ASSESSMENT OF EFFICIENT AND SUSTAINABLE TOOLS FOR CHOLERA DETECTION AND INTERVENTION IN LOW RESOURCE SETTINGS

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Abstract

Objective

Cholera is it is estimated to infect millions of people every year resulting in over 100,000 deaths annually. There are two WHO pre-qualified oral cholera vaccines that are recommended for use in conjunction with other prevention and control strategies in endemic and outbreak areas. There is currently a limited availability of vaccine supply, therefore, its use must be administered strategically. This study sought to improve the understanding of cholera disease burden and outbreak risk worldwide through the development of simplified and sustainable tools for use in low resource settings.

Methods

Data from 949 clinical diarrhea cases and 1,102 environmental specimens from 7 Health Facilities were analyzed using simplified laboratory diagnostic methods to assess the cholera burden in the Far North of Cameroon. *V. cholerae* 01 positive specimens were analyzed to determine the genetic relationship between geographically distinct areas in Cameroon. To further evaluate tools for determining disease burden, a rapid risk assessment tool (RAT) for cholera was developed and evaluated using surveillance data from the Republic of Kenya.

Results

In the sentinel surveillance study, the simplified laboratory diagnostics identified outbreaks early and with no false positive results. Sequencing revealed that outbreak specimens from the Bourrha Health district in June of 2014 were related to outbreak specimens from Darak and Blangoua Health districts in October of 2014. The cholera RAT

identified a few key districts in Kenya where implementation of cholera interventions, to include vaccination, may be targeted.

Conclusions

The simplified laboratory diagnostics demonstrated improved specificity and feasibility of use in the remote areas in our surveillance study. While *V. cholerae* was minimally present in the first year of surveillance, the outbreaks were detected early due to the application of our epidemiological and laboratory methodologies in the study area. We found that while the outbreaks in Bourrha, Cameroon and Darak, Cameroon were from distinct clonal complexes, there was a genetic relationship among the genotypes suggesting that the strain mutated between the geographic areas. The cholera RAT demonstrated the value of a risk factor weighting system to identify areas of heightened cholera risk for consideration of cholera intervention programs.

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List of Abbreviations and Acronyms

DOVE	Delivering Oral cholera Vaccine Effectively
OCV	Oral Cholera Vaccine
V. cholerae	*Vibrio cholerae*
ORS	Oral Rehydration Solution
WHO	World Health Organization
UNICEF	United Nations Children's Fund
MoH	Ministry of Health
MSF	Médecins Sans Frontières
LHF	Local Health Facility
DMC	District Medical Center
FNC	Far North Cameroon
CFR	Case Fatality Rate
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
mL	milliliter
PBS	Phosphate Buffered Saline
APW	Alkaline Peptone Broth
TCBS	thiosulfate citrate bile salt sucrose
Wt	weight
Vol	Volume
µL	Microliter
U	Unit
pmol	Picomole
bp	base pair
dNTP	deoxynucleotide triphosphate
Mg	Magnesium
rDNA	ribosomal DNA
LPS	Lipopolysaccharide
CT	Cholera toxin
MLVA	Multi-locus variable-number tandem-repeat (VNTR) analysis
VNTR	Variable-number tandem-repeat
ST	Sequence type
rRNA	ribosomal ribonucleic acid
PFGE	pulsed-field gel electrophoresis
MLST	Multilocus Sequence Typing
rfb	O-specific lipopolysaccharide biosynthesis region
ctxA	cholera toxin A
ompW	Outer membrane protein W
DHS	Demographic Health Surveys
SES	socioeconomic status
U5M	Under-five mortality rate
CTC	Cholera Treatment Center
OR	Odds Ratio
CIR	cumulative index case
HDSS	Health and Demographic Surveillance Site
DTC	Diarrheal Treatment Center

icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
TCP	toxin-coregulated pilus
VPI	TCP pathogenecity island
LPS	lipopolysaccharide
RDT	Rapid Diagnostic Test
ctxϕ	Cholera toxin phage
ctxA	cholera toxin A gene
ACF	TCP-accessory colonization factor
Cyclic AMP	Cyclic Adenosine monophosphate
Hib	*Haemophilus influenzae* B
WaSH	Water, Sanitation and Hygiene
KAP	Knowledge, Attitude and Practice
bp	base pairs

Chapter One: Literature Review:

1.1 History

Cholera has been recognized for its deadly dehydrating illness throughout recorded human history, with the first recorded pandemic occurring in 1817 throughout the Indian subcontinent (1, 2). To date there have been seven cholera pandemics, most lasting between 5-20 years. However, the most recent pandemic began in 1961 in Sulawesi, Indonesia and subsequently spread to Southeast and South Asia, the Middle East, Europe, reaching sub-Saharan Africa in the 1970's and Latin America in 1991 (3). The 7th pandemic continues to thrive today as cholera outbreaks are occurring around the world with increasing frequency and severity (4). The strain responsible for the current pandemic is the El Tor biotype of *V. cholerae* serogroup 01, having gradually replaced the classical biotype of the first six pandemics and now has spread throughout most of the developing world (4, 5). El Tor is known to persist longer in the environment and causes more asymptomatic cases which shed extensively into excrement, creating further spread of infection (6). These two characteristics of the El Tor biotype enable the pathogen to spread into new locations unnoticed and once the pathogen has found a new location it will likely persist and become a cholera endemic area. This is one reason that the current epidemic continues still today (4). Beginning in 1992, novel variants of O1 serogroup began to emerge with the first being O139 Bengal which spread rapidly throughout Bangladesh and into neighboring countries, displacing *V. cholerae* O1 El Tor (7). While it was anticipated to start a new pandemic, *V. cholerae* O1 El Tor re-emerged in 1994. *V. cholerae* O139 continues to coexist with O1 El Tor, however, it is extremely rare and has not been reported even in the Indian subcontinent in recent years (8). More recent research has shown that

variant or altered strains of El Tor strains producing classical cholera toxin (CT) have completely replaced the original 7th pandemic EL strain producing El Tor CT. It is hypothesized that this altered strain is an evolutionary change in the El Tor biotype to create a new, more virulent and efficient El Tor Biotype (5, 9).

