neither be seen as an acceptable background of cases nor justify a cutback in preventive actions. On the contrary, during this period of low incidence, control activities targeting residual foci are more likely to be effective. Unfortunately, community health activities appear insufficient and poorly suited. The present analysis, mildly severe but objectively documented, should be received as an incentive to maximize efforts to prevent new outbreaks during the rainy season and ultimately eliminate cholera from Haiti and the island of Hispaniola. Not long ago, other Latin America countries were able to achieve this radical goal (Harvez and Ávila 2013). Cholera must remain a health emergency and not a development issue. Cholera will not become endemic in the country if the disease is not endemic in the minds of the people.

10 Analysis of *V. cholerae* isolates from the Democratic Republic of the Congo

The eastern DRC represents one of the major cholera hotspots on the planet. Together with our collaborators, we analyzed a comprehensive panel of *V. cholerae* isolates using antibiotic resistance assays and MLVA typing. Both clinical and environmental samples were included. Overall, our analysis of strain characteristics and fluctuations coupled with existing epidemiological studies and field investigation reports shed light on the underlying dynamics of cholera in this country. Our analyses also answered specific questions concerning the 2011/2012 cholera epidemic that spread out of the eastern endemic zone in eastern DRC and crossed the country in just a few months to affect the capital of Kinshasa, which had previously been untouched by cholera outbreaks for an entire decade.

10.1 Integrated analysis of antibiotic susceptibility and genetic profiles of 1093 *Vibrio cholerae* isolates from the Democratic Republic of the Congo

Cholera is an acute intestinal infection caused by *V. cholerae* (Sack et al. 2004b). Although hydration remains the primary treatment for cholera, antibiotic therapy is recommended for severely ill patients (Centers for Disease Control and Prevention 2013). However, multi-drug resistant *V. cholerae* strains have long been observed in Africa (Finch et al. 1988), and strains exhibiting new resistance phenotypes have emerged during recent epidemics (Ngandjio et al. 2009). It is therefore critical to carefully monitor changes in strains' susceptibility to antimicrobial drugs in each African country and adapt treatment recommendations accordingly.

Few longitudinal studies assessing shifts in the resistance of *V. cholerae* to antimicrobial drugs in Africa have been established. The available studies are limited either to a restricted area (Mandomando et al. 2007) or a short time period (Materu et al. 1997). We describe the long-term evolution of antimicrobial drug susceptibility of an extensive set of *V. cholerae* isolates collected in the Democratic Republic of the Congo (DRC). We applied whole-genome sequencing and multiple locus variable-number tandem-repeat analysis (MLVA) to clarify the mechanisms behind the aggressive epidemic of 2011–2012 that spread throughout the country, affecting regions to which cholera was not endemic (Bompangue et al. 2012).

10.1.1 The Study

Sample collection included all available isolates from major outbreaks in the DRC during 1997–2012, which were stored at the National Institute of Biomedical Research. The **Table 6** shows the locations where the 1,093 tested isolates were collected.

| Year | Province | | | | | | | | | | Total |
|-------|----------|-----------|----------|----------------|---------|----------|---------|------------|----------|------------|-------|
| | Bandundu | Bas-Congo | Equateur | Kasai-Oriental | Katanga | Kinshasa | Maniema | North Kivu | Oriental | South Kivu | |
| 1997 | 0 | 4 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 6 |
| 1998 | 0 | 2 | 0 | 0 | 15 | 14 | 0 | 4 | 7 | 0 | 42 |
| 1999 | 8 | 0 | 0 | 0 | 1 | 22 | 0 | 0 | 0 | 0 | 31 |
| 2000 | 3 | 0 | 10 | 0 | 0 | 13 | 0 | 0 | 0 | 0 | 26 |
| 2001 | 0 | 0 | 0 | 0 | 16 | 0 | 0 | 0 | 0 | 0 | 16 |
| 2002 | 0 | 0 | 0 | 0 | 44 | 0 | 0 | 0 | 0 | 0 | 44 |
| 2003 | 0 | 0 | 0 | 19 | 21 | 4 | 0 | 0 | 0 | 0 | 44 |
| 2004 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 3 | 0 | 0 | 6 |
| 2005 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 3 |
| 2006 | 2 | 3 | 0 | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 14 |
| 2007 | 0 | 0 | 0 | 0 | 15 | 1 | 0 | 1 | 5 | 0 | 22 |
| 2008 | 0 | 0 | 0 | 0 | 30 | 0 | 5 | 4 | 17 | 0 | 56 |
| 2009 | 0 | 0 | 0 | 0 | 112 | 0 | 0 | 2 | 0 | 11 | 125 |
| 2010 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 5 | 15 |
| 2011 | 46 | 0 | 17 | 0 | 0 | 73 | 4 | 151 | 45 | 0 | 336 |
| 2012 | 11 | 25 | 16 | 0 | 1 | 27 | 0 | 196 | 31 | 0 | 307 |
| Total | 70 | 34 | 43 | 19 | 276 | 158 | 9 | 363 | 105 | 16 | 1093 |

Table 6. Distribution of 1,093 *V. cholerae* isolates, by year and province, Democratic Republic of the Congo, 1997–2012.

V. cholerae O1 strains stored in nutrient agar during 1997–2012 were cultured on thiosulfate citrate bile salts agar and nutrient agar at the National Laboratory, Kinshasa, DRC. The strains were enriched in alkaline peptone water liquid medium and incubated at 37°C for 18–24 h. Biochemical and serogroup characterization was subsequently performed according to standard protocols (Bopp, Ries, and Wells 1999).

Antimicrobial drug susceptibility testing of 1,093 confirmed *V. cholerae* isolates was performed by using the Kirby-Bauer disk diffusion method (Jorgensen and Turnidge 2007) with Mueller agar (bioMérieux, Marcy l'Etoile, France). *V. cholerae* O1 Ogawa and Inaba reference strains (ATCC) served as controls. Isolates were tested against nine antimicrobial drugs as follows: ampicillin (10 µg), chloramphenicol (30 µg), sulfamethoxazole/trimethoprim (1.25 + 23.75 µg), tetracycline (30 µg), doxycycline (30 µg), norfloxacin (5 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), and erythromycin (30 µg) (bioMérieux). Interpretation of inhibition diameters (sensitive, intermediate,

and resistant) was performed according to Clinical and Laboratory Standards Institute guidelines (Clinical and Laboratory Standards Institute 2006). When no interpretive criteria for *V. cholerae* were available from these guidelines, breakpoints for *Enterobacteriaceae* were applied by using *Escherichia coli* ATCC 25922 for quality control. The few intermediate results were categorized as resistant for this study. The strains were then regrouped into 21 resistance profiles.

Seventy-four clinical isolates from the 2011–2012 epidemic, spatiotemporally representative of outbreak diffusion, were subcultured and transported to Marseille, France. In Marseille, the strains were recultivated and identified as previously described (Rebaudet et al. 2014). For DNA extraction, a 50-colony aliquot of cultured cells was suspended in 500 µL NucliSENS easyMAG lysis buffer (bioMérieux). DNA was extracted by using a NucliSENS easyMAG platform (bioMérieux) according to the manufacturer's instructions. MLVA-based genotyping of the *V. cholerae* isolates and eBURST analysis (<http://eBURST.mlst.net>) were performed as previously described (Rebaudet et al. 2014). To perform a phylogenetic assessment of the core *V. cholerae* genome on the basis of genome-wide single nucleotide polymorphisms, whole-genome sequencing was performed on an isolate (L286) collected at epidemic onset by using a HiSeq Illumina System (Illumina, San Diego, CA, USA) as previously described (Mutreja et al. 2011).

A spatiotemporal analysis was performed on the basis of the antibiogram profiles of *V. cholerae* isolates collected in the DRC during 1997–2012. The strain profiles were plotted by year (**Figure 10**) and then mapped by year and province. Using these data, we regrouped them into five representative periods (**Figure 11**). The *V. cholerae* strains displayed an increasingly complex resistance phenotype to various antimicrobial drugs. Sulfamethoxazole/trimethoprim resistance was observed initially, followed by resistance to nalidixic acid, erythromycin, and chloramphenicol during the early 2000s. Although sensitivity to fluoroquinolones seemed to be preserved, strain resistance patterns continued to evolve with the circulation of isolates resistant to tetracyclines and ampicillin from 2007–2010. Finally, isolates collected during 2011–2012, which was marked by the westward spread of a major epidemic (Bompangue et

al. 2012), displayed a single antimicrobial drug susceptibility profile: resistance to most antimicrobial drugs except cyclines and fluoroquinolones.

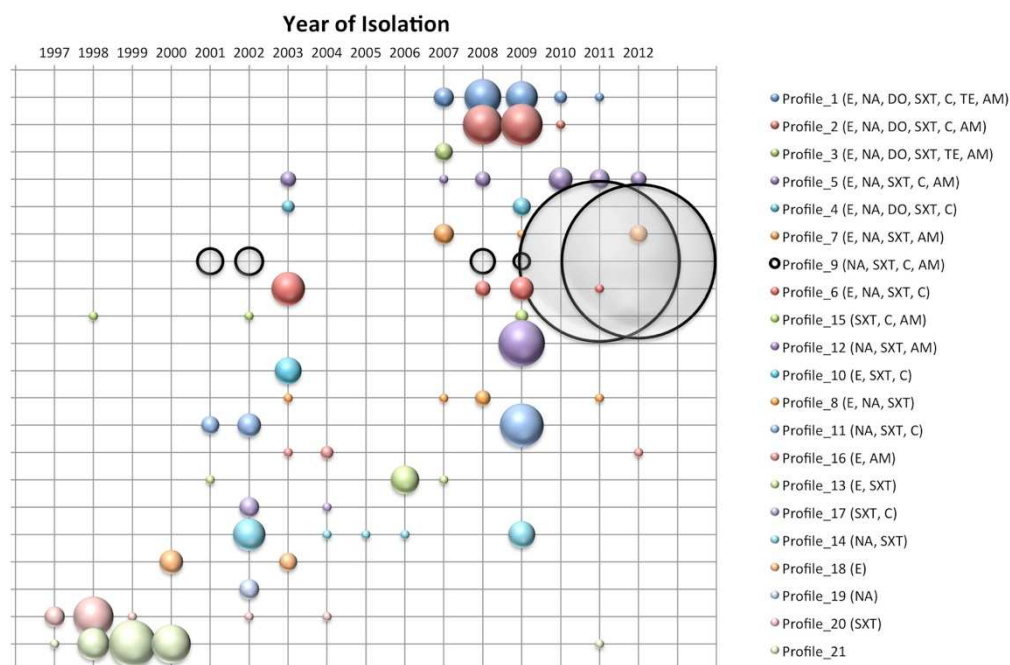


Figure 10. *Vibrio cholerae* strain antimicrobial drug resistance profiles plotted by year, Democratic Republic of the Congo, 1997–2012. On the basis of the antibiogram results, strains were grouped into 21 antimicrobial drug resistance profiles. The antimicrobial drugs to which the strains displayed resistance are indicated on the right. Circle circumference represents the relative number of isolates per profile. AM, ampicillin; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; DO, doxycycline; NOR, norfloxacin; CI, ciprofloxacin; NA, nalidixic acid; E, erythromycin.

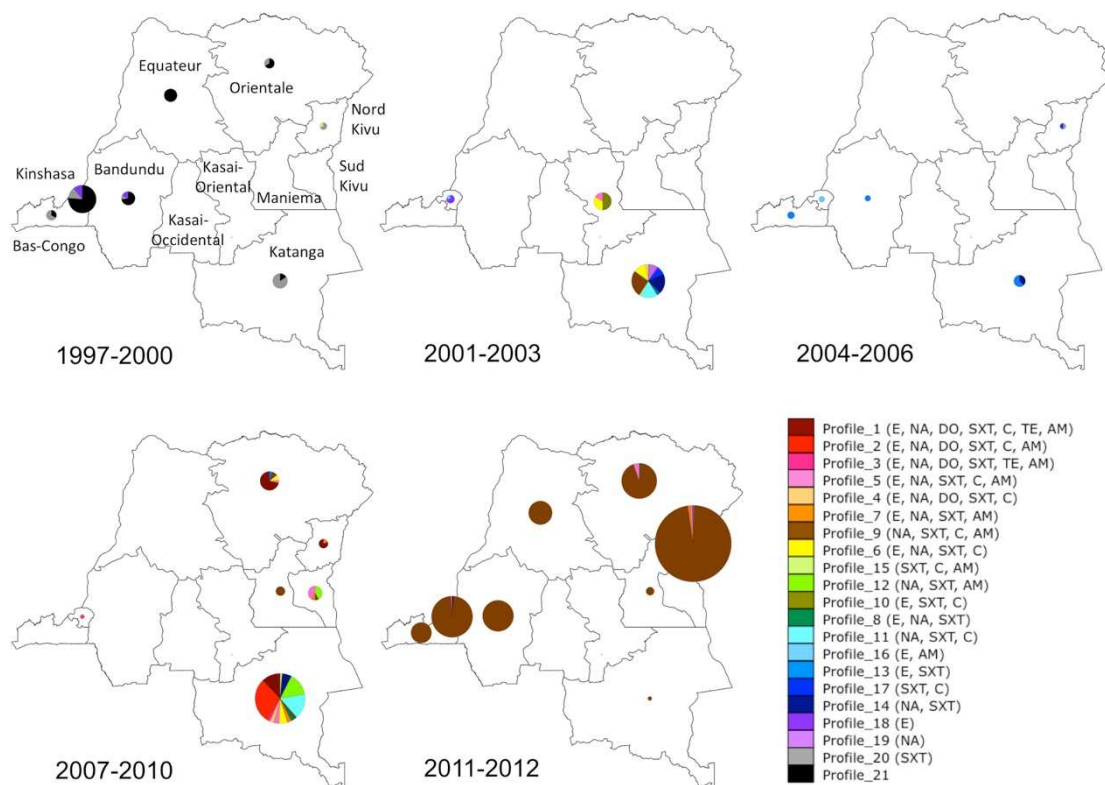


Figure 11. Spatiotemporal localization of isolate antimicrobial drug resistance profiles by time period and province, Democratic Republic of the Congo, 1997–2012. Strains were grouped into 21 antimicrobial drug resistance profiles. The antimicrobial drugs for which the strains displayed resistance are indicated in the lower right panel. Patterns of antimicrobial drug resistance were further grouped into five periods. Circle circumference represents the relative number of strains, while the colors correspond to the different antimicrobial drug resistance profiles. Provinces are indicated in the 1997–2000 map. The maps were generated by using QGIS version 2.4.0-Chugiak (<http://qgis.org/api/2.4/>). AM, ampicillin; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; DO, doxycycline; NOR, norfloxacin; CI, ciprofloxacin; NA, nalidixic acid; E, erythromycin.

Serotype analysis of the 1,093 *V. cholerae* isolates showed that Inaba strains were restricted to the western region of the country, Ogawa strains were isolated in the east and south, and Hikojima strains were restricted to Oriental Province, in the northeastern region of the country. During 2001–2010, Inaba and Ogawa serotypes

were observed, but Ogawa predominated; during 2011–2012, these serotypes switched, and the Ogawa serotype was almost completely replaced by Inaba.

To examine the particular 2011–2012 epidemic that spread throughout the DRC (Bompangue et al. 2012), 74 *V. cholerae* isolates were assessed by using MLVA and eBURST analysis. Overall, the isolates displayed 19 different MLVA genotypes, of which 18 grouped into one clonal complex. eBURST analysis indicated that the clonal complex likely arose from a founder strain identified at the beginning of the epidemic. Furthermore, whole-genome sequence analysis of an isolate identified in March 2011 in Lubunga, Oriental Province (L286), revealed that the strain was an El Tor variant with CTX-3 type phage and a RS1 satellite phage. Phylogeny analysis situated this DRC strain close to the major Kenyan clade in the most recent wave of the seventh pandemic (data not shown).

10.1.2 Conclusions

Analysis of a panel of *V. cholerae* clinical isolates from the DRC from 1997–2012 highlighted a loss of sensitivity to leading antimicrobial drugs, although strains remain susceptible to fluoroquinolones. However, a risk for emergence and spread of fluoroquinolone-resistant strains exists, as has been shown elsewhere in Africa (Islam et al. 2009). Because resistance to nalidixic acid is frequently associated with decreased susceptibility to fluoroquinolones, nalidixic acid resistance must be detected to monitor the emergence of highly resistant strains (Ray et al. 2006).

Our findings also provide new insight regarding the cholera epidemic of 2011–2012. This epidemic appears to have been caused by the expansion of a specific *V. cholerae* subpopulation, which rapidly diffused countrywide. Furthermore, sequence analysis showed that the clone responsible for this epidemic, an El Tor variant with CTX-3 type phage, falls close to the major Kenyan clade in wave 3 of the seventh pandemic. This observation correlates with a 2011 study demonstrating that the seventh cholera pandemic had been caused by specific strains originating from a unique ancestral clone that have spread globally in successive waves (Mutreja et al. 2011). The 2011–

2012 isolates displayed a specific antimicrobial drug resistance pattern, characterized by the return of tetracycline and doxycycline sensitivity. The outbreak strain also represented a serotype switch from Ogawa to Inaba. However, further MLVA genotyping of preoutbreak isolates is required to determine whether these strains were already present in the region or if they represent a new *V. cholerae* population.

This study demonstrates that molecular and microbiological analyses of *V. cholerae* isolates provide extensive insight into the mechanisms of cholera epidemics. MLVA and whole-genome sequencing are powerful tools for elucidating epidemic dynamics because these methods have been used to link distinct outbreaks and identify the origin of certain epidemic *V. cholerae* strains (Hendriksen, Price, and Schupp 2011). Improved sampling of clinical isolates is essential to monitor changes in pathogen antimicrobial drug resistance and elucidate the dissemination pathways of toxigenic strains to ensure proper management of patients requiring antimicrobial drug treatment and to appropriately direct the public health response.

10.2 Relationship between Distinct African Cholera Epidemics Revealed via MLVA Haplotyping of 337 *Vibrio cholerae* Isolates

10.2.1 Introduction

Since 1817, seven cholera pandemics have plagued humans worldwide (Echenberg 2011). During the current pandemic, the disease first appeared on the African continent when *Vibrio cholerae* was imported by migrants traveling to the Guinean capital of Conakry in 1970 (Echenberg 2011). Following this initial importation of the bacterium, cholera cases have been reported every year in Africa, and many regions in Sub-Saharan Africa have been deemed cholera-endemic (Gaffga, Tauxe, and Mintz 2007).

Although Africa currently has the highest incidence of cholera globally, the disease affects the continent in a heterogeneous manner. African cholera outbreaks primarily cluster at certain hotspots including (1) the African Great Lakes Region and (2) West Africa, stretching from Cameroon and the Lake Chad region along the coast to Guinea (Nkoko et al. 2011; Oger and Sudre 2011). Cholera outbreaks commencing in the African Great Lakes Region have been found to spread to neighboring countries, such as Sudan in 2006 and Kenya in 2009 (Rebaudet, Sudre, et al. 2013a). Likewise, epidemics have progressively moved along the West African coast, as observed in 2003-2007 when outbreaks spread from Liberia and Sierra Leone to Guinea (Rebaudet, Sudre, et al. 2013b). Indeed, cholera appears to spread in a highly dynamic manner that poses a significant public health threat at a regional level across Africa.

To design effective public health strategies to combat the disease, it is critical to understand the mechanisms of cholera emergence and diffusion in a region-specific manner. Epidemiological analysis of outbreaks is critical to identify hotspots and patterns of disease spread. However, molecular biological methods can provide further insight into the relationship between pathogenic strains and epidemic populations (Ramisse et al. 2004). Indeed, isolate typing is useful to differentiate

between different isolates, identify clusters, establish phylogeny, and track bacterial transmission. Lam et al. (Lam et al. 2012) have recently shown that MLVA (Multi-Locus VNTR (Variable Number Tandem Repeat) Analysis) represents a highly discriminatory technique to distinguish between closely related seventh pandemic isolates. They have also emphasized that the method is best applied for outbreak investigations or to identify the source of an outbreak. Our research group has recently demonstrated that MLVA-based analysis of clinical *V. cholerae* isolates combined with an epidemiological assessment was instrumental in deciphering the origin of the 2012 Guinean epidemic (Rebaudet et al. 2014).

In the current study, we applied MLVA-based typing of 337 *V. cholerae* isolates from recent cholera epidemics in Sub-Saharan Africa to assess the relationship between outbreaks. We identified 89 unique MLVA haplotypes across our isolate collection. When coupled with corresponding epidemiological data, we revealed the short-term divergence and microevolution of these *V. cholerae* populations to provide insight into the dynamics of cholera outbreaks and the relationship between distinct epidemics in West Africa and the African Great Lakes Region.