1.2 Disease Burden

Worldwide:

While the numbers reported globally vary due to the fact that only around 1% of cholera cases are actually reported (10), it is estimated that cholera continues to infect millions of people every year resulting in over 100,000 deaths annually (4). Under-reporting is likely a result of lack of proper disease surveillance and a lack of adequate laboratory capacity especially in remote/rural areas, additional hindrances in reporting include fear of economic and social repercussions (11). The most recently publicized outbreak occurred in Haiti in 2010, infecting ~ 500,000 people within one year and killing nearly 7,000 (12). As a result of the devastating events in Haiti, much needed attention has shifted to addressing the needs for better strategies for the detection, prevention and treatment of cholera cases. In their 2010 position paper on cholera vaccines, the WHO modified their recommendation stating that the WHO pre-qualified oral cholera vaccines should be used in conjunction with other prevention and control strategies in endemic and outbreak areas (6). Due to the lack of adequate vaccine supply, the vaccine must be administered in a strategic manner to maximize protection of vulnerable people in both epidemic and endemic settings (13).

Disease Burden in South Asia

Cholera is often referred to as "Asiatic Cholera", as historically cholera is traced back to its presence in the Indian subcontinent. A description of a cholera-like disease is described in the Sushruta Samita, which is estimated to have been written between 400-500BC (14), after which the earliest recorded records of its presence in India are recorded in 1769 (2). In spite of records of cholera at this time, little information about the disease exists, and the first recorded cholera pandemic began in 1816, as the disease began to spread from India to China, the Philippines, Mauritius and Turkey, among other places (2). 5 more pandemics continued through 1960, spreading globally to almost all continents; however, cholera did not persist in any of the new geographical areas as it did in the Ganges Delta where it became an endemic disease. The current and 7th pandemic strain has differed in that it has become endemic globally, particularly in south Asia and Africa (15), however, all seven distinct pandemics have spread from Asia to other countries.

Disease Burden in Africa:

Except for the 2010 cholera epidemic in Haiti, the area globally reporting the worst cholera epidemics is sub-Saharan Africa. In 2007, the World Health Organization (WHO) Regional Committee for Africa officially recognized the resurgence of cholera in the African Region, and in 2010 a review of the situation in the African region found that the recurring cholera epidemics remained "alarming" (16). The spread of the 7th pandemic strain of cholera reached sub-Saharan Africa in August 1970 with the first cases identified in Guinea. The subsequent epidemic resulted in a reported over 150,000 cases and 20,000 deaths as it spread throughout the continent affecting 29 countries within 2 years (17).

Cholera has maintained a consistently high case-fatality rate reporting thousands of deaths every year on the continent (18). Unlike the classical waterborne disease, cholera in Africa spread not only through coastal areas but it also moved inland to savannahs, freshwater lakes and deserts which are not normally suspected to be conducive to vibrio survival (17). This may be due to transient refugees in overcrowded camps during the 1970's and 1980's; their migration along with other forms of trade and travel likely helped establish the endemic presence where it would not have been otherwise expected (17). As stated above, issues with surveillance and reporting in Africa have also resulted in the likely underestimation of reported cases and deaths. In the past decade, the number of countries reporting cholera has increased in sub-Saharan Africa, with more than 94% of the total global cholera cases reported in sub-Saharan Africa since 2001(18). According to the WHO, only five countries have reported cholera outbreaks every year since 1990, all of which are in Africa: Burundi, the Democratic Republic of the Congo, Ghana, Tanzania, and Cameroon (19). Alarmingly, recent developments show that cholera epidemics in sub-Saharan Africa are not only becoming more frequent, but are larger in size and persist for longer durations of time. For example, the 2008-2009 epidemic in Zimbabwe lasted for 11 months (8). Factors warranting a more comprehensive, multi-faceted approach to prevent, detect and treat cholera include its increasing endemicity throughout Africa, the lack of adequate water and sanitation infrastructure, the lack of access to adequate healthcare, and the lack of access to education on life-saving treatments such as oral-rehydration solution.

Disease Burden in Cameroon:

The Republic of Cameroon has a population of approximately 20,000,000 people, with approximately 40% of the population under the age of 15 (20). Cameroon is organized into 10 provinces, and further subdivided into 58 departments (21); the provinces have varied geographies. This includes several climatic zones: two main types climatic regions are the Equatorial domain and the Tropical domain. The Equatorial domain is broken down into the Guinea type, the Cameroon type, the maritime Cameroon type, and the montane Cameroon type. The Tropical domain is broken down into the Sudan or humid climate zone and the Sahel climate zone. The Far North of Cameroon (FNC), in the Sahel climate zone, experiences a very short rainy season of approximately four months; while the maritime zone includes Debundscha which is the second rainiest place in the world; in contrast to the Guinea type in the Southern Plateau of the country where there are four seasons, two rainy and two dry (22). Per the Ministry of Health of Cameroon, there are 10 health regions, with 181 functional health districts as of 2012 (J.Ateudjieu – personal communication). Major infectious diseases affecting the region include malaria, yellow fever, HIV/AIDS as well as food and waterborne diseases as a result of bacteria or protozoa (20). Only about half of the rural population of Cameroon has access to improved drinking water (47%) (23), and less than half have electricity (47%) (20). Cholera cases are increasingly abundant in Cameroon, specifically in the Far North Region with 9 outbreaks in the region since 1996, with the cholera outbreak in 2010 being the most serious outbreak in decades with almost 10,000 cases and 599 deaths (a 6.37% CFR) reported just 238 days after the outbreak began (24). It is likely that these numbers are an underestimate of the true incidence rate as barriers to care, lack of trained health personnel, and lack of

laboratory diagnostic capabilities hinder the ability to adequately estimate the true disease burden (Figure 1.1).