10.2.2 Materials and Methods

***V. cholerae* isolates**

Overall, we analyzed 337 *V. cholerae* isolates derived from epidemics in the Democratic Republic of the Congo (DRC), Guinea, Togo and Zambia. Our panel included six isolates from environmental samples and 331 clinical samples. A total of 237 *V. cholerae* isolates from epidemics in the DRC occurring in 2008 (3 isolates), 2009 (108 isolates), 2011 (60 isolates), 2012 (44 isolates) and 2013 (22 isolates) were provided by the INRB (French acronym for the National Institute of Biomedical Research), Kinshasa, DRC. Of the 60 DRC isolates from 2011, two were isolated from environmental water samples collected from Lake Tanganyika by staff at the Centre de recherche en Hydrobiologie (in Uvira, RDC) and analyzed at the Hôpital Générale de Référence in Uvira by Hilde de Boeck. The Guinean reference laboratory of the

Public Health National Institute (INSP—Institut National de Santé Publique), with support from the AFRICHOL Consortium (<http://www.africhol.org/>), provided 36 *V. cholerae* isolates collected throughout Guinea during the 2012 epidemic as previously described (Rebaudet et al. 2014). The National Institute of Hygiene in Lomé, Togo provided 35 *V. cholerae* isolates from Togo, which corresponded to epidemics in 2010 (13 isolates), 2011 (10 isolates) and 2012 (12 isolates provided by the bacteriology laboratory of the National Institute of Hygiene in Togo via the project AFRICHOL). A total of 27 *V. cholerae* clinical isolates from the 2012 Zambian epidemic were analyzed; the isolates were collected by the staff at the Cholera Treatment Center at the Mpulungu Health Centre during the CHOLTIC project together with the Institute of Tropical Medicine in Antwerp, Belgium. Two environmental isolates were also collected during the CHOLTIC project in association with the Department of Fisheries, Lake Tanganyika Research Unit. The Mpulungu Health Centre performed the initial characterization of the *V. cholerae* environmental samples.

Ethics statement

Regarding the isolates from Zambia, suspected cholera cases were routinely cultured to test for the presence of *V. cholerae*. The study, including a waiver of written consent, was approved by the University of Zambia Biomedical Research Ethics Committee, the Institute of Tropical Medicine institutional review board, and the ethical committee of the University of Antwerp, Belgium (study registration number B300201317249). Patients, children's parents and/or legal guardians were informed and approved via oral consent before enrollment into the study. Oral consent was registered by the ward nurse, and participant samples received a study ID number to anonymize the data.

Concerning the Guinean isolates, the sampling of suspected cholera cases for culture confirmation of *V. cholerae* is included among the routine procedures in accordance with the policies of the Ministry of Health Guinean. The Ministry of Public Health and Public Hygiene, Conakry (Ministre de la Santé Publique et de l'Hygiène Publique) approved the use of these *V. cholerae* isolates for research and publication purposes.

In Togo, to confirm suspected cholera cases, patient samples are routinely cultured to test for the presence of *V. cholerae*. The directorate of the National Institute of Hygiene, Lomé, Togo (the laboratory director and the head of the bacteriology department) approved the study of these isolates for research purposes, including the comparison of these Togolese isolates with other African *V. cholerae* samples.

In the Democratic Republic of the Congo, samples from suspected cholera cases are routinely collected and analyzed for the presence of *V. cholerae* in the framework of the epidemiological surveillance program of the Ministry of Health. The ethics committee at the University of Kinshasa approved the analysis of these isolates for research purposes.

All data analyzed in the study were anonymized.

***V. cholerae* culture and DNA extraction**

The isolates were subcultured and inoculated into *Vibrio cholerae* Enrichment Broth vials (Bio-Rad). The Bio-Rad vials were subsequently expedited (2–3 days) at ambient temperature to L'Hôpital d'Instruction des Armées Laveran in Marseille, France. In Marseille, the strains were recultivated on non-selective trypticase soy agar medium (Difco Laboratories/BD) for 24 hours at 37°C. Suspected *V. cholerae* colonies were identified via Gram-staining, oxidase reaction and agglutination assessment with *V. cholerae* O1 polyvalent antisera (Bio-Rad).

For DNA extraction, an aliquot of cultured cells (approximately 50 colonies) was suspended in 500 µL NucliSENS easyMAG lysis buffer (bioMérieux, Marcy l'Etoile, France). Total nucleic acid was extracted from *V. cholerae* cultures using a NucliSENS easyMAG platform (bioMérieux) according to the manufacturer's instructions. Nucleic acid concentration and 260/280 ratio were measured using a NanoDrop 3300 fluorospectrometer (Thermo Scientific, Villebon sur Yvette, France). The supernatants (100 µL) were stored at -20°C for downstream applications.

MLVA-based typing

Genotyping of the *V. cholerae* isolates was performed via MLVA of six VNTRs, including five previously described assays and a novel VNTR assay, VCMS12, specifically designed for this study to improve the discriminative power of the analysis (**Table 7**) (Rebaudet et al. 2014; Olsen et al. 2009; Kendall et al. 2010). The VCMS12 assay was designed based on the reference strain El Tor N16961 (GenBank accession numbers AE003852.1 and AE003853.1) using Perfect Microsatellite Repeat Finder (currently unavailable). VCMS12 is located within the cholera toxin A subunit promoter region at position 1568189 on chromosome 1 of El Tor N16961. This polymorphic tandem heptanucleotide repeat region has been previously identified by Naha et al. (Naha et al. 2013). Specific primer pairs were subsequently designed using Primer3 (<http://simgene.com/Primer3>). The fluorescent-labeled primers (**Table 7**) were purchased from Applied Biosystems.

| VNTR Locus name | Repeated pattern | Chr. ¹ | Position ² | Primer sequence (5'→3') | Ref. |
|-----------------|------------------|-------------------|-----------------------|--------------------------------|--|
| VC1 | AACAGA | 1 | 137106 | fw: CGGATACTCAAACGCAGGAT | (Olsen et al. 2009; Kendall et al. 2010) |
| | | | | rv: 6FAM-CTTTCGGTCGGTTTCTCTTG | |
| VC4 | TGCTGT | 2 | 187759 | fw: TGTTTGAGAGCTCGCCTCTT | (Olsen et al. 2009; Kendall et al. 2010) |
| | | | | rv: PET-TCATCAAGATGCACGACACA | |
| VC5 | GATAATCCA | 1 | 1915539 | fw: AGTGGGCACAGAGTGTCAAA | (Olsen et al. 2009; Kendall et al. 2010) |
| | | | | rv: VIC-AATTGGCCGCTAACTGAGTG | |
| VC9 | GACCCTA | 1 | 467111 | fw: CGTTAGCATCGAAACTGCTG | (Olsen et al. 2009; Kendall et al. 2010) |
| | | | | rv: NED-AGAAAACAATCGCCTGCTTG | |
| LAV6 | ACCAGA | 2 | 303939 | fw: NED-GCCTCCTCAGAAGTTGAGAATC | (Rebaudet et al. 2014) |
| | | | | rv: CCGATGAACTCTCTGAACTGG | |
| VCMS12 | TTTTGAT | 1 | 1568189 | fw: VIC-GAGGTCTAGAATCTGCCCCGA | Present study |
| | | | | rv: AAGCGCTGTGGGTAGAAGTG | |

Table 7. Characteristics and primer sequences of the 6 tested *V. cholerae* VNTRs. ¹

Chr.: chromosome. ² Based on the reference strain El Tor N16961 (GenBank accession numbers: AE003852.1 and AE003853.1).

Each VNTR locus was amplified separately. DNA amplification was carried out by preparing a PCR mix containing the following components: 0.375 µL of each primer (20 µM), 1 X LightCycler 480 Probes Master (Roche Diagnostics) and approximately 100 ng of template DNA. The PCR mix was brought to a total volume of 30 µL with H₂O. PCR was performed using a LightCycler 480 System (Roche Diagnostics). All PCRs were performed using the thermal cycling conditions as follows: 95°C for 5 min; followed by 30 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec; 72°C for 5 min.

Aliquots of the PCR products were first diluted 1:30 in sterile water. Next, 1 µL of the diluted PCR reaction was aliquoted into a solution containing 25 µL Hi-Di Formamide

3500 Dx Series (Applied Biosystems) and 0.5 µL GeneScan 500 LIZ Size Standard (Applied Biosystems). The fluorescent end-labeled PCR amplicons were separated via capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with POP-7 Polymer (Applied Biosystems). Finally, amplicon size was determined using GeneMapper v.3.0 software (Applied Biosystems).

Data analysis

The MLVA results were exported to Microsoft Excel 2008 v. 12.2.0. Allele numbers were derived directly from the fragment sizes, and MLVA types were determined from the combined profile of alleles (i.e., each unique combination of six allele numbers was assigned a novel MLVA type number). The absence of allele at a given locus (i.e., no amplification, despite repeated attempts) was assigned “999” for further analyses. No amplification was observed for 22 of the 2022 examined alleles. To perform the Minimum Spanning Tree (MST) analysis, the isolates were further assigned into 10 epidemic populations as follows: DRC 2008, DRC 2009 A (collected in January-May), DRC 2009 B (collected in July-November), DRC 2011, DRC 2012, DRC 2013, Guinea 2012, Zambia 2012, Togo 2010, Togo 2011 and Togo 2012. The DRC 2009 epidemic isolates were sub-grouped by time period as two distinct epidemics were observed in the region during field investigations.

Cartography

The maps were generated using QGIS version 2.4.0-Chugiak with shapefiles obtained from DIVA-GIS (<http://www.diva-gis.org/gdata>).

Minimum Spanning Tree

Based on allelic profiles the evolutionary relationship between all 337 isolates was assessed with the MST algorithm in BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) using the default settings according to the manufacturer’s recommendations. The MST was constructed using a categorical coefficient as previously described (Choi et al. 2010).

goeBURST analysis

To identify clonal complexes and founder MLVA types among the 331 clinical isolates (i.e., excluding the six environmental isolates), MLVA-types were compared at each of the six VNTR loci and genetic relatedness between the strains was assessed using goeBURST version 1.2.1 (<http://goeburst.phyloviz.net/>) (Francisco et al. 2012). The goeBURST algorithm identifies mutually exclusive groups of related MLVA types in a population. The algorithm also predicts the presumed founder(s) of each clonal complex and any single locus variant (SLV) and double locus variant (DLV) derivatives. The primary founder of a group is defined as the MLVA type that has the greatest number of SLVs. goeBURST then constructed a spanning forest in which each MLVA type is a node and two MLVA types are connected if they are SLVs. An MLVA cluster was defined as a group of isolates that share identical alleles at five of the six loci with at least one other member of the group. Accordingly, singletons were defined as MLVA types having at least two allelic mismatches with all other MLVA types. The number of re-samplings for bootstrapping was set at 1000.

Population genetics calculations

Population genetic analyses were performed on each set of epidemic isolates for which there were 10 or more isolates per set; therefore, the three DRC 2008 isolates were excluded from the statistical analyses. F_{st} (F-statistics; also known as fixation indices) and p-values were calculated pair-wise for all epidemic populations of the 328 clinical isolates using the Nei (1987) method implemented in GenoDive 2.0b25 (Meirmans and Van Tienderen 2004) as were the allelic diversity of all clusters and loci.

Field investigations

Several field investigations were conducted in the DRC, Guinea and Togo by members of our team (Renaud Piarroux, Stanislas Rebaudet, Berthe Miwanda, Didier Bompangue, Aaron Aruna Abedi, Jean-Jacques Depina and Sandra Moore). Field investigations were performed at sites affected by cholera, in which the data collected included number of cases/deaths, laboratory results, the locales affected and the spatiotemporal evolution of the epidemics. Further details of the field investigations are provided in the corresponding studies of the specific epidemics (concerning the Togo field investigation, please see **Suppl Text**) (Rebaudet et al. 2014; Bompangue et al. 2012).

In Zambia, strains collected in the context of the national surveillance system and the CHOLTIC project with the objective of comparing strains collected in the vicinity of Lake Tanganyika (at sites of varying distances from the coast). Strains were collected in Mpulungu, Northern Province, between the 12th and 24th of August 2012, during an outbreak that occurred at this time. The following data corresponding to the collected strains was also compiled: patient name, sex, age, date seen at facility, date of symptom onset, laboratory results (if any were performed) and date of patient discharge. No corresponding field investigation was performed during this outbreak.

10.2.3 Results

10.2.3.1 Minimum Spanning Tree and goeBURST analyses of 337 V. cholerae isolates from West and Central Africa

A total of 337 *V. cholerae* isolates from recent cholera epidemics in the DRC, Zambia, Guinea and Togo were subjected to MLVA. Each country is localized on the map of Africa in **Figure 12**. Analysis of the six VNTRs yielded 89 MLVA types. The VNTR loci and epidemic populations (grouped by country and year of isolation) corresponding to each MLVA type are outlined in **Suppl Table 1**.

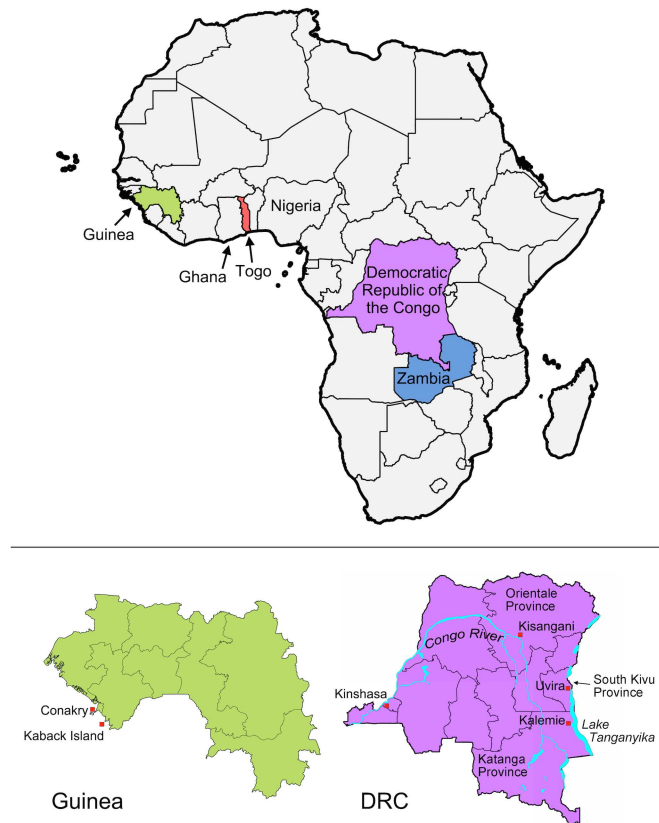


Figure 12. Map of African continent indicating countries mentioned in study. The four countries from which *V. cholerae* isolates were collected are indicated in different colors (i.e., Guinea, green; Togo, red; DRC, violet, and Zambia: blue). A zoom on the DRC and Guinea are shown.

A MST was constructed using the combined MLVA data to assess the relationships between the 337 *V. cholerae* isolates and the epidemic populations. On a continental scale, the MST revealed strong geographical clustering with isolates from the African Great Lakes Region, including the DRC and Zambia, clustering together. Furthermore, the isolates collected in the West African countries of Guinea and Togo formed a separate group (**Figure 13**). All but seven clinical isolates from the DRC and Zambia 2012 were linked by one- or two-VNTR variations. Likewise, all clinical isolates from Guinea 2012 and Togo were linked by one- or two-VNTR divergences. At a country-level scale our analyses revealed several distinct clonal groups, most notably (1) DRC 2011/2012, (2) DRC 2009, (3) Zambia 2012 and (4) Guinea 2012. We used goeBURST

to identify a potential founder MLVA haplotype for each MLVA cluster. Each epidemic complex was characterized by a central founder MLVA haplotype and closely related derivative SLV or DLV MLVA haplotypes, which branched from the founder. In contrast, all six of the typed environmental isolates were found to be singletons, unrelated to the main clinical epidemic isolate clusters (**Figure 13**).

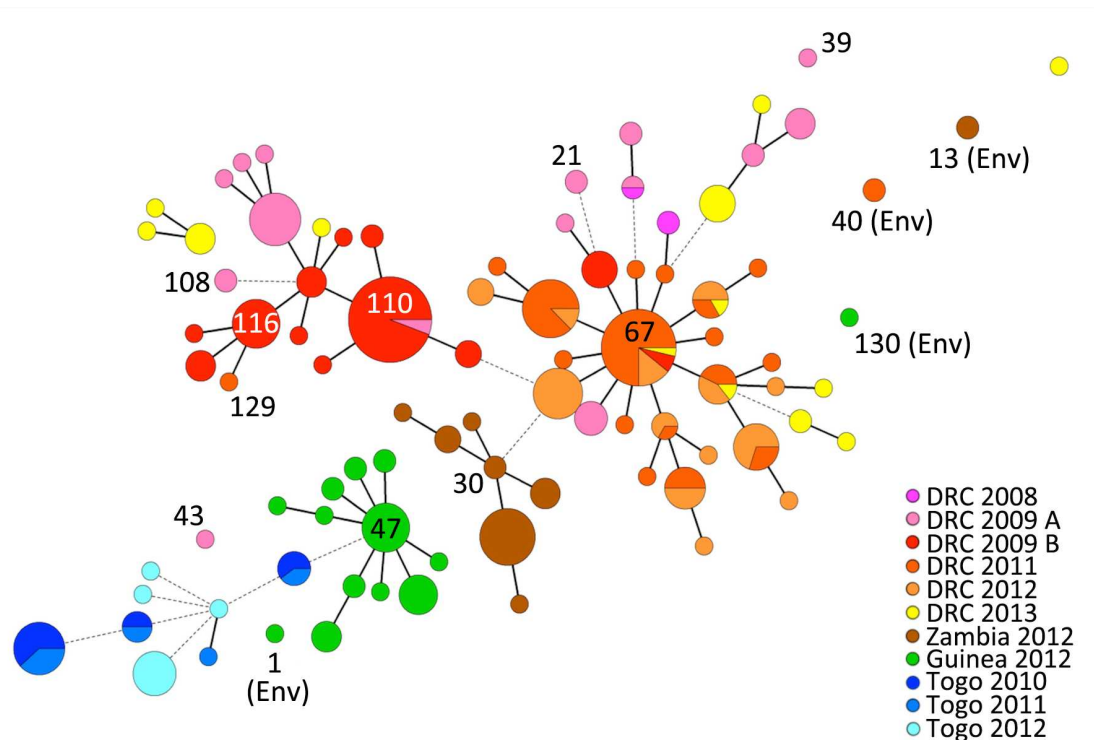


Figure 13. Minimum Spanning Tree based on the MLVA types of 337 *V. cholerae* isolates derived from several recent African epidemics. Each MLVA type is represented by a node, and the size of the nodes reflects the number of isolates with the indicated MLVA type. The relationships between isolate MLVA types are indicated by the type of connecting segments and the length of the segment between nodes. The solid lines indicate the most likely single-locus variation, and the dashed lines represent double-locus variations. The distance between the nodes represents the number of varying VNTRs. The colors reflect the distinct country and period of isolate origin (grouped by epidemic populations). Pie charts were used to indicate the distribution of strains isolated from different time periods or countries displaying identical an MLVA type.

Our analysis showed that the DRC 2011 and DRC 2012 isolates grouped together as one discrete complex (**Figure 13**). During this two-year period in the DRC, cholera was

caused by the extensive expansion and diversification from a single MLVA haplotype. The isolate found at the beginning of the 2011/2012 epidemic in Kisangani, Orientale Province in March 2011 was haplotype #67, which was designated the founder of the DRC 2011/2012 complex. MLVA type #67 and a SLV of this haplotype were the only types identified during the first week of the outbreak. This MLVA cluster then diversified in parallel with the spatiotemporal spread of the epidemic (Bompangue et al. 2012), as the most distant MLVA haplotypes within this cluster were identified in distant provinces in 2012. These findings correlate with an epidemiological report of the cholera epidemic that struck the DRC in 2011. This epidemic aggressively diffused from the onset point in Kisangani, Orientale Province across the country in less than 130 days. Strikingly, outbreaks followed the Congo River and quickly reached non-endemic zones in the West that had not experienced an epidemic for approximately 10 years (Bompangue et al. 2012). Kisangani and the Congo River are localized on the detailed map of the DRC (**Figure 12**, lower right).

Interestingly, the predicted founder of the 2011/2012 DRC epidemic, persisted in the country over the course of several years, as haplotype #67 was represented in isolates collected in the DRC during the 2009, 2011, 2012 and 2013 epidemics. Only one DRC isolate (MLVA type #129) collected in 2011 did not belong to the major DRC 2011/2012 MLVA cluster. Instead, haplotype #129 was a SLV of the DRC 2009 haplotype #116 cluster. In stark contrast, the *V. cholerae* non-O1 strain isolated at the same period from a water sample in Uvira, South Kivu was a genetically unrelated singleton (MLVA type #40) (**Figure 13**). Uvira is located on the northern shores of Lake Tanganyika as indicated in **Figure 12** (lower right panel).

Overall, the panel of DRC 2009 isolates displayed a high level of genetic diversity. In fact, four DRC 2009 clinical isolates (MLVA types #39, #43, #21 and #108) collected in February and March 2009 were designated distantly related singletons (**Figure 13**). The MST was then analyzed in further detail considering the date of sample isolation. The isolates collected during the first half of the year were highly diverse (indicated as “DRC 2009 A” in pink; **Figure 13**). In contrast, 63 of 66 isolates (95.5%) collected from July to November of 2009 in Katanga Province formed a tight clonal complex

(indicated as “DRC 2009 B” in red; **Figure 13**). This bottleneck effect was concomitant with an epidemic rebound in Katanga Province after a complete lull in cholera transmission in May and June 2009. In July 2009, cholera first appeared in Kalemie, a city located on the shore of Lake Tanganyika, and then spread throughout the rest of the province (based on field investigations in the DRC; Renaud Piarroux). MLVA types #110 and #116 were designated potential founders of this DRC 2009 B MLVA complex. Interestingly, MLVA type #110 was the first isolate collected on February 5, 2009, and isolates harboring this haplotype were collected up to November 20, 2009. Notably, MLVA type #110 was also found in August 2009 in Uvira, a city located approximately 360 km north of Kalemie on the shore of Lake Tanganyika (all sites are labeled in the lower right panel of **Figure 12**). Therefore, we hypothesize that this strain likely persisted in the region following the early-2009 outbreaks and a subsequently gave rise to the late-2009 DRC epidemic.

In Zambia, all clinical isolates from the 2012 epidemic formed a restricted clonal complex, which derived from the predicted founder MLVA #30. Once more, the two non-O1 environmental isolates collected from the shores of Lake Tanganyika in Mpulungu, Northern Province were singletons (MLVA haplotype #13) unrelated to the clonal complex (**Figure 13**).

The Guinea 2012 clinical isolates formed a solid clonal complex, with an MST of closely related derivative isolates that stemmed from the founder haplotype (MLVA type #47) (**Figure 13**). In a previous study, our group has shown that the Guinea 2012 epidemic appears to be due to the importation of a toxigenic clone from Sierra Leone. Using MLVA typing, we have demonstrated progressive genetic diversification of the strains from the founder type correlated with spatiotemporal epidemic spread (Rebaudet et al. 2014). The founding MLVA type was also the first and only MLVA type identified during the onset of the epidemic, on Kaback Island (**Figure 12**, lower left), Guinea in February 2012 (Rebaudet et al. 2014). In contrast, the two Guinean environmental strains (MLVA types #1 and #130) isolated from water samples at the site of the initial outbreak (Kaback) were unrelated singletons (**Figure 13**).

Our data showed that the Togo isolates represent a diverse set of MLVA haplotypes, as they were only related to the Guinean isolate MLVA types by a single DLV (**Figure 13**). Most of the isolates collected in Togo were designated singletons.

10.2.3.2 Population genetics

To provide statistical power to the observed relationships between 328 clinical isolates, population genetic analyses were performed. The six unrelated environmental isolates and the three isolates from DRC in 2008 were excluded from this analysis. Taking each MLVA VNTR locus in turn, this analysis revealed that there were 5, 17, 6, 6, 17 and 5 alleles, for the VNTR loci denoted VC1, VC4, VC5, VC9, LAV6 and VC12, respectively (**Table 8**). The discriminatory power of the six tested VNTRs was calculated via an index of genetic diversity (IOD) analysis (Nei, 1987) using GenoDive 2.0b25. The IOD and PCR product size range for each VNTR is outlined in **Table 8**. Accordingly, the corrected indices of diversity per locus were 0.758, 0.927, 0.54, 0.512, 0.871 and 0.579 for VC1, VC4, VC5, VC9, LAV6 and VCMS12, respectively. The overall corrected IOD for the six VNTRs combined was 0.698. The two most variable VNTRs were located on the small chromosome, which correlates with the observations reported by Lam et al. (Lam et al. 2012) (**Table 8**).

| Locus | Chromosome | Num | Eff num | Pop div | IOD | Corr IOD | PCR product size range (base pairs) |
|---------|------------|-------|---------|---------|-------|----------|-------------------------------------|
| VC1 | 1 | 5 | 1.516 | 0.357 | 0.717 | 0.758 | 179-203 |
| VC4 | 2 | 17 | 2.302 | 0.592 | 0.893 | 0.927 | 169-284 |
| VC5 | 1 | 6 | 1.157 | 0.142 | 0.5 | 0.54 | 160-215 |
| VC9 | 1 | 6 | 1.226 | 0.193 | 0.48 | 0.512 | 160-202 |
| LAV6 | 2 | 17 | 2.052 | 0.537 | 0.838 | 0.871 | 0, 209-329 |
| VC12 | 1 | 5 | 1.053 | 0.053 | 0.526 | 0.579 | 0, 258-286 |
| Overall | | 9.333 | 1.551 | 0.312 | 0.659 | 0.698 | - |

Table 8. Indices of genetic diversity per locus and observed PCR product size range. Genetic diversity (Nei, 1987) of 328 *V. cholerae* isolates, considering 6 loci, was calculated in GenoDive 2.0b25. Num = number of alleles; Eff num = effective number

of alleles; Pop div = gene diversity within populations; IOD = Index of Diversity; Corr IOD = corrected Index of Diversity.

The IOD (based on Nei, 1987) per population was calculated to determine the extent of genetic diversity of each designated population. The epidemics with the highest degree of genetic diversity were DRC 2009 A (IOD = 0.545) and DRC 2013 (IOD = 0.586). In contrast, the epidemic isolates derived from Togo 2012 (IOD = 0.152) displayed the lowest diversity. Epidemics in DRC 2011 (IOD = 0.218), DRC 2012 (IOD = 0.242), Zambia 2012 (IOD = 0.206) and Guinea 2012 (IOD = 0.222) also showed relatively low gene diversity (**Table 9**).

| Population | Number of samples | Number of alleles | Effective number of alleles | Diversity within populations |
|-------------|-------------------|-------------------|-----------------------------|------------------------------|
| DRC 2011 | 58 | 3.333 | 1.398 | 0.218 |
| DRC 2012 | 44 | 2.333 | 1.723 | 0.242 |
| DRC 2009 A | 39 | 5.5 | 2.698 | 0.545 |
| DRC 2009 B | 69 | 3 | 1.476 | 0.256 |
| Guinea 2012 | 34 | 2.5 | 1.397 | 0.222 |
| DRC 2013 | 22 | 4.667 | 2.962 | 0.586 |
| Togo 2010 | 13 | 2 | 1.582 | 0.325 |
| Togo 2011 | 10 | 2.167 | 1.764 | 0.374 |
| Togo 2012 | 12 | 2 | 1.238 | 0.152 |
| Zambia 2012 | 27 | 2 | 1.313 | 0.206 |

Table 9. Indices of genetic diversity per population. Genetic diversity (Nei, 1987) per population of the 328 *V. cholerae* isolates was calculated in GenoDive 2.0b25.

Pairwise differentiation analysis was performed to understand the statistical relationship between the epidemic populations. The F_{st} values for all pairs of populations were calculated considering all 328 *V. cholerae* clinical isolates. All F_{st} and p-values are outlined in **Table 10**. The closest statistically significant relationship was between the epidemics of DRC 2011 and DRC 2012 ($F_{st} = 0.125$, $p = 0.001$), which is coherent with the rapid diversification and expansion of an epidemic clone (Bompangue et al. 2012).

| Epidemic population | DRC 2011 | DRC 2012 | DRC 2009 A | DRC 2009 B | Guinea 2012 | DRC 2013 | Togo 2010 | Togo 2011 | Togo 2012 | Zambia 2012 |
|---------------------|----------|----------|------------|------------|-------------|----------|-----------|-----------|-----------|-------------|
| DRC 2011 | -- | 0.125 | 0.371 | 0.607 | 0.743 | 0.37 | 0.706 | 0.692 | 0.759 | 0.637 |
| DRC 2012 | 0.001 | -- | 0.319 | 0.571 | 0.732 | 0.33 | 0.707 | 0.69 | 0.737 | 0.592 |
| DRC 2009 A | 0.001 | 0.001 | -- | 0.276 | 0.464 | 0.107 | 0.444 | 0.423 | 0.53 | 0.429 |
| DRC 2009 B | 0.001 | 0.001 | 0.001 | -- | 0.66 | 0.353 | 0.655 | 0.643 | 0.714 | 0.661 |
| Guinea 2012 | 0.001 | 0.001 | 0.001 | 0.001 | -- | 0.516 | 0.486 | 0.475 | 0.693 | 0.662 |
| DRC 2013 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | -- | 0.447 | 0.418 | 0.551 | 0.476 |
| Togo 2010 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | -- | -0.076 | 0.525 | 0.658 |
| Togo 2011 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.829 | -- | 0.461 | 0.65 |
| Togo 2012 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | -- | 0.773 |
| Zambia 2012 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | -- |

Table 10. Pairwise differentiation. The F_{st} values for all pairs of populations were calculated with GenoDive 2.0b25, considering 328 clinical *V. cholerae* isolates and 6 loci. The F_{st} values are listed on the top triangle, while the corresponding p-values are listed in the lower triangle.

The DRC 2013 epidemic was closest related to the early DRC 2009 isolates ($F_{st} = 0.107$, $p = 0.001$), which suggests that the 2013 epidemic was likely due to an expansion of strains circulating in the country during previous epidemics. The pairwise analysis also demonstrated a statistically significant relationship between the early and late 2009 isolates from the DRC (2009 A and 2009 B, respectively) ($F_{st} = 0.276$, $p = 0.001$). These observations further support the hypothesis that the late 2009 epidemic came about following a bottleneck of the early 2009 DRC epidemic isolates.

10.2.4 Discussion

Overall, our results provide novel insight into the epidemic phenomena of cholera in West and Central Africa. At the sub-regional level, MST analysis revealed two distinct African clusters: (1) the African Great Lakes Region group, comprising DRC and Zambia isolates, and (2) the West African cluster with isolates from Togo and Guinea. At the country level, the epidemic *V. cholerae* populations from DRC 2011/2012, Zambia 2012, Guinea 2012 and late-2009 DRC were each designated tight complexes. The expansion of the isolate populations coincided with the progression of each epidemic, as the founder MLVA types corresponded to isolates collected at outbreak onset and more distantly related derivatives represented isolates found later during the epidemic.

Analysis of isolates from the DRC revealed that certain strains appear to remain in circulation in the country over a period of several years and eventually engender explosive outbreaks with diversification of founding isolates, as observed in 2011. This phenomenon is distinct from that observed in Togo, where isolates were grouped into a loosely connected quasi-complex without a founder MLVA type. The Togo isolate results rather indicate that when outbreaks occur, the isolates fail to diversify or diffuse throughout the country. A field assessment of outbreaks in Togo has revealed that the country is vulnerable to importation of cholera cases from neighboring countries, although outbreaks are then quickly extinguished (UNICEF field investigation (Sandra Moore) and personal communication with Dr Adodo Yao Sadji). From 2010

to week 48 of 2014, the country only recorded 551 suspected cholera cases (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). We hypothesize that the Togo isolates rather represent the descendants of a much larger epidemic cluster from a neighboring country experiencing severe cholera epidemics, such as the nearby countries of Ghana and Nigeria. From 2010 to week 48 of 2014, Ghana reported 48,546 suspected cholera cases (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). During that same five-year period, Nigeria signaled a staggering 110,904 suspected cases (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Isolates from these affected countries would have to be analyzed to test this hypothesis.

As *V. cholerae* is autochthonous in the coastal aquatic ecosystem, it has been widely presumed that cholera epidemics are triggered by environmental factors that promote growth of local bacterial reservoirs (Colwell 1996). However, all six of the environmental isolates collected from a range of countries were genetically unrelated singletons. We acknowledge that additional environmental isolates of *V. cholerae* should be included in the panel to affirm the relationship (or lack of) between clinical outbreak strains and those found in water bodies. Indeed, to examine the diversity of environmental strains, efforts should be made to collect further samples. Nevertheless, our preliminary analysis of the environmental samples correlates with two recent reviews elucidating the environmental determinants of cholera outbreaks in Africa. These reviews by Rebaudet et al. (Rebaudet, Sudre, et al. 2013a; Rebaudet, Sudre, et al. 2013b) found that at least 76% of cholera cases in Sub-Saharan Africa occurred in non-coastal regions located over 100 km from the coast. From 2009 to 2011, annual incidence rates of cholera were three times higher in inland Africa compared with the coastal region. In fact, toxigenic *V. cholerae* isolates have only been recovered from the environment during an outbreak, when patient-derived contamination of water sources is expected (Rebaudet, Sudre, et al. 2013b; Rebaudet, Sudre, et al. 2013a).

Our findings are also consistent with the phylogenic assessment of an extensive panel of seventh pandemic *V. cholerae* isolate. Mutreja et al. (2011) have revealed that a specific *V. cholerae* El

Tor clonal lineage appears to be responsible for the current pandemic. Their study demonstrated that the seventh pandemic is monophyletic and originated from a single ancestral clone that has radiated globally in distinct waves (Mutreja et al. 2011). Lineages of the current pandemic appear to emerge, diversify and eventually become extinct (Mutreja et al. 2011). Notably, we observed a similar phenomenon at a smaller scale with the DRC 2009, DRC 2011/2012, Guinea 2012 and Zambia 2012 epidemics. As isolates from the African Great Lakes Region were not included in the seventh pandemic phylogeny, whole-genome sequencing and phylogenetic analysis of this panel of African strains would provide even further insight into the mechanisms of cholera epidemics in the region. Together, MLVA and whole-genome sequencing-based phylogeny represent complementary approaches to better understand epidemic dynamics. MLVA is useful to elucidate the short-term microevolution of clonal complexes, while sequence-based phylogeny enables the identification of distant ancestors and related strains at a global level.

Concerning the limitations of the study, our findings would be bolstered by increased isolate sampling of several years in these and neighboring affected countries. Indeed, we could not verify that MLVA type #67 isolates found in the DRC persisted in the country throughout the 2010 epidemic, as few isolates were collected in 2010 due to a lack of funding for the epidemiological surveillance and prevention of cholera. Likewise, the analysis of the Togolese epidemics would benefit from additional isolate sampling in neighboring countries. Finally, although environmental *V. cholerae* samples are difficult to isolate, this study would be enhanced by including additional isolates found in water bodies located in cholera-endemic areas.

Further studies should address the detailed mechanisms of cholera in the zones where cholera appears to persist. If the cholera dilemma in Africa can be narrowed down to a few locales, secondary affected areas (such as perhaps Togo and Guinea) may be largely protected by targeted interventions in cholera epicenters such as the DRC, Ghana and Nigeria. Therefore, with a clear understanding of cholera dynamics in the region, public health resources would be most effectively and efficiently applied.

Overall, our results show that cholera is indeed a regional public health dilemma in Africa. With varying dynamics in each country, certain strains are able to persist in a given region over a period of several years and occasionally spread into non-endemic areas or neighboring countries. Indeed, several elements play a role in cholera epidemics including climate, geography, economy, hygiene, sanitation, access to potable water and population movement, as addressed in the corresponding epidemiological reports (concerning the Togo field investigation, please see **Suppl Text**) (Rebaudet et al. 2014; Bompangue et al. 2012). Therefore, public health strategies should be optimized according to the dynamics and scale of cholera epidemics in each region. These findings also demonstrate the importance of monitoring the circulation of the bacterium among human populations, which appear to serve as the principal reservoir of toxigenic *V. cholerae*. Combined with classical epidemiological investigations, MLVA represents a rapid and discriminatory tool to track outbreak evolution at an epidemic and regional level.

10.2.5 Supporting Information

| Epidemic | MLVA type | Locus | | | | | | Num of isolates |
|------------------|-----------|-------|-----|-----|-----|------|--------|-----------------|
| | | VC1 | VC4 | VC5 | VC9 | LAV6 | VCMS12 | |
| Guinea 2012 | 1* | 0 | 0 | 185 | 188 | 281 | 0 | 1 |
| Zambia 2012 | 2 | 179 | 203 | 197 | 188 | 245 | 272 | 15 |
| Zambia 2012 | 3 | 179 | 203 | 215 | 188 | 245 | 272 | 1 |
| DRC 2013 | 4 | 179 | 226 | 178 | 160 | 0 | 0 | 1 |
| Togo 2010, 2011 | 5 | 179 | 243 | 188 | 188 | 251 | 272 | 5 |
| Zambia 2012 | 13* | 185 | 192 | 157 | 160 | 0 | 0 | 2 |
| DRC 2009 A | 21 | 185 | 198 | 197 | 181 | 233 | 265 | 2 |
| DRC 2013 | 22 | 185 | 198 | 197 | 188 | 257 | 265 | 6 |
| DRC 2013 | 23 | 185 | 198 | 197 | 188 | 263 | 265 | 1 |
| DRC 2009 A | 25 | 185 | 198 | 197 | 188 | 287 | 265 | 2 |
| DRC 2009 A | 26 | 185 | 198 | 197 | 188 | 293 | 265 | 4 |
| DRC 2011 | 29 | 185 | 203 | 197 | 188 | 233 | 265 | 1 |
| Zambia 2012 | 30 | 185 | 203 | 197 | 188 | 245 | 272 | 2 |
| Zambia 2012 | 31 | 185 | 203 | 197 | 188 | 251 | 0 | 1 |
| Zambia 2012 | 32 | 185 | 203 | 197 | 188 | 251 | 272 | 3 |
| Zambia 2012 | 33 | 185 | 203 | 197 | 188 | 263 | 272 | 1 |
| DRC 2008, 2009 A | 35 | 185 | 203 | 197 | 195 | 287 | 265 | 2 |

| | | | | | | | | |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|----|
| Zambia 2012 | 36 | 185 | 209 | 197 | 188 | 245 | 272 | 4 |
| DRC 2009 A | 37 | 185 | 209 | 197 | 195 | 287 | 265 | 2 |
| DRC 2011 | 38 | 185 | 215 | 197 | 188 | 233 | 265 | 1 |
| DRC 2009 A | 39 | 185 | 215 | 197 | 202 | 287 | 258 | 1 |
| DRC 2011 | 40* | 185 | 215 | 203 | 195 | 0 | 0 | 2 |
| Togo 2010, 2011 | 41 | 185 | 220 | 188 | 160 | 233 | 272 | 13 |
| DRC 2013 | 42 | 185 | 220 | 197 | 188 | 239 | 265 | 1 |
| DRC 2009 A | 43 | 185 | 232 | 160 | 167 | 329 | 286 | 1 |
| Guinea 2012 | 44 | 185 | 243 | 188 | 181 | 287 | 272 | 4 |
| Guinea 2012 | 45 | 185 | 243 | 188 | 188 | 269 | 272 | 2 |
| Guinea 2012 | 46 | 185 | 243 | 188 | 188 | 275 | 272 | 1 |
| Guinea 2012 | 47 | 185 | 243 | 188 | 188 | 281 | 272 | 11 |
| Guinea 2012 | 48 | 185 | 243 | 188 | 188 | 287 | 272 | 2 |
| Guinea 2012 | 49 | 185 | 243 | 188 | 188 | 311 | 272 | 1 |
| Guinea 2012 | 50 | 185 | 243 | 188 | 195 | 281 | 272 | 2 |
| Guinea 2012 | 51 | 185 | 249 | 188 | 188 | 281 | 272 | 2 |
| Guinea 2012 | 52 | 185 | 255 | 188 | 188 | 275 | 272 | 1 |
| Guinea 2012 | 53 | 185 | 255 | 188 | 188 | 281 | 272 | 7 |
| Guinea 2012 | 54 | 185 | 272 | 188 | 188 | 281 | 272 | 1 |
| DRC 2009 B | 62 | 191 | 198 | 197 | 188 | 233 | 265 | 6 |
| DRC 2011 | 63 | 191 | 198 | 197 | 188 | 239 | 265 | 1 |
| DRC 2012, 2011 | 65 | 191 | 203 | 197 | 188 | 227 | 265 | 3 |
| DRC 2011 | 66 | 191 | 203 | 197 | 188 | 233 | 0 | 1 |
| DRC 2011, 2012, 2009 B, 2013 | 67 | 191 | 203 | 197 | 188 | 233 | 265 | 28 |
| DRC 2011, 2012, 2013 | 68 | 191 | 203 | 197 | 188 | 239 | 265 | 7 |
| DRC 2012 | 69 | 191 | 203 | 197 | 188 | 245 | 265 | 12 |
| DRC 2011 | 70 | 191 | 203 | 197 | 188 | 257 | 265 | 1 |
| DRC 2009 A | 71 | 191 | 203 | 197 | 188 | 281 | 265 | 5 |
| DRC 2011 | 72 | 191 | 203 | 206 | 188 | 233 | 265 | 1 |
| DRC 2012 | 73 | 191 | 209 | 197 | 181 | 227 | 265 | 1 |
| DRC 2011, 2012 | 74 | 191 | 209 | 197 | 188 | 227 | 265 | 8 |
| DRC 2011 | 75 | 191 | 209 | 197 | 188 | 233 | 0 | 1 |
| DRC 2011, 2012 | 76 | 191 | 209 | 197 | 188 | 233 | 265 | 16 |
| DRC 2011, 2012 | 77 | 191 | 209 | 197 | 188 | 239 | 265 | 10 |
| DRC 2012 | 78 | 191 | 209 | 197 | 188 | 245 | 265 | 3 |
| DRC 2012 | 80 | 191 | 209 | 197 | 195 | 239 | 265 | 1 |
| DRC 2012 | 81 | 191 | 215 | 197 | 188 | 227 | 265 | 1 |
| DRC 2011, 2012, 2013 | 82 | 191 | 215 | 197 | 188 | 233 | 265 | 6 |

| | | | | | | | | |
|-----------------|------|-----|-----|-----|-----|-----|-----|----|
| DRC 2012 | 83 | 191 | 220 | 197 | 188 | 239 | 265 | 1 |
| Togo 2010, 2011 | 84 | 191 | 232 | 188 | 160 | 233 | 272 | 4 |
| DRC 2008 | 85 | 191 | 232 | 197 | 188 | 257 | 265 | 2 |
| Togo 2012 | 88 | 191 | 238 | 188 | 160 | 275 | 272 | 9 |
| Togo 2012 | 89 | 191 | 243 | 188 | 160 | 251 | 272 | 1 |
| Togo 2011 | 90 | 191 | 243 | 188 | 160 | 257 | 272 | 1 |
| DRC 2009 B | 96 | 191 | 260 | 188 | 188 | 245 | 265 | 3 |
| Togo 2012 | 97 | 191 | 266 | 188 | 160 | 239 | 272 | 1 |
| Togo 2012 | 98 | 191 | 272 | 188 | 160 | 245 | 272 | 1 |
| DRC 2013 | 99 | 197 | 169 | 188 | 181 | 221 | 265 | 1 |
| DRC 2009 A | 100 | 197 | 198 | 197 | 188 | 233 | 265 | 1 |
| DRC 2011 | 101 | 197 | 203 | 197 | 188 | 227 | 265 | 1 |
| DRC 2011 | 102 | 197 | 203 | 197 | 188 | 233 | 265 | 1 |
| DRC 2013 | 105 | 197 | 215 | 197 | 188 | 239 | 265 | 2 |
| DRC 2013 | 106 | 197 | 215 | 197 | 188 | 245 | 265 | 1 |
| DRC 2009 A | 108 | 197 | 238 | 188 | 188 | 209 | 265 | 2 |
| DRC 2009 B | 109 | 197 | 260 | 188 | 188 | 245 | 0 | 1 |
| DRC 2009 A & B | 110 | 197 | 260 | 188 | 188 | 245 | 265 | 35 |
| DRC 2009 B | 111 | 197 | 260 | 188 | 188 | 257 | 265 | 2 |
| DRC 2013 | 112 | 197 | 266 | 188 | 188 | 227 | 265 | 1 |
| DRC 2009 B | 113 | 197 | 266 | 188 | 188 | 239 | 265 | 1 |
| DRC 2009 B | 114 | 197 | 266 | 188 | 188 | 245 | 265 | 4 |
| DRC 2009 B | 115 | 197 | 272 | 188 | 188 | 245 | 0 | 1 |
| DRC 2009 B | 116 | 197 | 272 | 188 | 188 | 245 | 265 | 11 |
| DRC 2009 B | 117 | 197 | 272 | 188 | 188 | 251 | 265 | 4 |
| DRC 2009 B | 118 | 197 | 278 | 188 | 188 | 245 | 265 | 1 |
| DRC 2013 | 119 | 197 | 284 | 188 | 181 | 221 | 0 | 1 |
| DRC 2013 | 120 | 197 | 284 | 188 | 181 | 221 | 265 | 4 |
| DRC 2009 A | 125 | 203 | 249 | 188 | 188 | 245 | 265 | 1 |
| DRC 2009 A | 126 | 203 | 266 | 188 | 188 | 245 | 0 | 1 |
| DRC 2009 A | 127 | 203 | 266 | 188 | 188 | 245 | 265 | 13 |
| DRC 2009 A | 128 | 203 | 266 | 188 | 188 | 251 | 265 | 1 |
| DRC 2011 | 129 | 203 | 272 | 188 | 188 | 245 | 265 | 1 |
| Guinea 2012 | 130* | 209 | 278 | 197 | 202 | 0 | 0 | 1 |