The Far North of Cameroon (FNC) is part of the Lake Chad Basin region of Africa. The Basin region includes parts of Chad, Nigeria, Niger and Cameroon. The center of the Basin is a shallow freshwater lake that is 22,000km^2. The lake is surrounded by wetlands which are affected by the seasonal variation in the hydrology of the rivers that flow into the Lake Chad Basin (16). This rural area is also unique in that in spite of its isolation it is a crossroads for communication among the bordering countries and thus is a type of center for commercial activities. Additionally, as a result of the seasonal variation in water flow, populations are often displaced. Food security, water availability, and access to health care are the poorest in the country in the FNC, likely further exacerbated by the extreme climate situation (25). These characteristics, particularly the economic activities, demographic changes, and the additional lack of access to protected water sources make this region vulnerable to cholera outbreaks in which the attack rates are high and case fatality rates (CFRs) are above 5% (16).

The region was not spared when cholera arrived in Africa in 1971. Outbreaks have continued to affect the region today due to the same demographic movement/displacements, as well as lack of access to medical care and lack of access to safe water. Then in 1991 there was an unusual intensity in the cholera outbreak in the Lake Chad Basin with 80,600 cases and 9800 deaths reported among the four countries. The spread of the epidemic, similar to the first outbreak of cholera in 1971, followed trade routes by both land and water (16). In 2010, the Lake Chad Basin countries suffered one of the largest epidemics in the history of the region with an estimated 58,000 cases and 2300 deaths. The FNC, or

"L'Extreme Nord" in French, has had consistent outbreaks in recent years. In 2009, of the 814 cases reported in the one outbreak, there were 39 deaths with 395 cases presenting in the FNC and 419 in the North of Cameroon (case fatality rate 11.07%). A second outbreak in 2009 reported 407 cases of cholera and 65 deaths in the FNC (case fatality rate 16%). In 2010, the first outbreaks occurred in Makary and Mada at the start of the rainy season in May. By late November 2010, a total of 9,712 cases and 610 deaths (case fatality rate 28%) were registered in the FNC, the North, the Littoral Region (coastline) and the Centre (Figure 1.2) (26).

Disease Burden in Kenya:

The Republic of Kenya has a population of over 45 million people, with over 43% of the population 15 years of age and younger (27). It is organized into 8 provinces, and 69 districts (28). Kenya's geography is varied including coastal, lake, highland, (29) and 80% of the country's land area is semi-arid or arid. The country is comprised of two regions: the lowlands including the coastal region and the highlands, which extends on both sides of the Great Rift Valley. The four seasons in Kenya include a dry season from January to March, a rainy season from March to May, a dry season from May to October and a rainy season from October to December (27). According to the 2009 census, while ~90% of urban households have access to improved water sources, only approximately 50% of rural households have such access. In addition the type of improved water source greatly differs with urban households predominantly having piped water into the dwelling while rural households primarily have access to dug wells as an improved water source. Access to adequate sanitation is also a significant issue in Kenya, with the 2009 census reporting that

only 30% of urban and 20% of rural households have access to an improved toilet facility (27). Per UNICEF's 2012 Update on the Progress of Drinking Water and Sanitation, Kenya is among ten countries with the largest population without access to an improved drinking water source (30).

Cholera first appeared in Kenya in 1971, with fifteen distinct outbreaks from 1971 through 2010. These outbreaks have ranged in size as well as morbidity and mortality, but in comparison to Case Fatality Rates (CFRs) reported in Asia, the CFRs in Kenya have been markedly higher and cholera is a significant cause of death from diarrhea (31). According to the Weekly Epidemiological Reports (WER) from the WHO, the cholera outbreaks in Kenya have been increasing in size as well as mortality rates since 2007 (32). Several regions of the country are reportedly more prone to cholera outbreaks, including the Nyanza province which reported outbreaks in 1997-1999 and again from 2007-2010, the refugee camps in urban Nairobi, the Turkana province and the North East province where large refugee camps exist (29). Shikanga et al reported on the 2008 outbreaks in Nyanza during a period of civil unrest in Kenya. The study reported significant underreporting of cases and deaths; through active-case finding they found a 200% increase in the number of deaths and a 37% increase in the number of cases reported (33).

1.3 Vibrio cholerae

1.3.1 Epidemiology:

The history of cholera is often tied to John Snow's historic epidemiological search to demonstrate the link between contaminated water and infection in England in 1854, and as such it is often described as the classic water-borne disease (17). However, it is

important to remember that this is not the sole means of transmission as *V. cholerae* can also be transmitted via contaminated food, particularly inadequately cooked seafood. Additionally, cholera can persist in leftover foods such as rice and millet for days (17). Direct person-to-person transmission is rare due to the large quantity of *V. cholerae* that is required to result in disease (8).

In areas where cholera is endemic, it is known to display seasonality with one or two annual peaks (34). However, the annual rates in endemic areas often vary widely, likely due to environmental and climate variation. It remains unclear as to what is the determining factor in the seasonal appearance of epidemic cholera; it has been suggested that during inter-epidemic periods that toxigenic *V. cholerae* persist in association with aquatic organisms until an environmental trigger results in the multiplication and proliferation of the dormant bacteria (15).