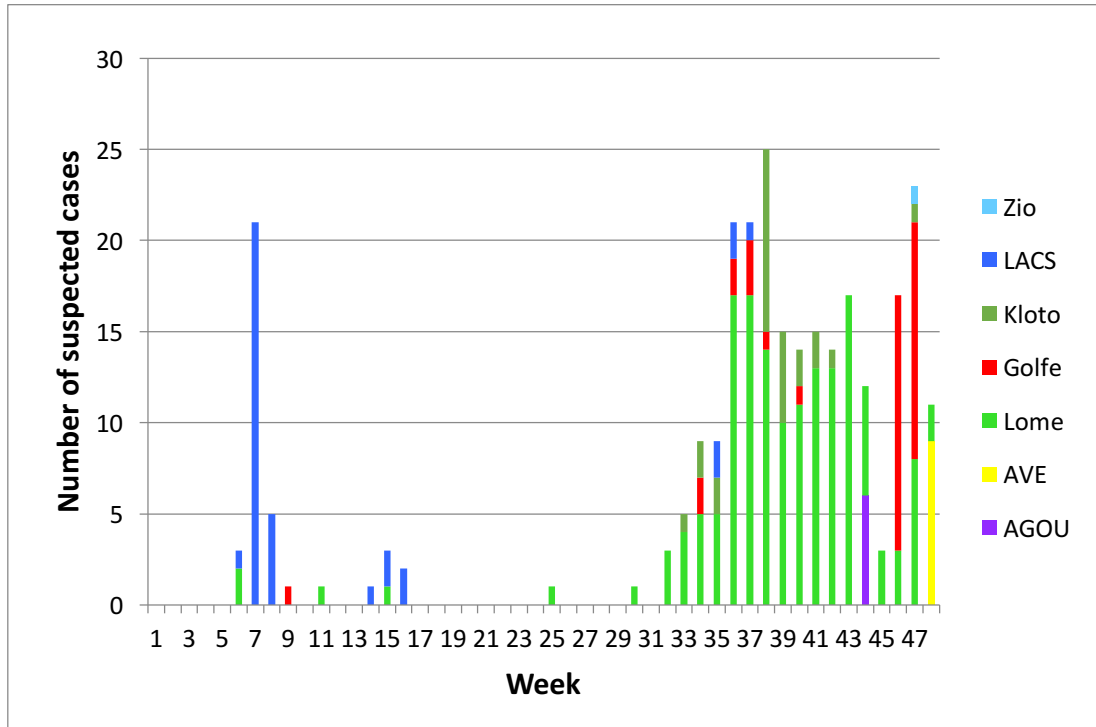
Suppl Table 1. The epidemic populations and PCR amplicon size of each allele corresponding to each MLVA type. The number of isolates corresponding to each MLVA type is indicated on the right. The environmental isolates are indicated with an asterisk.

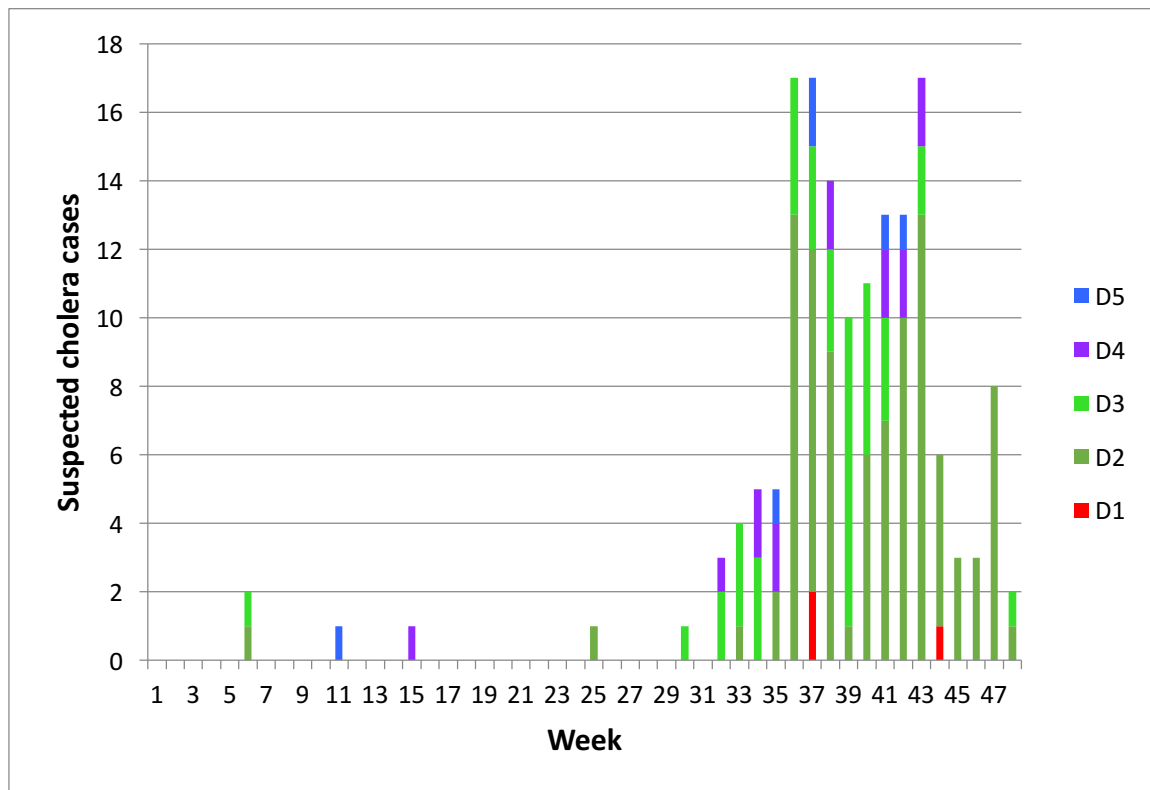
Suppl Text. Main findings of the field investigation of cholera performed in Togo in December 2014.

Togo has experienced cholera epidemics every year since 2000. However, the country has displayed a significant reduction in suspected cholera cases since the early 2000s. During the seven-year period from 2000 to 2006, a total of 7234 suspected cases were reported. Meanwhile, during the following seven-year period, only 983 cases were reported in the country, yielding an average of approximately 140 cases each year. As of week 48 of 2014, a total of 281 suspected cases were reported in Togo.

To understand the dynamics of cholera in the country we performed a detailed assessment of the 2014 epidemic. We found that an initial epidemic struck the Lacs district during weeks 6-8 (indicated in dark blue in **Suppl Figure 1**), during which three deaths were reported. Of note, the first cases reported at the same period in Lomé during week 6 proved to be negative for *V. cholerae*. During week 9 and 11, one lab-confirmed case was reported in the districts of Golfe and Lomé, respectively, although these cases failed to give rise to epidemic expansion. Five suspected cases and two cholera related deaths were again reported in Lacs during weeks 14-16.

The epidemic did not explode in Lomé until later in the year following a confirmed case on week 30. The epidemic peaked in the country on week 38. During this epidemic, a few suspected cases were also reported in Zio (1 case), Kloto (26 cases), Ave (9 cases) and Agou (6 cases). The epidemic then gradually subsided until week 45, when a second peak of cases occurred in Golfe during weeks 46-47 (**Suppl Figure 1**).



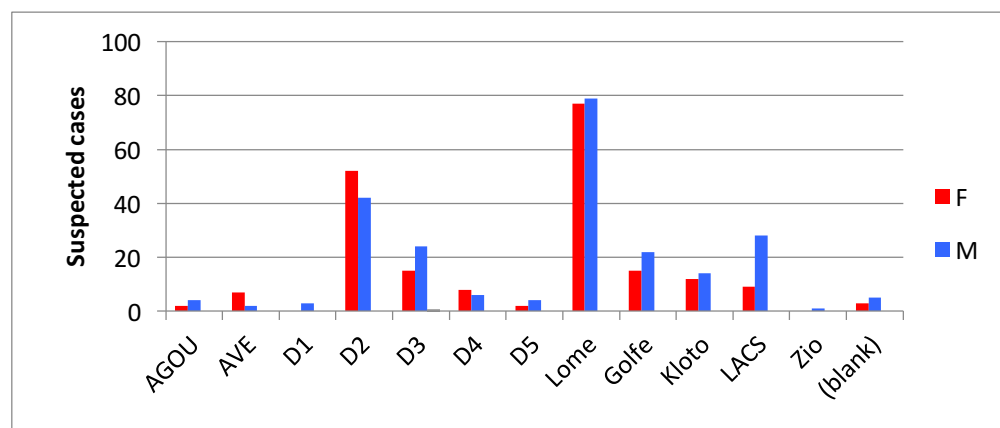


Suppl Figure 2. Evolution of the 2014 cholera epidemic in Lomé. Each district within Lomé reporting suspected cases is indicated in different colors.

Many of the cases in D3 were reported in Katanga. This site is primarily a fishing community with people of various ethnic origins. There is significant movement between Katanga and Ghana. Traditional wells are the primary source of water. The water is slightly brackish and the level varies with the tides. The wells (1 well per 5-10 households) provide access to a significant amount of water for domestic use. The exact number of fishermen affected by the disease is unknown, as this population is very mobile and may evade disease surveillance. For example, fishermen from Ghana that contract cholera in Lomé sometimes prefer to return to Ghana for treatment.

In Lacs, both epidemics from 2013 and 2014 were associated with people traveling from abroad (e.g., Nigeria and Benin) for large annual traditional animist ceremonies, which usually occur

during the dry season. For 2014, the first cases were people who attended the ceremony in Séko and then a few secondary cases occurred before the outbreak came to a halt. In Séko, the traditional animist ceremony was described to be rather “masculine”, which likely explains why males were more affected (**Suppl Figure 3**). Furthermore, the attendants of such ceremonies tend to be older, which correlates with the older average age of cases in Lacs (37.5 years), compared with Golfe (26.1 years), Kloto (31.9 years), D2 (27.6 years) and D3 (31.6 years). During the ceremony of 2014, it was stated that open defecation occurred adjacent to the site where animals were slaughtered for the meals. Activities also took place along a riverbank where attendants drink untreated water directly from the river, which could be easily contaminated by the open defecation practice.



Suppl Figure 3. Cholera epidemic of 2014 in Togo: sex distribution by district.

An unusual two-week peak of 27 cases was reported in Golfe during week 46-47. The majority of the cases (67%; 18 of 27 cases) were reported from Agoè zongo, in which many of the initial cases were a group of children aged and two teachers all living in close proximity. All of the six cases who were interrogated, except for an 8-year-old boy who died on Nov 15th (we spoke with his mother), obtain drinking water from the same borehole water station, although it is in very good condition.

The index case in Golfe during week 46-47 was likely a 17-year-old boy who first developed symptoms on Nov 13 at 2 AM. He stated that he had consumed water from the borehole water station and beans and cassava flour at home. He declared no history of travel or receiving visitors. Nobody in his immediate courtyard contracted the disease.

11 Integrated study of cholera epidemics in West Africa

Our group has previously shown that an integrated approach, combining spatiotemporal assessment of cholera outbreaks, field investigations and molecular analyses of *V. cholerae* isolates enables significant insight into the mechanisms behind cholera epidemics. When investigating the cholera epidemic that struck Guinea in 2012, we found that the strain was likely imported from neighboring Sierra Leone by fishermen traveling across the boarder. The first few modest outbreaks occurred in fishing villages along the coast. However, cholera case numbers rose dramatically in the city of Conakry with the onset of the rainy season, where it was found that flooding and latrine overflow instigated spread of the bacterium in crowded Conakry neighborhoods (Rebaudet et al. 2014). We extended this line of investigation throughout West Africa, from Benin to Mauritania. First, we continued to examine the link between the epidemics in Guinea and Sierra Leone in 2012 via MLVA applied using DNA directly extracted from filter paper stool samples. We then conducted spatiotemporal assessment of epidemics throughout the region between 2009 and 2015 and field investigations in Benin, Togo, Ghana, Côte d'Ivoire, Sierra Leone, Guinea, and Senegal. We performed MLVA and whole-genome sequencing of *V. cholerae* isolates from the majority of epidemics affecting West Africa since 2010. Taken together, these studies provide the first cohesive vision of cholera dynamics in a vast proportion of West Africa with novel insights that challenge the “cholera dogma”.

11.1 Direct dried stool sampling on filter paper for molecular analyses of cholera

Dear Sir:

We read with great interest the recent article by Debes et al. (Debes, Ateudjieu, Guenou, Ebile, et al. 2016) concerning simplified cholera surveillance methods performed in the Far North Region of Cameroon in 2013-2014. The authors describe the novel use of filter paper to preserve DNA specimens for PCR confirmation of cholera. In another recent article (Debes, Ateudjieu, Guenou, Lopez, et al. 2016), they employed the same sampling method to genotype *Vibrio cholerae* using multiple loci variable number of tandem repeats (VNTR) analysis (MLVA), in the same resource-constrained area. In both studies, stool specimens were initially enriched for 6–8 hours in alkaline peptone water (APW). One to 2 drops of the enrichment were subsequently aliquoted onto Whatman 903 Protein Saver Cards and allowed to air-dry.

While investigating the origin of a cholera epidemic in the Republic of Guinea (Rebaudet et al. 2014) in September 2012, we experimented direct dried stool sampling on filter paper in the neighboring Sierra Leone, without prior enrichment in APW. Indeed, analysis of surveillance data and field investigations suggested a recent importation of cholera from Sierra Leone. However, lacking laboratory capacities in this country impaired the inclusion of samples for genetic comparison with Guinean culture isolates (Chattaway et al. 2014). Dried blood or saliva specimens on filter paper are relatively easy to collect, store and transport, as they are not subject to United Nations biosafety regulations for transport of category B infectious substances (UNECE 2013). Moreover, direct PCR detection of *V. cholerae* on stool samples has been shown to represent a successful diagnosis method for cholera (Varela et al. 1994)

With official authorization of Sierra Leonean health authorities, 17 suspected cholera patients were thus anonymously sampled in cholera treatment units in several coastal districts (**Suppl Figure 4**). One or 2 drops of their watery diarrhea were directly collected from non-chlorinated buckets below cholera cots and then deposited onto each sample area of Whatman FTA Elute Micro Cards (GE Healthcare Bio-Sciences). Each sample was air-dried for approximately 5 minutes

in a shaded area, sealed with a Dessicant Packet into individual Multi-Barrier Pouches (GE Healthcare Bio-Sciences), transported to Marseille, France, and stored at ambient temperature. For technical reasons, DNA extraction was performed only in June 2015. Using a scalpel, a 1-cm² area of the filter paper was removed and suspended in 500 µL NucliSENS[®] easyMAG[®] lysis buffer (bioMérieux) overnight at 4°C. The DNA was then extracted using a NucliSENS[®] easyMAG[®] platform (bioMérieux) as previously described (Moore et al. 2015), and the supernatants were stored at -20°C for downstream PCR assays. To check for conserved DNA, a 16S ribosomal DNA PCR was performed as previously described (Paster et al. 2001). Six previously described VNTRs (Moore et al. 2015) were independently genotyped for each sample. All primer pairs were verified to be specific for *V. cholerae* using NCBI BLAST and one primer set is located within the cholera toxin A subunit promoter region. MLVA results of the Sierra Leonean samples were included within the genotype panel of 35 Guinean isolates isolated via conventional culture in February-September 2012 (**Suppl Figure 4**) and genotyped using the same method (Moore et al. 2015).

The presence of amplifiable bacterial DNA was confirmed by 16S PCR in all 17 filter paper samples (Supplementary Table). Toxigenic *V. cholerae* was confirmed and MLVA genotyping was completed for nine of them. All VNTR-specific PCRs were negative for the eight remaining samples, which suggests that the corresponding patients may have been affected by non-cholera acute watery diarrhea. The panel of 9 genotyped samples from Sierra Leone displayed four distinct MLVA types (1, 5, 6 and 7). Together with the samples from Guinea, these MLVA types formed a single clonal complex of 13 closely related MLVA types (**Suppl Figure 4**). Two MLVA types (5 and 6) were common to both Sierra Leonean and Guinean samples (**Suppl Figure 4**).

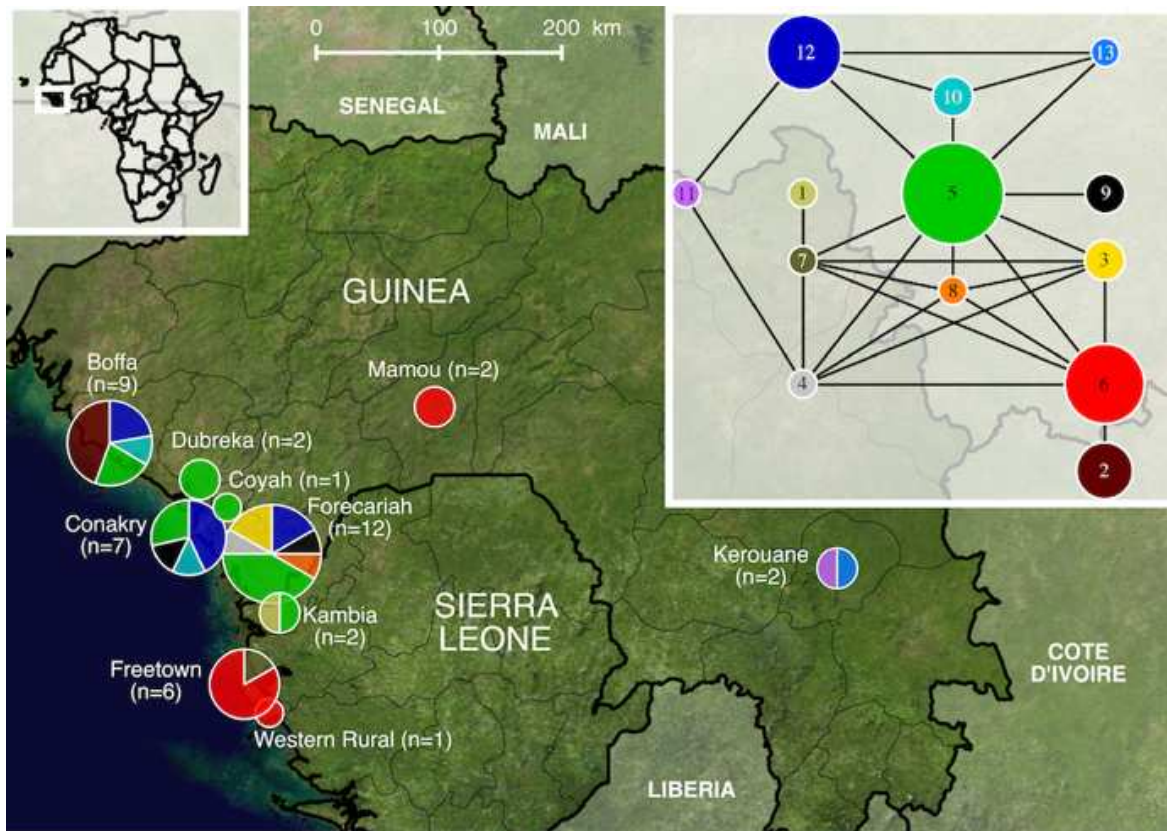
We thus confirm that filter paper is a very convenient, inexpensive and efficient tool to sample suspected cholera cases for delayed molecular studies, including *V. cholerae* MLVA genotyping, even after several years of storage at room temperature. Moreover, we demonstrate that prior stool enrichment in APW is not necessary to perform specific *V. cholerae* PCRs, which renders sampling even easier. Further experiments are required to determine whether this method of

DNA collection and conservation is sufficient for *V. cholerae* whole-genome SNP-based phylogenetics.