Humans are the only known vertebrate hosts of *V. cholerae*, and of the 25% of persons infected who develop symptoms, only 10-20% will develop severe disease after an incubation period of 2 hours to 5 days post-infection. The remaining 75% of persons infected with *V. cholerae* who do not become symptomatic may still shed the bacteria, potentially exposing other people(35). Infection severity is dependent on local intestinal immunity, the size of the inoculum, the patients gastric-acid barrier, and the patient's blood group (15).

1.3.2 Clinical Presentation:

Cholera is identified often by the rapid onset of acute watery diarrhea and vomiting. The disease can progress rapidly from the first watery stool to shock in as little as 4-12 hours (11). The symptoms include the painless purging of large quantities of rice-water

stool and clear, watery vomit. The rate of the loss of fluid plays a role in risk of death; if extremely rapid the patient may die within a few hours of onset. Without treatment the case-fatality rate (CFR) for severe diarrhea can be as great as 50%. However, if fluids are given promptly, the case fatality rate can be reduced to as little as <1% (34).

Treatment options depend on the severity of cholera infection, utilizing fluids comprised of a similar electrolyte composition to those that have been purged. Severe cases require intravenous fluid followed by oral rehydration solution (ORS) in order to compensate quickly for the volume of fluid that has already been lost. Severe cases should also receive antibiotics (doxycycline) for 1-3 days to shorten the illness and reduce diarrhea. For patients of lesser degrees of dehydration, ORS provides sufficient rehydration. Antibiotic therapy is not needed unless there is dehydration. (15).

1.3.3 Microbiology/Pathology:

Cholera's acute diarrheal infection is the result of direct fecal-oral contamination or ingestion of contaminated food or water with *Vibrio cholerae*. *V. cholerae* are facultatively anaerobic, asporogenous, motile, gram-negative rods ranging from 1.4 to 2.6μm in length (36). Unlike most bacteria, *V. cholerae* has its genome divided into two circular chromosomes, a discovery made in 1998 by Trucksis et al (37). Chromosome I is the larger chromosome with 2,961,146 base pairs, containing crucial genes for essential cell functions and pathogenicity. *V. cholerae* requires two regulated factors for full virulence: cholera toxin (CT) and toxin-coregulated pilus (TCP) (38). The production of the toxin CT, encoded by the ctxAB gene, is located within the integrated genome of a temperate filamentous phage, ctxϕ, on chromosome I (39). The receptor for ctxϕ into the

cell is the surface organelle TCP which is required for intestinal colonization. All strains capable of causing cholera must also possess a regulatory protein, ToxR, which co-regulates the expression of CT and TCP (40). Therefore, there are two regions of the *V. cholerae* chromosome I in which virulence factors are clustered: the CTX element and the TCP pathogenecity island (VPI), which is composed of the TCP-accessory colonization factor (ACF) gene cluster (39). Research suggests that the horizontal transfer of these gene clusters may be responsible for the origination of new pathogenic *V. cholerae* strains. The ctxϕ plays a critical role in this transfer of genes from one *V. cholerae* strain to another. For example, the transformation of O1 El Tor strains to O139 occurred as a result of one or more horizontal gene a transfer event in which there was a deletion and replacement of gene clusters encoding lipopolysaccharide O-side chain synthesis enzymes (41).

The smaller chromosome II with 1,072,315 base pairs is plasmid-like but due to its size and functions is considered a chromosome that may have once been a megaplasmid captured by a Vibrio species. This chromosome encodes potential toxins including *hap* and *hlya* virulence factors (Figure 1.3) (39).

The ability of cholera to reach and subsequently colonize the small intestine of humans and produce CT is a complex process. CT consists of five binding (B) subunits and one active (A) subunit (15). The B subunits bind to GM1 ganglioside receptors in the small intestine mucosa, the A subunit is then transported into the cell where it activates adenylate cyclase, leading to an increase in cyclic AMP (42). The resulting ion fluxes lead to in an increase in chloride secretion and a reduction to zero absorption of sodium. The end result is discharge of fluid into the small intestine that exceeds the normal absorptive capacity of the bowel resulting in watery diarrhea. The fluid lost is electrolyte-

rich resulting in low blood pressure and shock. Additionally, the fluid contains high concentrations of cholera vibrios that are highly infectious and can further contaminate environmental sources (15).

The organism is classified by biochemical tests for which it ferments glucose, sucrose and mannitol and is positive in the lysine and ornithine decarboxylase tests. It is then further subdivided into serogroups based on the polysaccharides of the somatic O antigen, of which there are over 200 serogroups (15). However, only 01 and 0139 have been associated with epidemic disease (43). Strains that test positive for *V. cholerae* but do not agglutinate with O1 or O139 antisera are referred to as non-O1/non O139 *V. cholerae;* while not involved in epidemics these strains can still be pathogenic (6). Serogroup 01 can be further divided into two biotypes, El Tor and classical; both of which can be further classified into two serotypes: Ogawa and Inaba. Ogawa strains produce the A and B antigens and a small amount of C antigen, which can be differentiated from Inaba by biotype specific genes as it only produces A and C antigens. An additional serotype known as Hikojima produces both specific antigens but is very rare (Figure 1.4) (15, 44).

Identification of *V. cholerae* O1 or O139 is performed by culture of fecal specimens on thiosulphate citrate bile salts sucrose (TCBS) agar as it inhibits the growth of most normal flora while allowing the growth of vibrios. Alternatively, a fecal specimen may be incubated in alkaline peptone water, as it preferentially supports the growth of vibrios, for 6-12 before inoculating the TCBS plate. *V. cholerae* produces smooth yellow colonies on TCBS agar that have slightly raised centers, these colonies can be selected and tested for presumptive identification if they are oxidase-positive and agglutinate with either O1 or O139 anti-serum (15).