Additionally, MLVA genotyping of these cholera filter paper samples further bolsters the initial conclusion that Sierra Leone and the Republic of Guinea were affected in 2012 by the same transborder cholera epidemic (Rebaudet et al. 2014), which was genetically related to outbreaks in Togo between 2010 and 2012 (Moore et al. 2015).

Such genotyping results can provide valuable insight to optimize control and prevention strategies. Direct stool sampling on filter papers should therefore be included in the rapid response package to investigate cholera epidemics, especially when *V. cholerae* culture and strain storage facilities are not available or biosafety shipping to specialized genotyping laboratories is too complicated.

For **Supplementary Table. List of clinical cholera isolates included in this study.** Please see AJTMH online supplementary data.



Suppl Figure 4. MLVA types, relatedness and spatial distribution of 44 *V. cholerae* clinical isolates from the 2012 cholera epidemic in Sierra Leone and Guinea.

Nine samples were collected in Sierra Leone in September 2012 using dried spots of stool on filter papers, and thirty-five isolates were sampled via conventional stool culture in Guinea between February and September 2012. Six VNTRs (VC1, VC4, VC5, VC9, LAV6 and VCMS12) were genotyped as previously described (Moore et al. 2015). Thirteen MLVA types were identified. On the MLVA network, each MLVA type is represented by a node and is identified by a number. The size of the nodes reflects the number of isolates with each MLVA type. The solid lines indicate the single locus variants. On the map, pie charts indicate the spatial distribution of MLVA types in both countries. MLVA network was performed using RStudio version 0.98.994 for Mac (<http://www.rstudio.com/>, accessed 7 March 2016) with R version 3.1.1 (<http://www.r-project.org/>, accessed 7 March 2016) and igraph package (<https://cran.r-project.org/web/packages/igraph/index.html>, accessed 7 March 2016). The map was drawn using QGIS v2.12.1-Lyon (<http://www.qgis.org/en/site/>, accessed 7 March 2016).

11.2 Dynamics of Cholera Epidemics in West Africa

11.2.1 Introduction

Seven cholera pandemics have been documented since 1817 (Echenberg 2011). The disease has plagued every continent, spreading along trade routes via both land and sea (Echenberg 2011). Current epidemics are however localized to Southeast Asia, Haiti and Sub-Saharan Africa (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Since the ongoing pandemic first reached West Africa in 1970 (Echenberg 2011), outbreaks have been repeatedly reported throughout the region (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

Cholera is contracted by consuming food or water contaminated with toxigenic *Vibrio cholerae* O1 or the derivative O139 (Kaper, Morris, and Levine 1995; Morris 2003; Sack, Sack, and Chaignat 2006; Harris et al. 2012). Numerous *V. cholerae* non-O1 and some O1 serogroups lacking the cholera toxin are autochthonous in seawaters worldwide (Colwell, Kaper, and Joseph 1977; J. Kaper et al. 1979; Chowdhury et al. 1992; Rai, Tripathi, and Joshi 1991). *V. cholerae* non-O1 serogroups have been found associated with a variety of aquatic flora and fauna, notably copepods (Vezzulli et al. 2010). In the Bay of Bengal, elevated seawater temperatures, copepod and plankton blooms, and rainfall have been shown to correlate with increased concentrations of *V. cholerae* in the environment (Huq et al. 1984; A. S. Jutla et al. 2011a; Katia Koelle et al. 2005; Anwar Huq et al. 2005). In West Africa, a study has addressed the relationship between climate inter-annual variability and cholera in Nigeria, Benin, Togo, Ghana and Cote d’Ivoire over a 20-year period. From 1987-1994, they observed temporospatial synchrony between cholera incidence and rainfall in all countries except Cote d’Ivoire (Constantin de Magny et al. 2007). Cholera has thus been depicted as a waterborne disease driven by ecological factors (Morris 2003; Sack, Sack, and Chaignat 2006; Singleton et al. 1982). Current approaches to control the disease pivot on the assumption that cholera-causing *V. cholerae* is omnipresent in the aquatic ecosystem and that outbreaks are triggered by climatic conditions (Jutla et al. 2013). However, despite numerous surveys and technological improvements, a perennial aquatic reservoir of

cholera-causing *V. cholerae* O1 has yet to be identified in West Africa (Rebaudet, Sudre, et al. 2013b).

A recent phylogenetic analysis of clinical isolates has shown that the current pandemic is characterized by successive global clonal expansion of three waves of closely related serotype O1 El Tor lineages emanating from the Bay of Bengal (Mutreja et al. 2011). The rapid global spread of pandemic cholera, often not associated with coastal areas in Africa (Rebaudet, Sudre, et al. 2013a; Rebaudet, Sudre, et al. 2013b), suggests that *V. cholerae* spreads within and between human populations by direct or indirect human-to-human transmission. Indeed, social factors such as population density, migration, sanitation and hygiene also influence cholera transmission (“Cholera Fact Sheet (number 107)” 2015). As the seventh pandemic isolates were distinct from environmental isolates, which lack the pathogenicity islands encoding the cholera toxin (Mutreja et al. 2011), these findings challenge the hypothesis that a stable environmental reservoir is the primary source of epidemics. Whether cholera outbreaks in West Africa are triggered from an environmental or human reservoir requires drastically different control strategies.

We applied an integrated approach to elucidate the dynamics of cholera epidemics and the factors that influence the disease in coastal West Africa, from Benin to Mauritania. We conducted spatiotemporal assessment of epidemics throughout the region between 2009 and 2015 and field investigations in Benin, Togo, Ghana, Côte d'Ivoire, Sierra Leone, Guinea, and Senegal. We performed MLVA (Multi-Locus VNTR [Variable Number Tandem Repeat] Analysis) and whole-genome sequencing of *V. cholerae* isolates from the majority of epidemics affecting West Africa since 2010. We describe our findings country-by-country, from Benin to Mauritania.

11.2.2 Methods

Cholera case and rainfall data

Databases of all suspected cholera cases were collected from the epidemiological unit of Benin, Togo, Ghana and Guinea. National databases comprised weekly case/death numbers at the district level (Ghana and Togo), commune level (Benin), or prefecture level (Guinea) since 2009. For heavily affected areas, we also analyzed the cholera case line lists, which include data on age, sex, clinical outcome and residence. We obtained approval from the Ministry of Health (MoH) of each country to use these databases for epidemiological, research and publication purposes. The line lists were anonymized and cleaned prior to analysis.

Daily-accumulated rainfall data for GAR were obtained from satellite estimates (TRMM_3B42RT_DAILY.007) from NASA (<http://disc.gsfc.nasa.gov/precipitation/tovas>).

Field investigations

Field investigations of index cases and local conditions that supported cholera emergence and transmission were conducted in affected areas by epidemiologists with local counterparts. They included basic interviews among affected communities identified by the hospital- and community-based surveillance system and followed routine procedures of the corresponding MoH. We also evaluated ecological, social, water and sanitation conditions.

Field investigations were performed in Côte d'Ivoire (12/2013; SM and RP); Ghana, Togo and Benin (11-12/2014; SM, PC, and RP); Guinea and Sierra Leone (08-09/2012; SR); and Senegal (05-10/2013; GCDM).

Cartography

The country maps were generated using QGIS v2.8-Wien with shapefiles from DIVA-GIS (<http://www.diva-gis.org/gdata>). The shapefile of Accra was generated with QGIS in collaboration with the Ministry of local government, Division of environmental health, Accra.

***V. cholerae* isolate culture, DNA isolation, MLVA and whole-genome sequencing**

A total of 173 *V. cholerae* O1 clinical isolates collected throughout Ghana from 2010 to 2014 were provided by the National Public Health Reference Laboratory, Accra. The isolates were sub-cultured and transported in glycerol tubes at ambient temperature to Marseille, France. Aliquots of the culture were directly submitted for DNA extraction. We also analyzed three *V. cholerae* isolates from Senegal in 2011 following the same procedure. The MLVA results of the Ghanaian isolates were compared with those of previously analyzed strains from Togo (35 isolates), Guinea (37 isolates) and Sierra Leone (nine isolates) as previously described (Rebaudet et al. 2014; Moore et al. 2015).

DNA was extracted using a NucliSENS® easyMAG® platform (bioMérieux) (Sandra Moore et al. 2015). MLVA of the isolates using an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems) was performed using six VNTRs as described previously (Moore et al. 2015), and the relationship between the isolates was established using the goeBURST algorithm on PHYLOViZ v1.1 (<http://www.phyloviz.net/>). We performed a phylogenetic assessment of the core *V. cholerae* genome of the strains of the third wave of the seventh pandemic (Mutreja et al. 2011) based on genome-wide SNPs. Isolates from Togo-2010 (six), Togo-2011 (six), Togo-2012 (five), Ghana-2011 (one), Ghana-2014 (five), and Guinea-2012 (two) were also included in the analysis. DNA was sequenced using a HiSeq Illumina System® (Illumina) and analyzed as described (Mutreja et al. 2011).

11.2.3 Results

11.2.3.1 Cholera epidemics in West Africa

From 2009 to 2015, Benin and Togo accounted for a combined average of 694 cholera cases annually (**Table 11**). In Benin, the lakeside commune of Sô-Ava, which is directly connected to Nigeria via Lake Nokoué and Yewa River, reported cholera outbreaks every year since 2010 and was often the first and hardest-hit commune. Sô-Ava reported 40% of cases in 2013 and 30.4% of cases in 2014 (Ministry of Health - MoH). Cotonou, the economic center of Benin, was only affected by limited cholera outbreaks in 2010, 2011 and 2013, also in neighborhoods characterized by fishing activity and pronounced population movement (MoH). Overall, intense commercial activity via road and boat may represent a major pathway by which cholera is imported from outbreaks in Nigeria to susceptible waterfront communities in Benin. In neighboring Togo, most outbreaks in Lomé, the capital of Togo, occurred in flood zones or areas linked with fishing activity and intense population movement. Meanwhile, outbreaks in the eastern Lacs Prefecture, Togo were associated with travelers from Benin or Nigeria attending traditional animist ceremonies. Despite the regular importation of cholera into Togo, outbreaks in the country often remain small and contained (**Suppl Figure 5**).

| Country | Suspected cholera cases reported | | | | | | | |
|--|----------------------------------|------|------|------|------|------|------|-------|
| | 2015 | 2014 | 2013 | 2012 | 2011 | 2010 | 2009 | Total |
| Coastal West African countries included in the epidemiological study | | | | | | | | |
| Cote d'Ivoire | 200 | 248 | 56 | 424 | 1261 | 32 | 5 | 2226 |
| Guinea | 0 | 2 | 319 | 7350 | 3 | 0 | 42 | 7716 |
| Benin | 0 | 874 | 528 | 668 | 775 | 983 | 74 | 3902 |
| Togo | 50 | 329 | 194 | 61 | 33 | 72 | 218 | 957 |

| | | | | | | | | |
|----------------------|-----|-------|-----|-------|-------|------|------|-------|
| Liberia | 0 | 60 | 92 | 219 | 1146 | 1546 | 1070 | 4133 |
| Sierra Leone | 0 | 0 | 377 | 23124 | 0 | 0 | 0 | 23501 |
| Ghana | 687 | 28944 | 20 | 9563 | 10387 | 438 | 1294 | 51333 |
| Guinea-Bissau | 0 | 18 | 969 | 3068 | 0 | 0 | 5 | 4060 |
| Senegal | 0 | 0 | 0 | 1 | 5 | 3 | 4 | 13 |
| Mauritania | 0 | 0 | 0 | 0 | 46 | 0 | 0 | 46 |
| The Gambia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Countries neighboring the study region

| | | | | | | | | |
|---------------------|------|-------|------|------|-------|-------|-------|--------|
| Burkina Faso | 0 | 0 | 0 | 143 | 20 | 0 | 0 | 163 |
| Nigeria | 5913 | 35996 | 6600 | 597 | 23377 | 44456 | 13691 | 130630 |
| Niger | 51 | 2059 | 585 | 5284 | 2324 | 1154 | 0 | 11457 |
| Mali | 0 | 0 | 23 | 219 | 2220 | 0 | 0 | 2462 |

Table 11. The number of suspected cholera cases reported in each country included in the study per year. Reported cases in the countries neighboring the study region are also indicated. The data is based on Weekly Epidemiological Records (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

Ghana accounted for 52.4% of all cholera cases in coastal countries from Benin to Mauritania, from 2009 to 2015 (51,333 suspected cases in Ghana / 97,887 total suspected cases in the 11 countries) (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Since 2011, cholera epidemics have significantly intensified in Accra, the capital of Ghana. The majority (73.6%) of cases from 2011 to 2014 was reported in the Greater Accra Region (GAR) (35,985 cases GAR/ 48,914 cases Ghana) (MoH). During this period, each epidemic started in GAR, with the exception of the 2011 epidemic, which started with small outbreaks in Central Region in late 2010 before intensifying in GAR in January 2011. **Figure 14** displays the sharp increase in

cases at the onset of each epidemic, indicating a rapid early expansion of the bacterium within Accra. Strikingly, from the end of 2012 through mid-2014, Ghana experienced an 18-month lull in cholera cases, despite rainfall and flooding. All 20 suspected cholera case samples in 2013 tested negative for *V. cholerae*. After this significant lull, Ghana experienced the largest epidemic (28,944 cases in 2014) since 1991 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

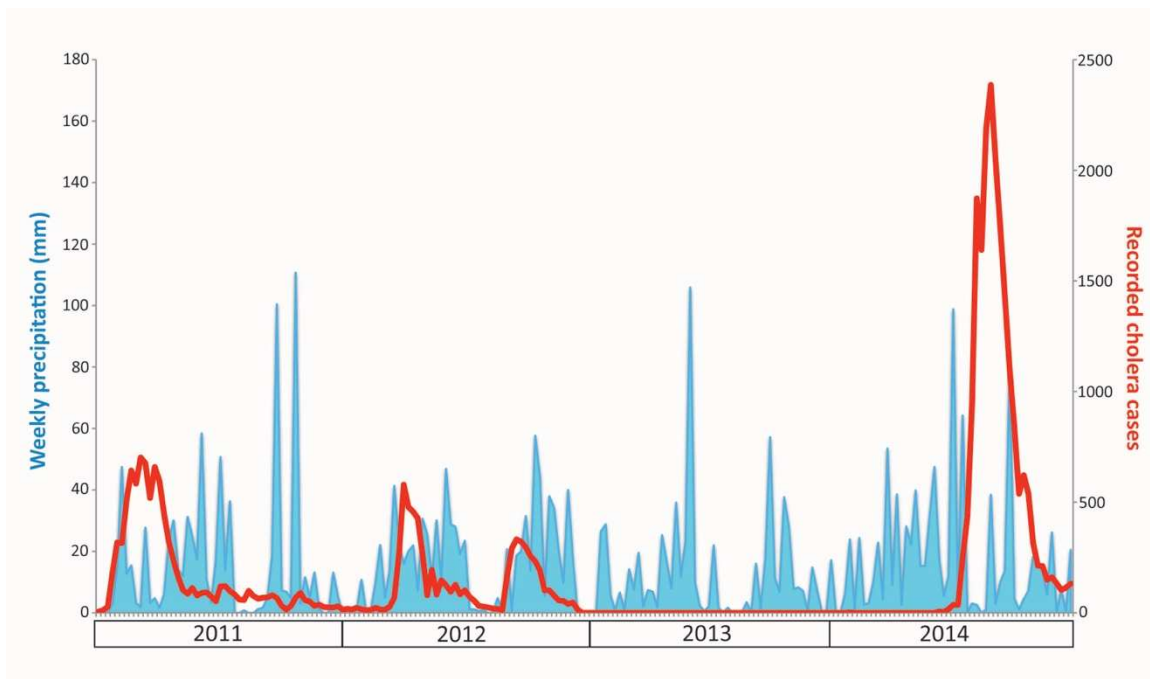


Figure 14. Weekly evolution of cholera epidemics and rainfall levels in Greater Accra Region from 2011 to 2014. Suspected cholera cases are indicated in red (right y-axis), and rainfall is indicated in blue (left y-axis). The corresponding year is labeled on the x-axis.

Once outbreaks erupted in Accra, cholera rapidly diffused throughout the majority of the city. This rapid spatial diffusion pattern was observed during the onset of the 2011, 2012, and 2014 epidemics. Many Accra neighborhoods were severely affected by cholera each year. However, certain nearby residential areas remained largely cholera-free, despite outbreaks in adjacent neighborhoods (**Figure 15**). Once epidemics erupted in Accra, outbreaks spread to other districts

in Ghana several weeks later. Field investigations revealed that 2014 index cholera cases in Ho, Volta Region were associated with people traveling from Accra (**Suppl Figure 5**).

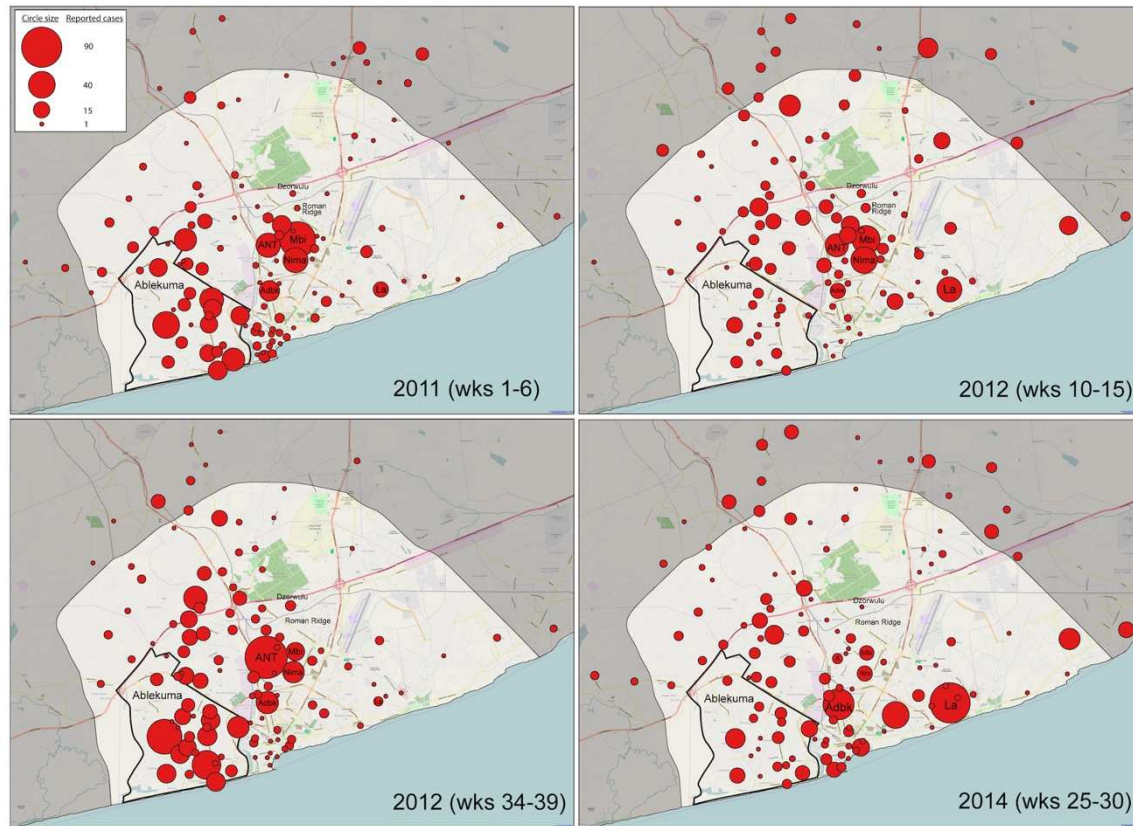


Figure 15. Distribution of cholera cases during the initial six weeks of epidemic escalation in Accra Metropolis from 2011 to 2014. The cumulative cases reported in the Accra Metropolis line list for each neighborhood during the first six weeks of each epidemic are indicated with red circles. Circle size represents the relative number of cases reported in each neighborhood. Approximately 90% of all neighborhoods in the line lists were localized. The neighborhoods of Maamobi, Nima, Accra New Town, and Adabraka often reported many cases during the first six weeks of each outbreak. Many parts of Ablekuma reported several cholera cases during outbreak onset. Labadi was the hardest-hit zone during the beginning of the 2014 epidemic. In contrast, certain nearby residential areas remained largely cholera-free (e.g., Dzorwulu and Roman Ridge), despite major outbreaks in adjacent neighborhoods. The total cases accounted for and percentage coverage of sites localized for each epidemic are as follows: 2011 (897 cases; 88%), first 2012 epidemic (780 cases; 92%), second 2012 epidemic (932 cases; 94%), and 2014 (913 cases; 86%). The districts adjacent to Accra Metropolis are grayed out. Abbreviations: ANT/A, Accra New Town; Mbi, Maamobi; Adbk, Adabraka; La, Labadi; Nm, Nima.

Field investigations revealed that water distribution was often interrupted for several days in many Accra neighborhoods. Water network pipes were often visibly damaged and running along the ground through roadside gutters. Many residents perform open defecation into these gutters, due to lack of proper latrine facilities. In locales with functioning latrines, septic tanks were sometimes connected to open drains. We thus hypothesize that ground water and human waste could seep into broken pipes, especially during the frequent water shortages, thereby allowing *V. cholerae* to enter the water network and spread when water pressure is restored. Increased rainfall and flooding markedly exacerbated this effect (**Figure 14**).