In the absence of laboratory capabilities, rapid diagnostic tests (RDTs) have been developed to diagnose toxigenic *V. cholerae* in a field setting. Based on lateral flow immunochromatography, the RDT tests fecal specimens for a qualitative response to monoclonal antibodies specific for *V. cholerae* O1 or O139 lipopolysaccharide (LPS). As depicted in Figure 5, the dipstick works similarly to a pregnancy test. There are two antibodies present on the dipstick: one is the colloidal gold-labeled detection antibody and the second is the capture antibody (anti *V. cholerae* O1 and O139 antibody as there are separate identification bands for each). When the dipstick is placed into the test tube containing the fecal sample, there is a binding reaction forming a complex with the analyte in the liquid specimen. This complex moves forward continuously on the dipstick until it is eventually captured by the antibody(s) on the surface of the nitrocellulose membrane. The nitrocellulose membrane provides a pore to allow the liquid-solid interface necessary for antibody-antigen binding (45). After ten minutes, and within 15-20 minutes, the results can be read in which there is a color signal representing a positive for *V. cholerae* O1, O139, both or if negative, no color signal other than the control band is visible. There is also a control band in which an antibody specific to the detection antibody is used to demonstrate that the dipstick functioned properly. This band should be visible after all tests are run (Figure 1.5) (46).

1.3.4 Ecology:

While traditionally accepted that cholera spreads via fecal contamination of water or food, recent studies have demonstrated that *V. cholerae* including pathogenic O1 and O139 strains are normal inhabitants of surface water which survive and multiply in

association with plankton independently of infected human beings (15). However, Non-O1 and non-O139 are more commonly isolated from the environment; *V. cholerae* O1 isolated outside of epidemic areas have mostly been CT negative. The life cycle of *V. cholerae* consists of two distinct phases illustrated in Figure 1; during the environmental phase, vibrios can be found as free swimming or attached to plant surfaces. However, in the 2nd/human phase genetic factors for colonization of the mammalian gut are necessary. As previously described, these major pathogenic genes lie in several clustered regions of the *V. cholerae* chromosome. This suggests the theory that the natural marine vibrios adapt to the intestinal environment through horizontal acquisition of the virulence genes during phases of infection (40). This evolution to pathogenicity would require first that strains acquire the VPI and secondly, the now TCP-positive strains are infected with and lysogenized by CTXϕ (Figure 1.6).

The term "hyperinfectivity" is used to describe the fact that organisms from one infected person become more infectious as they are passed to the second/subsequent infected individuals during a cholera outbreak. However, it is difficult to isolate vibrios from suspected environmental or water sources; additionally, clinical isolates are genetically similar and difficult to differentiate among pathogenic *V. cholerae* strains (47). There are many current methods for differentiating strains including: rRNA restriction fragment length polymorphism (ribotyping), pulse-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST), among others. However, recent research has revealed limitations in many of these methods as most CT positive O1 and O139 isolates are genetically indistinct using these methods.

Ribotyping involves the use of E. coli rRNA gene probes to compare restriction fragment length polymorphisms of conserved rRNA genes, including 16S and 23S, in different strains to determine if different patterns are present (48). However, the application of this methodology to the study of *V. cholerae* has identified approximately 30 ribotypes, of which only a few are common in clinical isolates. Additionally, the ribotypes evolve slowly as isolates of a specific *V. cholerae* serotype over a period of many years in a single area belong to a single ribotype (47).

PFGE is performed by embedding organisms in agarose, lysing the organisms and then using restriction endonuclease digestion to cleave DNA fragments. The DNA fragments are run on an agarose gel to resolve the restriction fragments into discrete bands for comparison with other isolate restriction patterns to determine relatedness of the isolates (49). PFGE has been shown to be more sensitive in *V. cholerae* subtyping than ribotyping (50, 51), however, PFGE types change slowly and are beneficial primarily for distinguishing between pandemic strains (47).

MLST, first described in 1998, shifts towards a more sequence-based approach as a single gene may not be useful in determining genetic relatedness of isolates. MLST is used to characterize isolates by the nucleotide sequence of specific housekeeping genes. In comparison to PFGE which examines variations among specific restriction sites; MLST reveals all genetic variations within an amplified gene fragment (52). However, this method has been useful mainly for characterizing relationships among non-toxigenic strains as well as for linking regional outbreaks with the responsible pandemic strain (47).

To distinguish pathogenic O1 from O139 strains for epidemiological purposes, methods are needed that can differentiate the strains in spite of the genetic similarities. One

method that enables strains to be distinguished within a species in order to monitor epidemics as well as routes of contamination is Multi-locus variable-number tandem-repeat (VNTR) analysis (MLVA) (53). This methodology is used to assess variable-number tandem repeats or simple sequence repeats (SSR) are known as a class of short DNA sequence motifs that are tandemly repeated at a specific locus. The method uses the number of repeats at each different locus as a fingerprint for distinguishing among isolates (47). As demonstrated in studies by Noller et al, *E. coli* O157 isolates that were indistinguishable by MLST could be distinguished to some extent by PFGE but ultimately, MLVA was able to distinguish isolates that had the same PFGE type (54).

SSRs occur due to the fact that short arrays of repeat units may engage in modified or erroneous base-pairing when being copied by DNA polymerase. As a result, the polymerase introduces or deletes individual repeat units. Ultimately, the frequency of these variations depends on the DNA repair system, and the repeat variability affects genome functioning (55). The random modification of gene coding may be biologically advantageous for microorganisms; important epidemiological questions can be assessed through determination of these changes and assessing relatedness among isolates. Recently, 17 VNTR loci were shown to differentiate *V. cholerae* strains that were indistinguishable by PFGE (53). Subsequently, a study of a second group of VNTR loci demonstrated that environmental and clinical isolates from distinct Bangladeshi villages could be distinguished utilizing five VNTR loci (56).