From 2009 to 2010, Côte d'Ivoire was largely unaffected by cholera (37 suspected cases) (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). However, an epidemic erupted in Abidjan in January 2011 following the post-election crisis of November 2010 and a collapse in the health and sanitation systems (World Health Organization: Global Task Force on Cholera Control 2011). The 2011 epidemic was responsible for 1,261 cases (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). We found that a new epidemic emerged in May 2012 in Sud Comoé, adjacent to Jomoro District in Ghana, where an outbreak was ongoing. As observed in Ghana, Côte d'Ivoire also experienced a complete lull in cholera in 2013 and early 2014. Each of the 56 suspected cases in 2013 tested negative for *V. cholerae* (World Health Organization 2014). This lull was interrupted in early October 2014, when an outbreak occurred in Abidjan with the arrival of ill Ghanaian fishermen (**Suppl Figure 5**) (United Nations 2014).

Liberia has reported 4,133 suspected cholera cases from 2009 to 2015 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). However, as no cholera-related deaths were reported from 2010 to 2013, and only two deaths (1,070 cases) were reported in 2009, it remains unclear whether a significant proportion of these suspected cases were truly cholera.

Following a three-year lull in the incidence of cholera, both Sierra Leone and Guinea experienced a trans-border epidemic in 2012, with 22,932 and 7,351 cases, respectively (Rebaudet et al. 2014). The two epidemics progressed following a very similar pattern. In Guinea, the outbreak started in February on Kaback Island with a fisherman traveling from Sierra Leone. In Sierra Leone, possible events of *V. cholerae* importation by fishermen travelling from Liberia and Ghana have been reported (Dunoyer et al. 2013). During the rainy season, cholera exploded in the capitals, which recorded over half of the total cases (Freetown, 52%; Conakry, 64%) (**Suppl Figure 5**) (Rebaudet et al. 2014; Dunoyer et al. 2013).

As cholera rates declined in Sierra Leone and Guinea, they rose in Guinea-Bissau (Dunoyer et al. 2013; Reliefweb 2012; WHO 2013), which also followed a near three-year lull. The country reported 3,068 cases in 2012 and 969 cases in 2013. Eighteen and zero cases were reported in 2014 and 2015, respectively (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

The number of cholera cases reported in The Gambia, Senegal and Mauritania has been very low since 2009 to present. The Gambia has not reported a single suspected cholera case since 2008. Likewise, Mauritania has reported no cholera since 2008, with the exception of 46 cases in 2011. Since 2009, Senegal has reported only 13 suspected cholera cases (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016).

*11.2.3.2 Genetic analyses of *V. cholerae* isolates*

We performed MLVA of 255 clinical *V. cholerae* isolates from Ghana, Togo, Guinea, Sierra Leone and Senegal. Two environmental isolates from Guinea 2012 were also included (**Suppl Table 2**). Interestingly, the Minimum Spanning Tree (MST) shows that the 2010/2011 isolates from Ghana were closely related to and likely seeded the epidemic in Guinea and Sierra Leone in 2012. The MST also demonstrates that the 2011, 2012 and 2014 epidemics in Ghana were due to three distinct *V. cholerae* MLVA-type clusters. The three clinical isolates from Senegal in 2011 displayed

an identical MLVA type, closely related to strains from Togo in 2011 and 2012, which may represent imported cases from further south in West Africa (**Figure 16**).

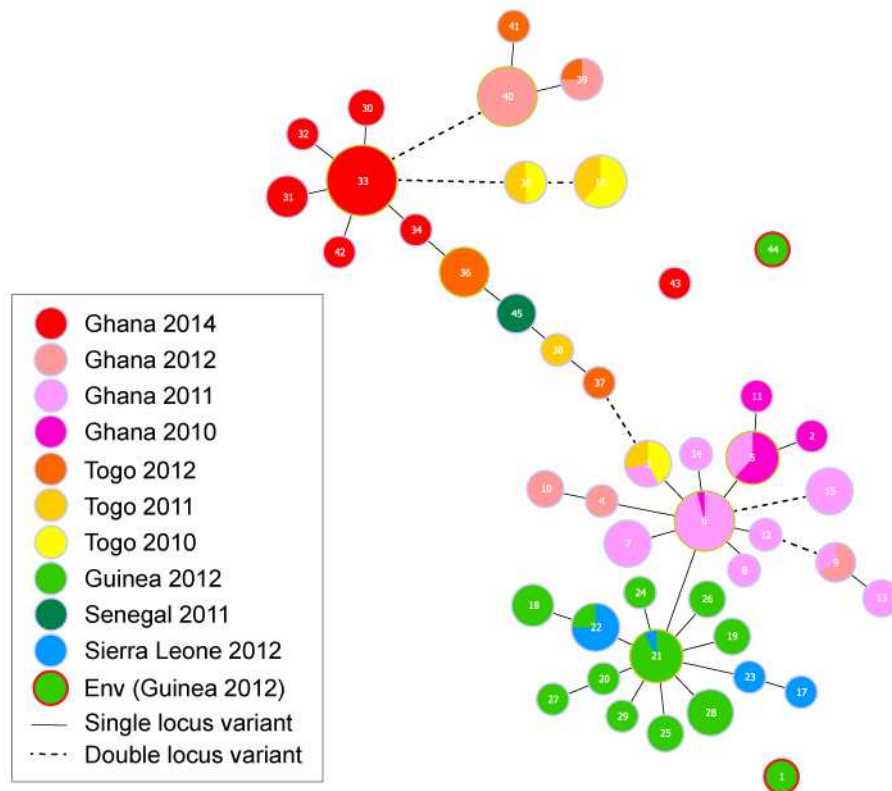


Figure 16. Minimum Spanning Tree based on the MLVA types of 257 *V. cholerae* isolates from several recent West African cholera outbreaks. Each MLVA type is represented by a node (and a unique number), and the size of the nodes reflects the number of isolates of each MLVA type. The solid lines indicate the most likely single locus variant, while dashed lines indicate the most likely double locus variant. The colors reflect the distinct country and year of isolate origin. Pie charts indicate strains from different time periods or countries displaying an identical MLVA type. The two strains represented by MLVA types #1 and #44 were isolated from environmental samples in Guinea (encircled in red).

Whole-genome SNP sequence analysis and phylogeny assessment of select strains from Ghana and Togo correlate with the MST. When the Ghana and Togo strains were included in the alignment of the seventh pandemic *V. cholerae* isolates (Mutreja et al. 2011), the Ghana 2011 and closely linked strains from Togo grouped with the Guinea 2012 strains on the third wave of

the current pandemic. In contrast, Ghana 2014 and other Togo strains clustered together in a separate clade, genetically distinct from the Ghana 2011 cluster (**Suppl Figure 6**).

11.2.4 Discussion

From 2009 to 2015, we found that Accra, the capital of Ghana, represented the hotspot of cholera in the entire region of coastal West Africa, west of Nigeria. Cholera outbreaks spread from Accra into other countries in a wave-like fashion. We show that the main cholera epidemic wave spread westward from Ghana in 2011 to Sierra Leone, Guinea and likely Guinea-Bissau in 2012, affecting every country in between (**Figure 17**). Genetic analysis confirmed that the Ghanaian isolates of 2011 likely seeded the 2012 epidemics in Guinea and Sierra Leone. Field elements suggest that the distinct outbreaks were linked via migration of specific populations, such as certain groups of fishermen who travel long distances at sea with limited access to improved sanitation and potable water. We noted that other cities (Conakry and Freetown) also appeared to function as amplifiers of cholera, when cases were present and rainfall increased. Strikingly, we found that many countries deemed cholera-endemic (Ali et al. 2015) actually suffered very few outbreaks, with multi-year lull periods during which no cholera cases were detected. Extended lulls in cholera incidence occurred despite increased rainfall, typical high temperatures, slums and population exposure to coastal environments.

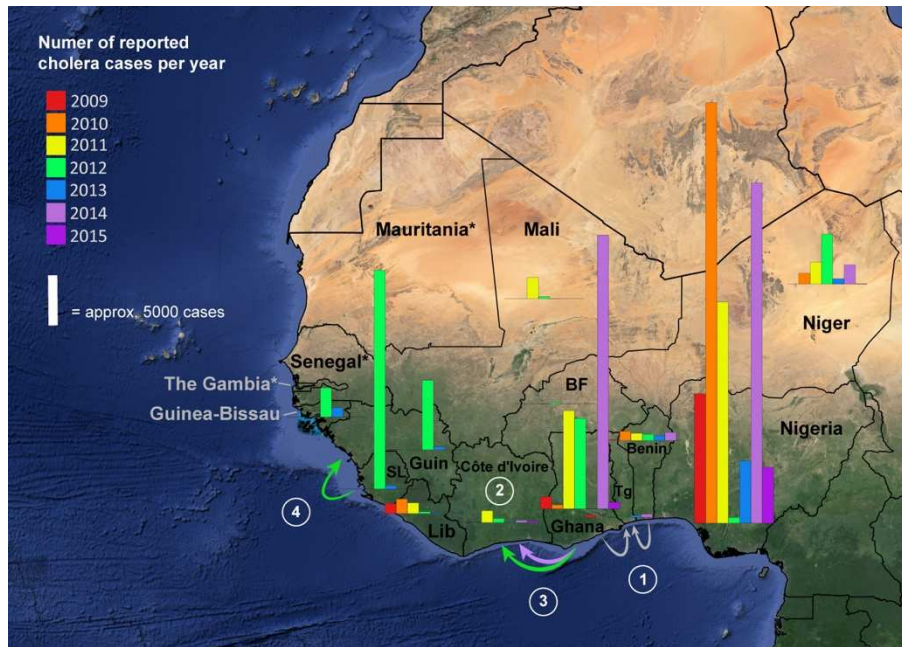


Figure 17. Annual cholera cases reported in West Africa from 2009 to 2015 and major identified events involved in the westward wave of epidemics from 2011 to 2014. Key events and observations involved in cholera epidemics: (1) events of *V. cholerae* importation from Benin and Ghana to Togo; (2) the 2011 cholera epidemic in Côte d'Ivoire broke out following the post-election crisis and public health breakdown; (3) in Côte d'Ivoire, 2012 and 2104 index cases had traveled from Ghana; and (4) a *V. cholerae* clone was imported to Guinea by a fisherman from Sierra Leone (index case of the 2012 epidemic in Guinea). Countries reporting less than 50 cases for the study period are indicated with an asterisk. Abbreviations: SL, Sierra Leone; Guin, Guinea; Lib, Liberia; BF, Burkina Faso; Tg, Togo.

Our findings and independent reports indicate that the Accra water network may play a role in rapid diffusion of cholera throughout a majority of the city and therefore proliferation of *V. cholerae* O1. A study in Accra (Osu Klottey) has shown that drinking community pipe-borne water (OR=2.15) was associated with cholera in 2012 (Davies-Teye et al. 2014). Furthermore, a separate study has revealed unsuitable residual chlorine levels and the regular presence of fecal coliform in the Accra network water during the dry season (Karikari and Ampofo 2013).

Molecular assessment of epidemic strains demonstrated that outbreaks in West Africa spread in a northwestward wave. As Ghanaian strains from previous years fail to reappear during

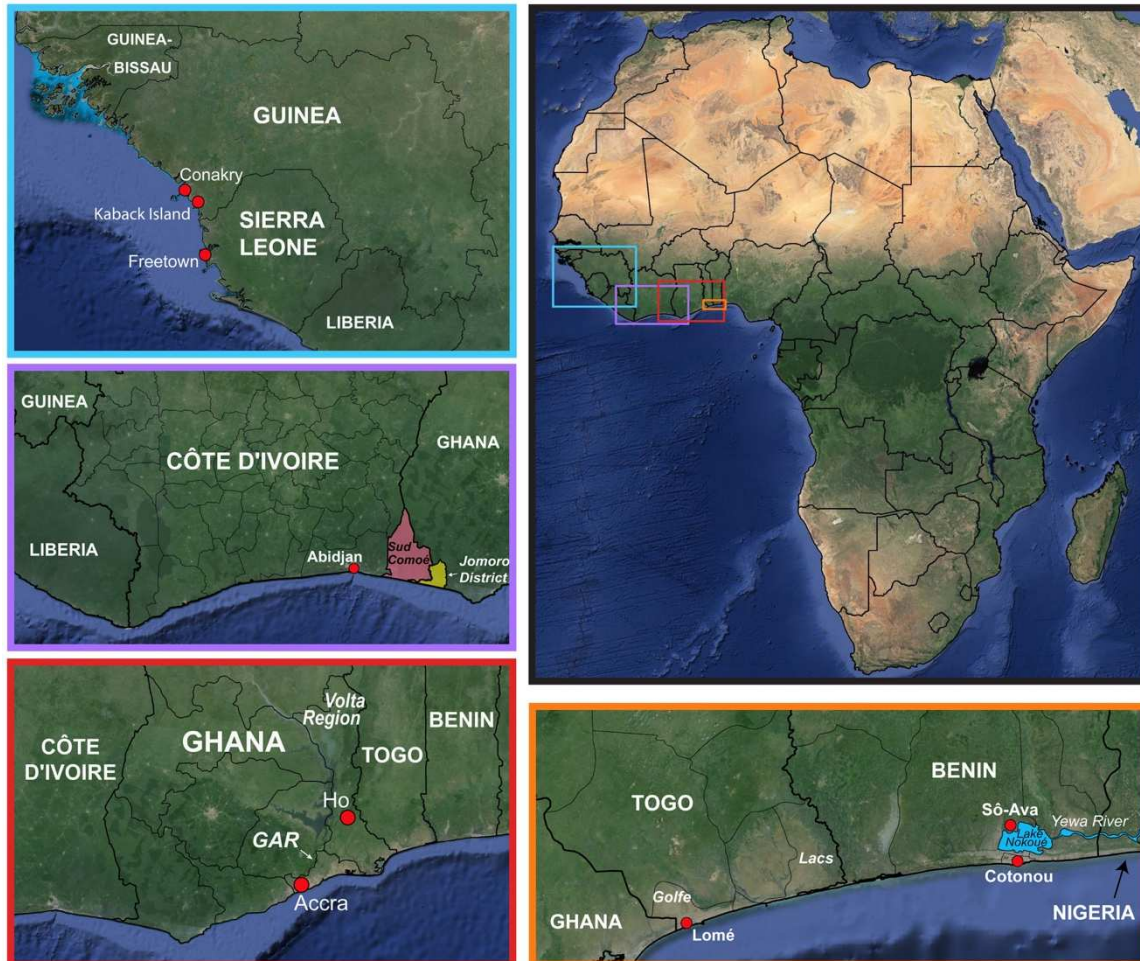
subsequent epidemics, we hypothesize that epidemics affecting Accra may likely originate due to imported cases from a nearby cholera focus. Neighboring Nigeria is currently one of the major cholera foci in the world (Piarroux and Faucher 2012), with 130,630 cases reported from 2009 to 2015 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). The country is also a likely source of outbreaks in the Lake Chad Basin (Piarroux and Faucher 2012). The lull in Ghana during 2013 paralleled a relatively low number of cholera cases reported in Nigeria in 2012 and 2013. The 2014 Ghanaian epidemic coincided with an epidemic rebound in Nigeria. Interestingly, a significant lull in cholera outbreaks is currently ongoing in West Africa, with only 94 cases in Nigeria and one case in So-Ava, Benin during the first six weeks of 2016 (UNICEF and WHO 2016a). This drop in coincides with zero cases reported in the rest of West African since week 49 of 2015 (UNICEF 2016).

To prevent expansion of cholera outbreaks in West Africa, epidemiological surveillance should be enhanced in identified vulnerable zones, such as Accra. Improved monitoring of the drinking water supply, ensuring water quality and proper chlorination, in vulnerable areas would mitigate epidemics and perhaps stop cholera propagation. Our findings also suggest that cholera may be controlled by targeted interventions or vaccination of susceptible populations that migrate extensively, such as certain fishermen.

Overall, this epidemiological overview also argues against any direct link between ecological factors, such as plankton and copepod blooms, and incidence rates of cholera, thereby raising serious doubts regarding the prevailing theory (Colwell 1996) stating that a stable ecological niche for *V. cholerae* in the aquatic environment and climate play a significant role in cholera prevalence. In contrast, our results show that cholera spread through West Africa via the movement of certain vulnerable populations, such as certain fishermen. Once cases arrive in urban settings with poor sanitation facilities, increased rainfall facilitates the infiltration of human waste, and therefore *V. cholerae*, into the water network via damaged water pipes, thus promoting a rapid increase in cholera incidence. Translating scientific findings into public health

strategies, UNICEF has recently adopted this vision of cholera dynamics to combat cholera in Africa.

11.2.5 Supplementary files



Suppl Figure 5. Map of Africa indicating the localization of sites discussed in field investigation analyses.

A zoom on the Guinea/Sierra Leone region (blue), southern Côte d'Ivoire (purple), southern Ghana (red), and southern Togo and Benin (orange) are shown.

| Isolate IDs | Epidemic population | MLVA type | Locus | | | | | | Num of isolates |
|---|--|--------------|-------|-----|-----|-----|------|--------|--------------------|
| | | | VC1 | VC4 | VC5 | VC9 | LAV6 | VCMS12 | |
| L247 | Guinea_2012 | 1* | 0 | 0 | 185 | 188 | 281 | 0 | 1 |
| G20-10 | Ghana_2010 | 2 | 179 | 232 | 188 | 188 | 269 | 272 | 1 |
| L387, L388, L390, L399, L400, G472-11, G470-11 | Togo_2010, Togo_2011, Ghana_2011 | 3 | 179 | 243 | 188 | 188 | 251 | 272 | 7 |
| G55-12 | Ghana_2012 | 4 | 179 | 243 | 188 | 188 | 263 | 272 | 1 |
| G28-11, G30-11, G704-11, G870-11, G15-10, G16-10, G17-10, G21-10, G25-10, G35-10, G13-10, G695-11, G14-10 | Ghana_2010, Ghana_2011 | 5 | 179 | 243 | 188 | 188 | 269 | 272 | 13 |
| G13-11, G60-11, G69-11, G427-11, G435-11, G68-11, G693-11, G699-11, G726-11, G781-11, G784-11, G800-11, G802-11, G807-11, G813-11, G869-11, G691-11, G18-10, G27-11, G33-11, G48-11, G50-11, G64-11, G760-11, G812-11 | Ghana_2010, Ghana_2011 | 6 | 179 | 243 | 188 | 188 | 281 | 272 | 25 |
| G762-11, G694-11, G714-11, G725-11, G732-11, G740-11, G860-11 | Ghana_2011 | 7 | 179 | 243 | 188 | 188 | 287 | 272 | 7 |
| G787-11 | Ghana_2011 | 8 | 179 | 243 | 188 | 188 | 293 | 272 | 1 |
| G56-12, G57-12, G755-11 | Ghana_2011, Ghana_2012 | 9 | 179 | 249 | 188 | 188 | 203 | 265 | 3 |
| G58-12, G54-12 | Ghana_2012 | 10 | 179 | 249 | 188 | 188 | 263 | 272 | 2 |
| G19-10 | Ghana_2010 | 11 | 179 | 249 | 188 | 188 | 269 | 272 | 1 |
| G810-11 | Ghana_2011 | 12 | 179 | 249 | 188 | 188 | 281 | 272 | 1 |
| G880-11, G867-11 | Ghana_2011 | 13 | 179 | 255 | 188 | 188 | 203 | 265 | 2 |
| G478-11 | Ghana_2011 | 14 | 179 | 255 | 188 | 188 | 281 | 272 | 1 |

| | | | | | | | | | |
|--|--------------------------------------|----|-----|-----|-----|-----|-----|-----|----|
| G689-11, G722-11, G820-11, G823-11, G719-11, G712-11, G816-11 | Ghana_2011 | 15 | 179 | 266 | 188 | 195 | 281 | 272 | 7 |
| L386, L389, L391, L392, L393, L394, L395, L398, L401, L402, L403, L405, L406 | Togo_2010, Togo_2011 | 16 | 185 | 220 | 188 | 160 | 233 | 272 | 13 |
| SL25 | SierraL_2012 | 17 | 185 | 238 | 188 | 188 | 299 | 272 | 1 |
| L220, L222, L227, L241 | Guinea_2012 | 18 | 185 | 243 | 188 | 181 | 287 | 272 | 4 |
| L235, L237 | Guinea_2012 | 19 | 185 | 243 | 188 | 188 | 269 | 272 | 2 |
| L234 | Guinea_2012 | 20 | 185 | 243 | 188 | 188 | 275 | 272 | 1 |
| L211, L212, L214, L215, L217, L218, L219, L236, L239, L242, L245, SL26, L219 | Guinea_2012, SierraLeone_ 2012 | 21 | 185 | 243 | 188 | 188 | 281 | 272 | 13 |
| L230, L231, SL30, SL32, SL34, SL36, SL37, SL38 | Guinea_2012, SierraLeone_ 2012 | 22 | 185 | 243 | 188 | 188 | 287 | 272 | 8 |
| SL41 | SierraL_2012 | 23 | 185 | 243 | 188 | 188 | 299 | 272 | 1 |
| L213 | Guinea_2012 | 24 | 185 | 243 | 188 | 188 | 311 | 272 | 1 |
| L225, L226 | Guinea_2012 | 25 | 185 | 243 | 188 | 195 | 281 | 272 | 2 |
| L216, L244 | Guinea_2012 | 26 | 185 | 249 | 188 | 188 | 281 | 272 | 2 |
| L223 | Guinea_2012 | 27 | 185 | 255 | 188 | 188 | 275 | 272 | 1 |
| L221, L228, L229, L233, L238, L240, L243 | Guinea_2012 | 28 | 185 | 255 | 188 | 188 | 281 | 272 | 7 |
| L224 | Guinea_2012 | 29 | 185 | 272 | 188 | 188 | 281 | 272 | 1 |
| G468-14, G372-14 | Ghana_2014 | 30 | 191 | 209 | 188 | 160 | 269 | 272 | 2 |
| G173-14, G169-14, G182-14, G487-14, | Ghana_2014 | 31 | 191 | 215 | 188 | 160 | 215 | 272 | 4 |
| G281-14 | Ghana_2014 | 32 | 191 | 215 | 188 | 160 | 269 | 265 | 1 |