1.4 Cholera Vaccines

1.4.1 Vaccine History:

The history of cholera vaccines dates back to the 1800's beginning with the discovery by Louis Pasteur that attenuated organisms could be used for immunizing domestic animals against infectious diseases, one of these diseases for which he developed a method of attenuation was for chicken cholera in 1880. In 1885, a Spanish physician, Jaime Ferran y Clua, was the first to immunize humans against any bacterial disease, vaccinating at least 30,000 Spaniards against cholera (57). Unfortunately, Ferran's work was disregarded at the time as being without scientific merit due to his unwillingness to detail his methods of attenuation. Subsequently, while working at the Pasteur Institute, Russian scientist Waldemar Haffkine vaccinated himself and 3 colleagues with two separate live cholera vaccines. The first attenuated vaccine was followed 6 days later by a second vaccine with enhanced virulence, as Haffkine's hypothesis was that the second enhanced vaccine would produce greater immunity. Haffkine continued to work out the best method of vaccination through trials in India and Bangladesh. While Ferran is recognized as the first to have inoculated humans, Haffkine is considered the creator of the controlled human testing of an acceptable cholera vaccine (57). Injected, or parenteral, attenuated cholera vaccines were used until the 1970's at which time a study in Bangladesh showed that the vaccines only conferred approximately 50% protection to the recipient for a period of less than six months post-vaccination (58).

Attention in the cholera vaccine field shifted toward oral vaccines with the understanding that immunity from cholera results mainly from mucosally secreted intestinal antibodies. Additionally, oral vaccines are easier to administer, more acceptable than needles to recipients, and reduce needle-associated costs and risks. There are two

types of oral cholera vaccines: killed whole cell (WC) vaccines and genetically attenuated live vaccines (11). Two of the killed whole cell vaccines are WHO qualified as of 2011: the first internationally licensed was the killed WC Vaccine with CT B Subunit (Dukoral) and the killed WC vaccine without the CT B subunit (Shanchol) (59).

1.4.2 Current Vaccines

Dukoral was developed in Sweden and first licensed in 1991; currently it is licensed in more than 60 countries but it is primarily used as a traveler's vaccine due to its high price (11)($5.25 per dose to the private sector) (60). The vaccine consists of a mixture either heat or formalin killed WC El Tor and Classical *V. cholerae* O1 biotypes representing both the Ogawa and Inaba serotypes with the addition of the B subunit of the cholera toxin. The vaccine must be given with a bicarbonate buffer in order to neutralize gastric acid and prevent the CT B-subunit from being destroyed. The vaccine works through the induction of antibodies against the bacterial components of the vaccine as well as the CT B. The antibodies produced against the bacterial components of the vaccine work by preventing *V. cholerae* from binding to the intestinal wall; ultimately impeding colonization of the bacteria. The antibodies to CT B prevent the cholera toxin from binding to the intestinal mucosal surface which prevents the toxin-induced diarrheal symptoms (61). The vaccine is licensed for use in persons 2 years of age and older; with persons 6 years and older requiring 2 doses given at least 7 days apart (but less than 6 weeks apart) and those between 2 and 5 years of age requiring 3 doses at least 7 days apart (but less than 6 weeks apart) (11). A randomized controlled trail in Bangladesh demonstrated a vaccine efficacy of 85% for 4-6 months following vaccination, with an indirect protective effect of ~50% in older children and adults (62). Additionally, a study in Mozambique

demonstrated more than 80% protection in the first year following vaccination in non-pregnant people greater than 2 years of age. This study also demonstrates that the vaccine is protective against the new variant El Tor expressing classical CT (63). Finally, since CT B is both structurally and functionally similar to heat-labile toxin of enterotoxigenic *Eschericia coli* (ETEC), there is cross protection against ETEC infections with this vaccine. This was demonstrated in a study in Matlab in which Dukoral provided short term protection against ETEC infection (59).

Shanchol is the killed WC vaccine without the CT B-subunit, which is the reformulated mORC-VAX by Shantha Biotechnics in India, where the national regulatory authority has WHO approval. The original 01 serogroup killed WC vaccine without CT B-subunit was developed and manufactured in Vietnam (mORC-VAX) in the 1980's and was subsequently made into a bivalent (O1 and O139) formulation (59). This low cost vaccine, ($0.75 per dose to the public sector) does not require oral buffer during administration since it does not contain the CT B-subunit. However, mORC-VAX had limitations making its prequalification by WHO unlikely including: production methods that were not adaptable to Good Manufacturing Practices (GMP), lack of compliance in standardization tests with recommendations and the vaccine was found to contain residual CT (60). Thus, a new bivalent (O1 and O139 serotypes) vaccine was created (Shanchol) using a heat-killed classical Inaba strain and a formalin-killed classical Ogawa strain. Similar to mORC-VAX, Shanchol requires no oral buffer for administration, is approved for persons greater than 1 year of age, requires 2 doses at two-week intervals, and is significantly cheaper in price than Dukoral ($1.85 per dose to the public sector) (60). Shanchol vaccine became licensed in India in 2009 following completion of an RCT which

demonstrated in more than 67,000 people that the vaccine was 66% efficacious during a 3 year period of follow up, and more recently shown to have a cumulative protective efficacy of 65% after five years (64). It subsequently received WHO prequalification in 2011 (59).