| | | | | | | | | | |
|--|--------------------------|----|-----|-----|-----|-----|-----|-----|----|
| G360-14, G180-14, G262-14, G269-14, G19-14, G30-14, G36-14, G40-14, G55- 14, G41-14, G93-14, G56-14, G59-14, G96-14, G114-14, G115-14, G131-14, G134-14, G135-14, G152-14, G154-14, G161-14, G204-14, G214-14, G224-14, G226-14, G239-14, G242-14, G273-14, G274-14, G278-14, G280-14, G282-14, G283-14, G286-14, G291-14, G292-14, G293-14, G297-14, G299-14, G303-14, G307-14, G309-14, G319-14, G322-14, G350-14, G381-14, G386-14, G393-14, G401-14, G408-14, G411-14, G413-14, G414-14, G455-14, G456-14, G471-14, G490-14, G494-14, G498-14, G34-14, G39-14, G171-14, G234-14, G312-14, G316-14, G330-14, G359-14, G403-14, G493-14 | Ghana_2014 | 33 | 191 | 215 | 188 | 160 | 269 | 272 | 70 |
| G271-14 | Ghana_2014 | 34 | 191 | 215 | 188 | 160 | 275 | 272 | 1 |
| L396, L397, L404, L407 | Togo_2010, Togo_2011 | 35 | 191 | 232 | 188 | 160 | 233 | 272 | 4 |
| L412, L413, L415, L416, L417, L419, L420, L421, L422 | Togo_2012 | 36 | 191 | 238 | 188 | 160 | 275 | 272 | 9 |
| L409 | Togo_2012 | 37 | 191 | 243 | 188 | 160 | 251 | 272 | 1 |
| L408 | Togo_2011 | 38 | 191 | 243 | 188 | 160 | 257 | 272 | 1 |
| L410, G10-12, G201-12, G426-12 | Togo_2012, Ghana_2012 | 39 | 191 | 266 | 188 | 160 | 239 | 272 | 4 |

| | | | | | | | | | |
|--|--------------|-----|-----|-----|-----|-----|-----|-----|----|
| G9-12, G462-12, G463-12, G471-12, G473-12, G475-12, G477-12, G478-12, G480-12, G481-12, G483-12, G487-12, G490-12, G491-12, G496-12, G498-12, G499-12, G520-12, G521-12, G525-12, G537-12, G543-12, G517-12 | Ghana_2012 | 40 | 191 | 272 | 188 | 160 | 239 | 272 | 23 |
| L418 | Togo_2012 | 41 | 191 | 272 | 188 | 160 | 245 | 272 | 1 |
| G146-14 | Ghana_2014 | 42 | 197 | 215 | 188 | 160 | 269 | 272 | 1 |
| G160-14 | Ghana_2014 | 43 | 209 | 238 | 170 | 181 | 0 | 0 | 1 |
| L246 | Guinea_2012 | 44* | 209 | 278 | 197 | 202 | 0 | 0 | 1 |
| S5091c, S5151, S5161 | Senegal_2011 | 45 | 191 | 238 | 188 | 160 | 257 | 272 | 3 |

Suppl Table 2. The epidemic populations, isolate IDs, and PCR amplicon size of each allele corresponding to each MLVA type. The number of isolates corresponding to each MLVA type is indicated on the right. The environmental isolates are indicated with an asterisk (MLVA type column).

12 Conclusion

Over the course of seven pandemics recorded since 1817, cholera has affected each continent. In the early 1800s, cholera was believed to spread via miasma: pollution or “bad air”. It was not until the London cholera epidemic of 1854 that John Snow showed that residents of the overpopulated city contracted cholera by consuming well water contaminated with human sewage. The bacterium only causes disease in humans, which were considered to represent the main reservoir of the bacterium. During the late 1800s, it was believed that the disease spread from person-to-person via transitory vectors such as water and food contaminated with the toxigenic bacterium (Echenberg 2011). However, when *V. cholerae* species were found in the Chesapeake Bay in the 1970s, it was suggested that the bacterium was a potential autochthonous inhabitant of brackish water and estuarine ecosystems (Morris 2003; Colwell, Kaper, and Joseph 1977). Over 200 serogroups of *V. cholerae* have since been identified in surveys of environmental samples, although only serogroups O1 and the derivative O139 cause cholera epidemics (Chun et al. 2009; Mutreja et al. 2011; Albert et al. 1993; Chatterjee and Chaudhuri 2003; Siddique et al. 1996; Shimada et al. 1994). The bacteria associates with a wide range of flora and fauna, and copepods are considered the major reservoir of *V. cholerae* strains (Vezzulli et al. 2010). Furthermore, certain correlations have been observed (in Bangladesh) between sea surface temperatures, copepod blooms, rainfall events and cholera outbreaks (Koelle 2009a; Huq et al. 2005; Constantin de Magny et al. 2008; Jutla et al. 2011a). The culmination of such observations ultimately led to the establishment of the “cholera paradigm”, which claims that cholera is contracted by exposure to environmental reservoirs of toxigenic *V. cholerae* and that outbreaks are driven directly by climate and environmental factors (Colwell 1996).

It has been thus widely suggested that current cholera epidemics ebb and flow in other “endemic” regions, such as Sub-Saharan Africa, due to an environmental reservoir of *V. cholerae* in coastal waters. Since the current seventh cholera pandemic appeared in Sub-Saharan Africa during the 1970s, cases have been reported on the continent every year, with cholera outbreaks primarily clustered at certain hotspots including the African Great Lakes Region (especially in eastern DRC)

and West Africa (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). In certain coastal West African countries, rainfall events have been shown to correlate with cholera outbreaks (Constantin de Magny et al. 2007). This logic has been extended to the ongoing epidemic in Haiti. Following the importation of a *V. cholerae* clone into this once cholera-free country, current outbreaks are believed to be due to toxigenic *V. cholerae* O1 supposedly settled in local waters. Many scientific studies and some public health policies thus pivot on the assumption that a permanent reservoir of *V. cholerae* in the aquatic environment is at the root of cholera epidemics (Lutz et al. 2013).

However, recent data argue against this theory that the environment hosts a perennial reservoir of cholera-causing *V. cholerae*. Therefore, we set out to gain a clear understanding of the dynamics of current cholera epidemics. We first performed a literature review to examine the clonal nature of cholera outbreaks, with a focus on the recent history of cholera in the Americas. We then investigated epidemics over an extended time period at three major foci: Haiti, the DRC and West Africa. An integrated approach was applied to assess epidemics at various scales, combining spatiotemporal assessment of contemporary epidemics, genetic and antibiogram analyses of *V. cholerae* strains, and a series of field investigations. Overall, our findings shed light on the dynamics of cholera epidemic emergence and diffusion. Our studies also reveal many elements that reinforce the theory that human populations rather than the aquatic environment serve as the main reservoir of *V. cholerae* O1, thereby strongly questioning the widely accepted environmental “cholera paradigm”.

Cholera-causing *V. cholerae* strains are adapted for inter-human transmission

Assessment of *V. cholerae* isolates by our group and others demonstrate that strains responsible for cholera epidemics are specifically adapted for inter-human transmission. Once the bacterium is ingested and reaches the small intestine, it produces and releases the cholera toxin complex. The toxin is internalized by epithelial cells, where the toxin A subunit indirectly induces constitutive cyclic AMP production (Gill and Kings 1975; Gill 1976b; Cassel and Pfeuffert 1978;

Gill and Meren 1978), which triggers secretion of water and electrolytes into the lumen of the small intestines (Cassel and Pfeuffert 1978; Gill and Meren 1978; Kaper, Morris, and Levine 1995). The cholera toxin thus facilitates transmission between humans by provoking severe discharge of the newly multiplied *V. cholerae* and eventual contamination of food or water sources, thereby infecting a subsequent host if local health and sanitation is not adequate.

The bacterium *V. cholerae* O1 causes disease exclusively in humans. Furthermore, of the vast spectrum of *V. cholerae* serotypes that have been identified, only a specific subset of *V. cholerae* O1 El Tor ‘types’, which have become increasingly specialized in inter-human transmission, have triggered epidemics of the on-going pandemic (Safa, Nair, and Kong 2009; Mutreja et al. 2011). The Vibrio seventh pandemic islands I (VSP-I) and II (VSP-II) are hallmark features specific to the seventh pandemic El Tor O1 lineage (Dziejman et al. 2001). At least VSP I plays a role in enhancing intestinal colonization and likely transmission of *V. cholerae* (O’Shea et al. 2004; Davies et al. 2013; Butler et al. 2006). Current epidemic strains have also acquired SXT-encoded antibiotic resistance elements, which also enhance inter-human transmission (Mutreja et al. 2011). In our assessment of 1,093 *V. cholerae* isolates from the DRC from 1997 to 2012, we found that *V. cholerae* strains displayed an increasingly complex resistance phenotype to various antimicrobial drugs over the course of the study time period (Miwanda et al. 2015). Strains exhibiting new resistance phenotypes have emerged during other recent epidemics in Africa, such as those in Cameroon and Zambia (Ngandjio et al. 2009; Mwansa et al. 2007). Overall, expression of the cholera toxin as well as the acquisition of both VSP-I-encoded factors and drug resistance elements has improved the fitness of the *V. cholerae* variant strains by enhancing human intestinal colonization and transmission.

***V. cholerae* strains responsible for epidemics are clonal**

Whole-genome sequence-based phylogeny has not only demonstrated that are cholera epidemics due to this specific subset of *V. cholerae* O1 El Tor ‘types’, but it has also shown that the current pandemic is monophyletic and originated from a single clone, with a common

ancestor dating to the 1950s. Lineages of the current pandemic appear to emerge, diversify and eventually become extinct. This study also showed that new epidemics paralleled major events of human migration (Mutreja et al. 2011).

In our review, we discuss the cholera epidemics of the 1990s in South America (Moore et al. 2014). The initial outbreak outbreak struck Peru in 1991, which was the first cholera outbreak to affect the region since 1895, and subsequently spread throughout most of South America and Mexico (Seas et al. 2000; “World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). The origin of the cholera epidemic was initially suggested to be due to *V. cholerae* species that had settled in the coastal environment (Seas et al. 2000). However, genetic assessment later showed that the responsible isolates formed a distinct genetic lineage, denoted WASA 1, which has evolved from an isolate collected in 1989 from Angola (Mutreja et al. 2011). Thus, these strains were all derived from a single clone sharing a common origin, which strongly suggests that the disease was imported by an individual traveling likely from Africa. Following the cholera epidemics in South America during the 1990s, many experts presumed that *V. cholerae* had settled in the coastal environment and would cause epidemic re-emergence for many years. However, Latin America remained almost completely unaffected by the disease from 2001 to 2010, until the cholera epidemic erupted in Haiti in October 2010 (“World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Likewise, microbiological analysis has also demonstrated that the Haitian cholera isolates were almost identical to isolates collected in Nepal a few weeks prior, thereby strongly confirming the epidemiological study that suggested the Haitian cholera strains were very recently imported from Nepal (Hendriksen, Price, and Schupp 2011; Frerichs et al. 2012b).

As the seventh pandemic phylogeny published by Mutreja et al. lacked strains from Central and West Africa, we analyzed a panel of *V. cholerae* strains from these regions via MLVA and whole-genome sequencing, while strains from the DRC were also assessed for antibiotic resistance. Using MLVA, we assessed 237 *V. cholerae* isolates from epidemics in the DRC occurring in 2008, 2009, 2011, 2012 and 2013. A total of 27 *V. cholerae* clinical isolates from the 2012 epidemic in

nearby Zambian were also analyzed. The MLVA study of African strains revealed two clusters: 1) West African isolates and 2) Central African isolates (DRC and Zambia). All but seven clinical isolates from the DRC and Zambia 2012 were linked by one- or two-VNTR variations. Furthermore, each epidemic complex was characterized by a central founder MLVA haplotype and closely related derivative SLV or DLV MLVA haplotypes, which branched from the founder (Moore et al. 2015).

In the DRC, the large majority of cholera cases are reported in the eastern lake *provinces* (Bompangue et al. 2008). However, in 2011, an epidemic aggressively diffused from the onset point in Kisangani, Orientale Province across the country in less than 130 days. Strikingly, outbreaks followed the Congo River and quickly reached non-endemic zones in the West that had not experienced an epidemic for approximately 10 years (Bompangue et al. 2012). This epidemic spilled over into 2012. Analysis of these *V. cholerae* isolates via MLVA shed light on the mechanisms behind the 2011-2012 epidemic. Our analysis showed that the 2011 and 2012 isolates from the DRC grouped together into one discrete complex that likely arose from a founder strain identified at the beginning of the epidemic. Interestingly, this founder MLVA-type was already present in the DRC since at least 2009. Phylogeny analysis situated this DRC strain close to the major Kenyan clade in the most recent wave of the seventh pandemic (Moore et al. 2015). The expansion of the isolate population coincided with the progression of the epidemic (Bompangue et al. 2012), as the founder MLVA types corresponded to isolates collected at outbreak onset and more distantly related derivatives represented isolates found later during the epidemic. The strains responsible for the 2011-2012 epidemic also displayed a single antimicrobial drug susceptibility profile: characterized by tetracycline and doxycycline sensitivity. This period was also marked by a serotype switch. During 2001–2010, although Inaba and Ogawa serotypes were both present, Ogawa strains largely predominated. However, during 2011–2012, the Ogawa serotype was almost completely replaced by Inaba (Miwanda et al. 2015). Overall, our results show that this epidemic was caused by the expansion of a specific *V. cholerae* El Tor variant subpopulation which likely crossed the country via population migration.

Assessment of *V. cholerae* isolates from epidemics in West Africa revealed a similar phenomenon. Our investigations have demonstrated that Accra is the major focus of cholera in West Africa, west of Nigeria, and that epidemics spread westward from Ghana in a wave-like pattern. From 2009 to 2015, we identified one major epidemic wave in which an epidemic commencing in Ghana in 2010 spread along the West African coast to reach Sierra Leone, Guinea and likely Guinea Bissau in 2012, affecting each country in between along the way. MLVA has shown that the isolates from Ghana in 2010 and 2011 are genetically closely related to the clinical isolates collected in Guinea in 2012 (Moore et al., unpublished). In a previous study, our group has demonstrated that the 2012 Guinean cholera epidemic was triggered by a single *V. cholerae* clone that was imported by a fisherman travelling from Sierra Leone (Rebaudet et al. 2014). In **Direct dried stool sampling on filter paper for molecular analyses of cholera**, we confirm via MLVA genotyping that the Sierra Leone and Guinea were indeed affected by the same transborder cholera epidemic in 2012 (Rebaudet et al. 2016).

Overall, our investigations of cholera epidemics at an intercontinental scale show that cholera appears to be largely caused by a subset of specific *V. cholerae* clones, which are disseminated via inter-human transmission, rather than by the vast diversity of *V. cholerae* strains found in the environment. Our observations correlate with the findings of Mutreja *et al.* demonstrating that the current pandemic is monophyletic and originated from a single ancestral clone.

Identification of direct transmission of cholera via population movement

Molecular analysis of *V. cholerae* strains indicates that cholera diffuses via population movement, and our field investigations in Haiti and West Africa as well as other reports further confirm this hypothesis.

When a cholera epidemic struck **Haiti** in 2010 for the first time since at least one hundred years (Jenson and Szabo 2011), many experts suggested that the epidemic was triggered by climatic events, claiming that a rise in the temperature of the Haitian waters induced the growth of *V.*

cholerae presumably residing dormant in the environment (Knox 2010). However, epidemiological studies and field investigations demonstrated that the epidemic strain was imported by UN Nepalese peacekeepers traveling from Nepal, where a cholera epidemic was ongoing at the time. An exact spatio-temporal correlation was found between the arrival of the soldiers in Haiti and the first reported cholera cases in Meille, a small village 2 km south of Mirebalais (Piarroux et al. 2011). Initial spread of the epidemic throughout the country correlated with mass contamination of the Artibonite River and subsequent movement of populations due to widespread panic (Piarroux et al. 2011).

Similar incidents have been repeatedly observed in Africa. The current pandemic first appeared on the African continent in 1970 when *V. cholerae* was imported by migrants traveling to the Guinean capital of Conakry from Crimea, where an epidemic was ongoing (Echenberg 2011). As cholera cases have continuously been reported in West Africa since this time, it has been presumed that *V. cholerae* has settled in the coastal waters and that current epidemics are caused by an environmental reservoir of the bacterium. However, during our field investigations in several West African countries, we found that the emergence of new cholera outbreaks was associated with movement of individuals traveling from a site where an epidemic was already ongoing. Here, we described our observations country-by-country, from Benin to Guinea.

In **Benin**, the lakeside commune of Sô-Ava, which is directly connected to Nigeria via Lake Nokoué and Yewa River, reported cholera outbreaks every year since 2010 and was often the first and hardest-hit commune. Sô-Ava reported 40% of cases in 2013 and 30.4% of cases in 2014. In Sô-Ava, many “floating” villages are located with houses built on stilts in the water, where the main occupation of the population was historically fishing-related activities. However, now that the lake has been overfished, we found that the people have resorted to a variety of alternative means to make a living, including both illegal and legal commercial activity between Benin and Nigeria via road and river. Cotonou, the economic center of Benin, was only affected by limited cholera outbreaks in 2010, 2011 and 2013, also in neighborhoods characterized by fishing activity and pronounced population movement. Overall, intense commercial activity between

populations of these neighboring countries represents a major pathway by which *V. cholerae* is likely imported from Nigeria to susceptible waterfront communities in Benin (Moore et al., unpublished).

In **Togo**, we found that cases in Lomé were often associated with areas linked with fishing activity as well as flood zones. In D2 (Lomé), cases appear to be associated with movement from Ghana and the large market close to the port. In D3 (Lomé), people of various ethnic origins are settled in the fishing community of Katanga, where there is significant movement between Katanga and Ghana. Along the border with Benin in Lacs Prefecture, the epidemics during 2013 and 2014 were both associated with people traveling from abroad (e.g., Nigeria, Ghana and Benin) for large annual traditional animist ceremonies, which usually occur during the dry season. During the ceremony of 2014, it was stated that open defecation occurred adjacent to the site where animals were slaughtered for the shared meal. Activities also took place along a riverbank where attendants drink untreated water directly from the river, which could be easily contaminated by the open defecation practice (Moore et al., unpublished).

The major cholera focus in coastal West Africa, west of Nigeria, was Accra, **Ghana**. Cholera epidemics in Accra eventually gave rise to outbreaks in neighboring areas. Field investigations conducted in other affected districts of Ghana (Ketu South and Ho) revealed that index cholera cases were associated with people traveling from Accra. Ketu South Municipality is located in the Volta Region, in the southeastern corner of Ghana. An episode of severe diarrhea and vomiting was reported in Ketu South on August 11, 2014. The index case had just returned from Accra and developed the condition on that same day and subsequently infected a family member. Ho is located further north in the Volta region, 158 km from Accra. High population movement occurs between Ho and the capital. Over the weekend of August 22-24, 2014, three *V. cholerae*-positive cases were reported in Ho. The first confirmed case from Ho municipal was a local hospital orderly who attended a female patient from Ho West. The second was a pastor from Ashaiman in the Greater Accra Region, who developed symptoms just prior to visiting his family in Ho (Moore et al., unpublished).

An epidemic erupted in Abidjan, **Côte d'Ivoire** in January 2011 following the post-election crisis of November 2010 and a collapse in the health and sanitation systems. The 2011 epidemic was responsible for 1,261 cases. Although an index case could not be identified due to the humanitarian crisis, we later found that subsequent index cases had traveled from a region of Ghana experiencing an ongoing cholera outbreak (World Health Organization: Global Task Force on Cholera Control 2011). In May 2012, a new epidemic emerged in Sud Comoé, adjacent to Jomoro District in Ghana, where an outbreak was ongoing. We also identified a cholera index case in San-Pédro, a young man who fell ill while traveling from Accra in 2012. Likewise, in early October 2014, a new outbreak occurred in Abidjan with the arrival of ill Ghanaian fishermen sailing from Ghana (Moore et al., unpublished).

Our group has previously conducted an analysis of the cholera epidemic that struck **Guinea** in 2012 following a three-year lull. The report showed that the epidemic strain was likely imported via fishermen traveling from Sierra Leone, where an epidemic was ongoing at the time (Rebaudet et al. 2014). It was thus hypothesized that the cholera epidemics affecting Guinea and Sierra Leone represented a single transborder epidemic. Using MLVA, we confirmed that the clinical *V. cholerae* strains from the 2012 cholera epidemics in Guinea and Sierra Leone were in fact genetically closely related and derived from the cholera epidemic that occurred in Ghana in 2010 and 2011 (Moore et al., unpublished).

Overall, our findings demonstrate that, at least in Haiti and West Africa, cholera outbreaks have emerged and diffused with population movement. Often index cases were those traveling long distances with limited access to both clean drinking water and proper sanitation, such as certain fisherman populations and merchants. Indeed, many fisherman populations were found to be vulnerable to cholera outbreaks along the coast of West Africa, from Benin to Guinea. Our observations and other reports have also shown that civil disruption, as observed in Côte d'Ivoire in 2011, and troop deployment to vulnerable locales, such as the case of Haiti in 2010, can play a major role in triggering a cholera epidemic.

The role that cities (and city water networks) play in amplification of cholera outbreaks

In West Africa, we found that the large majority of cases were reported from large cities, such as Accra (Moore et al., unpublished), Freetown and Conakry (Rebaudet et al. 2014). In each case, epidemics exploded when a few cases were present and rainfall increased. Our investigation and independent studies indicate that the Accra water network likely plays a major role in the rapid diffusion of the toxigenic bacterium throughout the city and therefore proliferation of *V. cholerae* O1. In Accra, we found that water distribution was often interrupted for several days in many neighborhoods, leading residents to collect unsecure water for household needs (**Figure 18**). In numerous locales, residents illegally connect their own pipes to the water network, which results in a tangled mass of plastic tubes termed “spaghetti pipes”. Poorly maintained water network pipes were often visibly damaged and running along the ground and through roadside gutters (**Figure 19**). Many residents perform open defecation into these gutters, due to lack of proper latrine facilities. In locales with functioning latrines, septic tanks were sometimes connected to open drains. Based on our observations, we believe that rainwater along with human waste could permeate broken pipes, especially during the frequent water shortages, thereby allowing *V. cholerae* to enter the water network. This contaminated water would then rapidly spread when water pressure is restored. Increased rainfall and flooding markedly exacerbated this effect (Moore et al., unpublished). A separate investigation has indeed revealed unsuitable residual chlorine levels and the regular presence of fecal coliforms in the Accra network water even during the dry season (Karikari and Ampofo 2013). In correlation with our hypothesis, a study in Accra sub-metro Osu Klottey has shown that drinking community pipe-borne water was associated with contracting cholera in 2012 (Davies-Teye et al. 2014). This situation is an unfortunate result of globalization, as populations migrate towards metropolises for employment at a rate quicker than public infrastructure can support increased population growth.



Figure 18. Children in La Municipality, Accra Metro, collecting water running along pipes. The children stated that the water would be used for household use, which highlights the extreme water shortages that many Accra residents experience. The collection buckets were also resting in the gutter water flowing just below the pipe, thereby further contaminating the water collected (Photo credit: Sandra Moore).



Figure 19. Self-installed water network pipes “spaghetti pipes” running along the ground near drainage flow in Agboghloshie, Accra. This situation is representative of many neighborhoods in Accra Metro (Photo credit: Paul Cottavoz).

We found that Abidjan, in contrast to Accra, was less susceptible to major cholera outbreaks. However, civil disruption that led to a complete (albeit temporary) degradation of the health, water and sanitation infrastructure was sufficient to render the city vulnerable to cholera. Regardless of the proximity with Ghana, only one major epidemic occurred in Côte d’Ivoire since 2009, which followed the post-election crisis of November 2010. In early 2011, thousands of

Ivoirians were displaced, health care and disease surveillance establishments were abandoned, the sewerage system was non-existent, and access to drinking water was severely restricted in Abidjan (World Health Organization: Global Task Force on Cholera Control 2011). Thus, any cholera cases in the city (such as Ghanaian fishermen arriving in the nearby fishing villages, as often observed) could easily contaminate unprotected drinking waters sources due to the breakdown in the sanitation system.

Similar patterns have been observed in other cities of Africa. Between 2000 and 2005, the majority of cholera outbreaks in Cameroon occurred in Douala, the country's economic capital. Outbreaks have typically started in densely populated slums, where the residents have limited access to proper sanitation facilities and clean drinking water (Akoachere, Masalla, and Njom 2013). In Guinea-Bissau, cholera outbreaks in 2005, 2008 and 2012 primarily affected the capital, Bissau (WHO 2013). One particular area of Bissau City, Bandim, where residents have limited access to improved drinking water and sanitation facilities, was found to be a major cholera hotspot during the 2008 outbreak (Azman et al. 2012). In 2008-2009 Zimbabwe was struck by a massive cholera epidemic, which was responsible for 128,208 cases and 5,634 deaths (WHO 2009). Approximately 50% of cases were reported from Budiro (a highly populated suburb of the capital, Harare), where it was noted that limited safe drinking water, a collapse of the waste management, water supply and sanitation systems likely played a major role in the epidemic. The report also stated that feces from cholera patients (from surrounding areas and burst sewage pipes) was washed into drinking water sources with the onset of the rainy season to aggravate the epidemic in the city (IOM International Organization for Migration 2008; Chirisa et al. 2015).

No evidence of an environmental reservoir of toxigenic *V. cholerae* O1

A variety of *V. cholerae* non-O1/non-O139 serogroups are autochthonous in coastal ecosystems, and it is presumed that cholera-causing *V. cholerae* O1 types are also resident to the aquatic environment (Morris 2003; Colwell, Kaper, and Joseph 1977). Certain climatic patterns, such as increased rainfall and elevated water temperatures, have been shown to coincide with cholera

outbreaks (Huq et al. 2005; Constantin de Magny et al. 2007). It has thus been hypothesized that cholera epidemics emerge due to fluctuations in *V. cholerae* O1 populations in the aquatic environment triggered by changes in ecological and climate conditions. Indeed, in each of our study sites, we did note that cholera outbreaks amplified in parallel with increased rainfall. However, the observations discussed below strongly suggest that the aquatic ecosystem does not host a perennial reservoir of toxigenic *V. cholerae* O1 and that the links between cholera, warm season, rainfall, climate and certain coastal areas are much likely due to other mechanisms.

As cholera outbreaks have persisted in **Haiti** following the importation of *V. cholerae* in 2010, outbreaks have continued in the country to this day, especially exacerbated during the rainy seasons. It has thus been suggested that cholera-causing *V. cholerae* O1 has settled into the coastal Haitian waters and represents an environmental source of current outbreaks (Knox 2010). In **No evidence of significant levels of toxigenic *V. cholerae* O1 in the Haitian aquatic environment during the 2012 rainy season**, we investigated whether the aquatic environment presents a major risk of cholera transmission to local populations. Various PCR assays were used to assess the levels of toxigenic *V. cholerae* O1 on a panel of water samples. The aquatic samples, which were collected during a warm period of the rainy season, were characterized by a wide range of salinity levels (freshwater to 40‰ salinity), water temperatures ranging from 27.7°C to 38.8°C, and pH levels ranging from 6.5 to 8.4. Sampling sites were located in areas profoundly affected by cholera. Although numerous non-toxigenic *V. cholerae* non-O1 isolates were widely isolated at each of the test stations, all water samples contained less than 10 toxigenic *V. cholerae* O1 bacteria per liter, a level far below that required for direct transmission to local human populations (Baron et al. 2013).

Other studies have either failed to identify *V. cholerae* O1 in the Haitian environment (Hasan et al. 2012) or identified only a couple toxigenic strains in freshwater canals during the peak of the epidemic (Hill et al. 2011). In 2012 and 2013, another study found toxigenic *V. cholerae* O1 El Tor strains in 3 of 179 water samples (Alam et al. 2014); however, with on-going cholera cases in neighboring locales, these strains (for which the details concerning collection time and location

were omitted) likely originated from cases lacking proper sanitation (Rebaudet and Piarroux 2015). It has also been hypothesized that the Haitian/Nepalese epidemic strain may exchange DNA fragments with environmental *V. cholerae* strains (Hasan et al. 2012). However, DNA sequence-based analysis of Haitian clinical isolates and environmental *V. cholerae* strains has clearly shown that the epidemic strains in Haiti exhibit no evidence of acquired genetic elements via gene transfer from environmental strains (Katz et al. 2013a). In fact, the epidemic strain was found to be poorly transformable (Katz et al. 2013a). Overall, our findings and other reports indicate that the *V. cholerae* O1 El Tor clone imported from Nepal has failed to settle into the Haitian waters, at least at a significant level. Furthermore, the environmental *V. cholerae* strains in Haiti likely play no role in the ongoing cholera epidemic.

In contrast to the “cholera paradigm”, the majority of cholera cases in **Sub-Saharan Africa** have been unassociated with the coastal environment. From 2009 to 2011, annual incidence rates of cholera were found to be three times higher in inland Africa compared with the coastal region (Rebaudet, Sudre, et al. 2013b; Rebaudet, Sudre, et al. 2013a). Indeed, the largest recorded epidemic in Africa struck the landlocked country of Zimbabwe in 2008-2009, far from any coastal waters (WHO 2009; “World Health Organization. Cholera, Number of Reported Cases (data by Country)” 2016). Outbreaks have also been reported during periods of drought and famine, as observed in Mali (Tauxe et al. 1988).

The **DRC** represents one of the major foci of cholera in the world. Cholera incidence never reaches zero in eastern DRC, and proximity to Lake Tanganyika, an African Great Lake in the east of the DRC, has been shown to be associated with increased risk of contracting cholera (Bompangue et al. 2008). Our MLVA results indeed indicate that strains have persisted in the country for an extended period (from at least 2009 to 2014), as certain MLVA-types were recovered every year of the study period (with the exception of 2010 due to limited sampling resources). However, all of the isolates collected from environmental samples in the DRC were genetically unrelated to the main clinical epidemic isolate clusters, according to our MLVA results (Moore et al. 2015). Furthermore, a recent study has aimed to determine whether cholera outbreaks at Lake

Tanganyika were induced by climate factors and an environmental reservoir of *V. cholerae* O1. Samples from neighboring Zambia were also included in the study. Fecal contamination, phytoplankton, zooplankton, and certain fish species in Lake Tanganyika were assessed for presence of *V. cholerae*; however, very few *V. cholerae* isolates were identified in environmental samples (Plisnier et al. 2015). All four environmental isolates from Uvira (DRC) and Mpulungu (Zambia) were found to be *V. cholerae* non-O1. According to the MLVA results, these environmental isolates were largely unrelated to the clinical *V. cholerae* isolates collected at the same time, which in contrast formed a closely related complex (Moore et al. 2015). Overall, these studies found no evidence of an environmental reservoir of *V. cholerae* O1 at these sites in eastern DRC and Zambia.

Our investigations in **West Africa**, from Benin to Senegal, revealed comparable findings. As observed in the DRC, clinical isolates from major epidemics in Guinea, Sierra Leone and Ghana formed closely related complexes upon Minimum Spanning Tree analysis of MLVA results. Although Togo only experienced relatively smaller outbreaks, several clinical isolates were also found to be closely related to those from Ghana, Guinea, Sierra Leone and Senegal. In contrast, the two environmental isolates recovered from Guinea during the epidemic in 2012 were largely unrelated to the *V. cholerae* strains isolated from clinical samples during the same period. During field investigations in Côte d'Ivoire and Togo, we also met with researchers who had conducted surveys of environmental samples to identify toxigenic *V. cholerae* O1 in local waters. In Abidjan, investigators from the Pasteur Institute had recovered several serogroups of non-O1/non-O139 from Lagune Ebrié, although they had been unable to identify *V. cholerae* O1 in the body of water believed to be the source of intermittent cholera outbreaks. Similarly, Togolese public health staff were unable to detect toxigenic *V. cholerae* O1 in the local waters of Katanga (the often affected fishermen neighborhood of Lomé) (Moore et al., unpublished).

Beyond our investigations, several surveys of environmental samples conducted by other groups have largely failed to identify toxigenic *V. cholerae* O1 strains in the aquatic ecosystem, recovering only non-O1/non-O139 isolates (Colwell, Kaper, and Joseph 1977; Kaper et al. 1979;

Chowdhury et al. 1992; Usera et al. 1994). The few studies that have managed to identify a small number of *V. cholerae* O1 strains either collected samples during an on-going cholera outbreak, during which patient-derived contamination of local water sources cannot be excluded (Huq et al. 1993; Tickner and Gouveia-Vigeant 2005; MS Islam et al. 1993), or used suboptimal identification techniques (Martins et al. 1993). For example, in the overcrowded slum of Bepanda in Douala, Akoachere et al. have isolated 23 *V. cholerae* O1 isolates from unprotected wells and nine O1 isolates from streams where latrine waste is disposed, the majority of which were identified during the rainy season. Tap water samples were free of *V. cholerae* O1 (Akoachere and Mbuntcha 2014). The same group found a similar pattern in the slums of New Bell (another slum of Douala) (Akoachere, Masalla, and Njom 2013). These findings again indicate contamination of unprotected drinking sources (well water) rather than a population of *V. cholerae* O1 thriving in the saltwater environment in symbiosis with copepods. In Nigeria, Eyisi et al. have identified *V. cholerae* O1 in a variety of shellfish collected from fishing villages, where residents had no toilet facilities and defecated directly into the sea (Eyisi, Nwodo, and Iroegbu 2013). As a cholera epidemic was ongoing in the country, with 13,691 cases (WHO 2012), it is likely that the strains recovered from shellfish originated from cholera cases lacking proper sanitation.

In contrast, non-toxigenic *V. cholerae* non-O1/non-O139 isolates have been readily isolated in our study and others from a variety of aquatic ecosystems (ranging from freshwater to seawater) throughout the world (many of which are cholera-free areas), such as Germany, Italy, California, Chesapeake Bay (Northeast USA), Taiwan and Columbia (Bockemühl et al. 1986; Barbieri et al. 1999; Louis et al. 2003; Wu et al. 1996; De Silva, Mantilla, and Agudelo 1997; Maugeri et al. 2004). As series of *V. cholerae* non-O1 strains have been repeatedly isolated from a variety of environments, it has thus been proposed that they may have the potential to convert into toxigenic strains and subsequently engender cholera outbreaks. However, a study of the molecular characterization of environmental and non-toxigenic strains of *V. cholerae* showed that these strains lack the cholera toxin genes and thus cannot cause cholera (Kaper, Moseley, and Falkow 1981; Faruque and Mekalanos 2012).

Not only have several surveys of environmental samples failed to identify a population of toxigenic *V. cholerae* O1 in the ecosystem, these cholera-causing species appear to be unfit for survival in environmental waters. A study examining the *in situ* elimination rate of *V. cholerae* O1 El Tor in two bodies of water in Mexico has shown that *V. cholerae* O1 were eliminated from samples derived from the brackish waters of Mecoacan Lagoon and the athalassohaline waters of Lake Alchichica at a daily rate averaging 32% and 63%, respectively (Perez, Macek, and Galvan 2004). Thus, even as water sources likely serve as a transient vector when patients inadvertently contaminate the local environment, these toxigenic O1 strains appear to have limited capacity to establish a long-term foothold in the aquatic ecosystem. Furthermore, in a pond microcosm experiment, Nelson et al. have shown that *V. cholerae* transmission is antagonized by lytic phage in the aquatic environment (Nelson et al. 2008). These observations correlate with the findings of the whole-genome sequencing-based phylogeny of the seventh pandemic *V. cholerae* isolates. Mutreja et al. have shown that present-day epidemics are caused by variant *V. cholerae* El Tor strains, which are derived from a single ancestral clone and most often spread via inter-human transmission (Mutreja et al. 2011). Environmental *V. cholerae* strains were also found to be largely unrelated to epidemic isolates. Overall, the whole gamut of epidemiological studies and genetic analyses of strains suggests that the aquatic environment does not represent a stable ecological niche for toxigenic *V. cholerae* O1.

A coherent perspective of cholera emergence and diffusion

Overall, our global reflection of cholera epidemics in these three distinct foci provides a coherent vision of the mechanisms of cholera emergence and diffusion. As the epidemiological and genetic data show that toxigenic *V. cholerae* O1 likely does not persist in the aquatic environment, we reject the hypothesis stating that cholera outbreaks are due to the proliferation of an environmental reservoir triggered by ecological events such as plankton blooms. Instead, cholera epidemics appear to be spread via inter-human transmission of a specific subset of *V. cholerae*

clones, which correlates with the clonal characteristic of the seventh pandemic strains (Mutreja et al. 2011; Moore et al. 2014).

In Africa, we found that cholera outbreaks did not occur in a homogenous pattern along the coast, as would be expected if the coastal waters hosted toxigenic *V. cholerae* O1. Instead, cases were largely concentrated in highly populated areas, such as certain cities (Moore et al., unpublished) (Rebaudet et al. 2014). Likewise, two of the major cholera hotspots in Sub-Saharan Africa are located inland far from the coasts, at the Lake Chad region and the eastern DRC *provinces* (Piarroux and Faucher 2012).

We have consistently seen that novel cholera outbreaks emerged when toxigenic *V. cholerae* was imported via index cases from a nearby (or not so nearby) focus. Certain populations who travel long distances and lack access to sufficient potable water and improved sanitation were especially at risk of contracting cholera and transmitting *V. cholerae*, such as certain West African fisherman populations who travel for several days at sea. Interestingly, although Accra represents the major cholera focus in West Africa (west of Nigeria), we found that fishermen populations based in the city had been largely unaffected by cholera. This is due to fact that these *Ga* fishermen do not travel long distances and rather fish in the local waters of Accra for only a few hours at a time, which correlates with our mechanism and argues against the theory that the coastal environmental waters represent the source of outbreaks. Indeed, if a reservoir of toxigenic *V. cholerae* O1 lingered and proliferated in the Accra waters, these *Ga* fishermen would be the first cases. Military troops are also at risk of transmitting *V. cholerae*, as they often travel from one disaster zone to the next with varying degrees of medical clearance between missions, as observed in Haiti in 2010.

Furthermore, epidemics in all the three study sites amplified under a comparable set of conditions. We found that cholera epidemics peaked subsequent to increased precipitation. Heavy rainfall and flooding played a role in amplification of ongoing outbreaks by contaminating drinking water sources, whether it be network pipes (e.g., Accra), river water (e.g., Artibonite

River in Haiti), lake water (e.g., Lake Tanganyika in eastern DRC), or other drinking water sources. Each major outbreak amplified in areas where populations consumed unprotected drinking and had limited access to proper sanitary facilities. With increased rainfall, drinking water could easily become contaminated with human feces from nearby cholera cases as latrines overflow due to flooding (Rebaudet, Gazin, et al. 2013). A public health breakdown, such as that following the civil crisis in Abidjan in 2011, could push a functioning sanitary and water system over the brink, thereby rendering the city vulnerable to cholera epidemics. Therefore, during a cholera outbreak, rainfall enables drinking water sources to function as a transient vector of *V. cholerae* O1, but not a permanent reservoir.

Populations persistently or repeatedly suffering from cholera outbreaks remain the main source of *V. cholerae*. These foci lead to sometimes explosive expansion of epidemics when a *V. cholerae* O1-carrying cholera case travels to a vulnerable locale, such as Accra, and rainfall levels increase. In the case of West Africa, this can produce a wave of cholera epidemics such as observed in 2011 to 2012 when an epidemic wave spread from Ghana to Guinea, affecting every country in between (Moore et al., unpublished). A comparable event occurred in the DRC in 2011-2012 when a persisting *V. cholerae* strain spread out of the endemic eastern *provinces* and crossed the country, likely via population movement, along the Congo River (Bompangue et al. 2012). Interestingly, the period 2011-2012 were not warmer El Niño years, but it rather a La Niña period, which is characterized by cooler water temperatures (Null 2016). In Haiti, we found a similar pattern in which remaining foci during the dry season, largely due to persisting cholera outbreaks insufficiently tackled by control activities, likely represent the source of larger outbreaks once the rainy season commences (Rebaudet, Gazin, et al. 2013). Overall, this mechanism explains the supposedly mysterious emergence of cholera, extensive lulls (despite rainfall and flooding) and heterogenous nature of cholera outbreaks.

13 Future directions

To continue the investigation of the ongoing epidemic in Haiti, a study headed by Renaud Piarroux and Stanislas Rebaudet, is assessing clinical isolates collected between November 2013 – November 2014 using MLVA. Such analysis would shed light on the mechanisms of cholera persistence and recurrence. The study will also address whether re-importation of *V. cholerae* strains or local low-grade transmission is at the root of cholera outbreak re-emergence in Haiti.

Our investigation and previously published elements suggest that the Accra water network may have played a major role in the rapid early diffusion of the toxigenic bacterium throughout a majority of the city. Additional assessment of the water network in Accra as well as localization of potential hotspots within the city, via GPS-based tracing of cases for example, are required to better understand why Accra is susceptible to explosive cholera outbreaks and identify an effective approach to control and prevent future epidemics. Lindsay Osei will continue working on this project.

Our investigation revealed that outbreaks in Ghana may be associated with epidemics occurring in neighboring Nigeria. Nigeria is currently one of the major cholera foci in the world (Piarroux and Faucher 2012), with 127,033 cases reported from 2009 to 2015 (week 26). The country is also a likely source of outbreaks in the Lake Chad Basin. In 2010, a massive epidemic started in Nigeria before spreading to Cameroon, Chad and Niger (Piarroux and Faucher 2012). Interestingly, the lull in Ghana during 2013 paralleled a relatively low number of cholera cases reported in Nigeria in 2012 and 2013. Similarly, the current lull in Nigeria since the beginning of 2016 coincides with a simultaneous lull in cholera outbreaks throughout all of West Africa (UNICEF and WHO 2016b). Additional investigations are required to ascertain the link between epidemics in Ghana and Nigeria and, if there is indeed a link, determine the mechanism by which *V. cholerae* is transmitted between these countries.

As the phylogeny study by Mutreja et al. lacked strains from Africa, it is important to continue the whole-genome sequencing analysis of the entire panel of *V. cholerae* isolates from Central and West. This study should help to understand to which extent recent African epidemics are connected, identify whether a recent shift has occurred among epidemic strains in Africa, and if so determine whether “ancestral” strains remain in circulation. Examining why some strains spread easier than others and determine how long strains persist before going extinct once they have emerged in a region may also provide insight into the development of antibiotic resistant strains.

Together with our collaborators at the Sanger Institute we also aim to assess whether DNA extracted directly from filter paper could be sequenced via whole-genome sequencing, thereby providing an easy tool for subsequent molecular epidemiology analyses.

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