CVD 103-HgR (Orochol or Mutachol) is a genetically engineered, live-attenuated *V. cholerae* O1 Inaba strain in which the gene for the cholera toxin A subunit was deleted and a gene for mercury resistance was inserted (60). A single dose of the vaccine has been shown to be 95% protection against classical strains and 65% protection against El Tor Strains (11). However, a phase III efficacy trail in Indonesia reported no protection detectable against cholera in the four years of follow-up to a single dose regimen. As a result, the vaccine has never been licensed for use in settings of endemicity (65). Its safety profile and protection did lead to its licensure as a traveler's vaccine in Switzerland in 1993 (11) but it is not currently being marketed. There are plans to re-introduce the vaccine by another company (PaxVax) soon (66).

Several other live OCV candidates are currently being developed, including Peru-15, a genetically engineered *V. cholerae* O1 El Tor Inaba strain isolated in Peru. Peru-15 is the most advanced OCV candidate as it was tested in over 500 children and adults and shown to be safe and immunogenic in both endemic (Bangladesh) and non-endemic (US) populations (59).

1.5 Prevention Strategies and Challenges

As evidenced by recent coverage of the cholera outbreaks in Cameroon, South Sudan, Guinea, and other countries; the disease persists as a major problem in many developing countries as large outbreaks continue to be reported yearly (67). As a result

cholera surveillance remains a vital component for determining patterns of cholera incidence and prevalence in different areas of the world. However, as previously noted, it is well documented by the WHO and others that the number of globally reported cholera cases is significantly underestimated. From 2007-2010, the annual global figures which were reported to the WHO claimed that there were between 178,000 – 237,000 cases of cholera, with 4,000 – 6,300 deaths (68). However, it is estimated that these figures only represent 5-10% of the actual number of cases occurring annually worldwide (69), with the actual burden of disease estimated to be between 3 – 5 million cases annually, with 100,000 – 130,000 deaths per year (59). The reasons previously discussed contribute to underreporting, such as fear of economic and societal consequences and political disincentives. However, a significant influence to the under-reporting is predominantly due to surveillance difficulties, owing both to lack of proper epidemiological surveillance systems as well as to a lack of adequate laboratory facilities (69).

Surveillance systems and the data that they produce are vital to determining the pattern of disease occurrence in a country or region of interest. This data is necessary to facilitate disease control efforts, and in situations where vaccines are available, can be instrumental in local and governmental decisions about vaccine introduction (70). Surveillance data can be collected using various methodologies. Population-based surveillance identifies ~100% of new cases of the targeted disease in a defined population. This provides information that can be used to calculate the disease incidence rate in the defined population. Unfortunately, population surveillance is often too costly and logistically time-consuming. An alternate strategy is to utilize sentinel surveillance methodologies in which the disease of interest is monitored and data collected at one or a

selected number of facilities. This method requires fewer resources and is generally less-costly than population based surveillance. While there are limitations in estimating incidence rates, the increased feasibility of sentinel surveillance as compared to population-based surveillance has led the WHO to recommend its use for monitoring the effect of newly implemented vaccines (71).

In remote areas of developing countries sentinel surveillance may still prove to be too costly and too resource intensive for the limited district and/or regional health budgets. In 2003, a study was published in which a sampling system was utilized to survey for cholera in 4 distinct geographic locations in Bangladesh. The surveillance was conducted in a local hospital in each of the 4 sites; a physician collected clinical data and specimens from patients presenting with acute water diarrhea during a 3 day period every fifteen days (72). This sampling methodology was implemented for its sustainability in resource-constrained environments. Experts on epidemiological surveillance have noted that it is not essential to have complete counts of diseases to implement disease-control efforts. Even sporadic reporting of cases can reveal unusual disease occurrence, stimulating investigations and disease-control activities (73).

The surveillance data recording and reporting the disease burden of cholera around the globe has likely hindered the consideration of OCV's by policy makers both at the international level by agencies such as the WHO advisory group, as well as decision makers in high risk countries. These factors have contributed to the lack of demand for OCV by manufacturers; ultimately resulting in a lack of available vaccine supply. While measuring disease burden is difficult with limited surveillance data; it is even more difficult to introduce new vaccines into developing countries when the population has little to no

awareness of the impact of the disease. In 2004, a standardized rapid assessment tool (RAT) was developed by the WHO to estimate the incidence of *Haemophilus influenzae* B (Hib) meningitis and Hib pneumonia. This tool illustrated that limited retrospective data on local rates of Hib disease could provide disease burden rates similar to those obtained from population-based studies in the same regions. This tool provides an inexpensive means of using locally obtained data while engaging health care workers and government officials to evaluate existing local surveillance data and to assess rates of disease (74). The framework established for assessment of Hib disease burden can be useful in conducting the same or similar assessment for cholera disease burden in high risk countries in order to highlight the need for disease-control efforts and engage policy makers in discussion on use of available OCV supply for targeted use in high-risk and susceptible populations.

1.6 Rationale

Several large field trials have been conducted to demonstrate safety and immunogenicity of both Dukoral and Shanchol (64, 75-78). A cluster-randomized phase III trial was conducted in Kolkata, India in 2006, with five years of follow-up

demonstrating vaccine efficacy of more than 65% after five years (64) Cholera intervention campaigns including OCV, training in Water, Sanitation and Hygiene (WaSH) and knowledge attitude and practice (KAP) about cholera were implemented in refugee camps in Tak Province, Thailand (78) and in urban (Mirpur and Dhaka) and rural (Keraniganj) settings of Bangladesh (79) , reactive vaccination in Guinea (80) In 2012, several additional vaccination implementation campaigns were conducted including a reactive vaccine campaign 2 years into the epidemic in Haiti combined with an assessment of feasibility of vaccine delivery initiated in Haiti in which the delivery of the vaccine improved the baseline knowledge and essential health practices necessary for cholera control (81). In 2012 another reactive campaign was conducted in Boffa and Fore'cariah, Guinea. Following the report of over 147 cases and 13 deaths between February and March of 2012, the Ministry of Health (MoH) of Guinea, with the support of Médecins Sans Frontières (MSF) implemented the first reactive OCV campaign in Africa (82). Despite a short time interval for implementation the campaign was successful with a high degree of acceptance.by the population and high vaccination coverage (80). A re-analysis of data collected during the field trial of Dukoral in Bangladesh demonstrated herd protection in the unvaccinated persons in the community, suggesting that high levels of vaccine coverage in a community can lead to increasing levels of indirect protection (herd protection) (62). These findings were supported in efficacy studies conducted following a mass vaccination campaign in Zanzibar[6], further highlighting the importance of herd protection benefits when considering vaccine implementation. In conjunction with such clear evidence supporting cholera vaccination's benefits, the WHO modified their recommendation in

2010 supporting the use of the 2 WHO approved OCV's in conjunction with other interventions for cholera's prevention and control in cholera affected areas (6).

While the evidence has been successfully presented for the implementation of OCV, there have been considerable obstacles to its implementation. These include the need for a cold chain for the vaccine, the large volume needed for the Dukoral vaccine, the multi-dose requirement within a short timeframe, the vaccine is not 100% efficacious, concern that its use might detract from other important interventions such as water and sanitation hygiene (WASH) efforts, and as seen in Haiti, concerns that it might interfere with other national vaccination efforts. These obstacles can be addressed through logistical planning as well as working well with other agencies such as WASH efforts to utilize OCV as a tool to complement water and sanitation activities to create a comprehensive approach. Additionally, the individual vaccine efficacy does not have to be extremely high if the vaccine also has demonstrated herd immunity (59); however, the obstacle that continues to heavily impede progress in vaccine implementation is the shortage of supply of vaccine (83). As a result of the current situation, it is necessary to improve surveillance in high risk areas in order to apply targeted vaccination strategies and optimize the available number of vaccines. The overall goal of the "Delivering Oral Cholera Vaccine" (DOVE) project funded by the Bill and Melinda Gates Foundation is to facilitate and focus the delivery of oral Cholera vaccine (OCV) in high burden countries. Unfortunately, there is currently a very poor understanding of both disease patterns and disease risks in most endemic countries, and few Ministries of Health in these affected countries are considering the implementation of OCV due to lack of knowledge about the vaccine, difficulties in

obtaining the vaccine, and logistical issues surrounding the distribution of the vaccine, among other obstacles.

The Far North of Cameroon is an area of interest for understanding cholera epidemiology in sub-Saharan Africa as well as in developing targeted approaches towards vaccination. As a result of repeated outbreaks with notably high case fatality rates (CFRs) since cholera first appeared on the African continent, the Lake Chad Basin is considered a "hot spot" of cholera disease. Unlike Asian countries such as Bangladesh that are known to be cholera endemic as a result of monsoons and seasonal flooding, this area of sub-Saharan Africa has entirely different environmental and climatic issues that may suggest a different set of risk factors to people living in the Lake Chad Basin. As a sub-study to the DOVE project, the "Sustainable Cholera Surveillance for Cameroon" study will be conducting surveillance in the Far North region of Cameroon for the next four years. The objective of this surveillance effort is to evaluate the use of low-cost and sustainable epidemiological and laboratory methods in remote and rural field settings. We will work within this area to apply efficient epidemiologic and laboratory tests to facilitate the understanding of disease transmission in this area and work to provide tools for Cameroon and other similarly affected countries to rapidly assess their cholera disease burden and options for cholera intervention implementation, particularly the use of OCV.

Infectious disease burden data is often minimally available or non-existent in developing countries. Cholera disease burden data is similarly absent due to lack of surveillance in endemic areas as well as a result of fear of economic repercussions from reporting outbreaks (84). The African continent has the most reported cholera cases. As the WHO looks to target use of OCV in high risk areas, local and regional data on cholera

disease burden will be vital in facilitating decisions by the Ministry of Health. The Risk Assessment Tool (RAT) will aid endemic countries such as Kenya in decisions regarding allocation of resources and implementation of intervention strategies, particularly targeted OCV campaigns.

1.7 Overall Goals and Specific Aims

1.7.1 Overall Goal of the Study

The overall goal of this dissertation was to assess sustainable and efficient tools for the epidemiological and laboratory surveillance of *V. cholerae* in low resource settings. In a remote area in the Far North of Cameroon, the goals were to evaluate novel epidemiological and laboratory surveillance tools for the sustainability of routine surveillance and the characterization of disease burden. In the endemic country of Kenya, the goal was to evaluate a rapid risk assessment tool that can provide disease burden information through the use of adjusted incidence and death rates with supplemental risk factor weighting that will highlight areas of high-risk for cholera intervention campaigns utilizing temporal, geographic and demographic factors from historical data.

Specific Aim 1

To evaluate the implementation of modified sentinel surveillance using low-cost and rapid lab diagnostics in a low-resource and remote setting for concurrent environmental and clinical surveillance in an effort to understand cholera transmission patterns in the area while potentially providing tools for early detection of cholera.

Specific Aim 2

To determine the temporal and geographic genetic relationship between environmental specimens and clinical specimens isolated from persons and water sources in the far North region of Cameroon.

Specific Aim 3

To conduct a review of historical cholera surveillance data to determine the burden of disease of Cholera in Kenya while evaluating a rapid risk assessment tool; identifying high-risk areas for potential cholera interventions, including vaccination.

Figure 1.1**:** Cameroon (85)

Figure 1.2: Extrême-Nord, Cameroon **(**personal communication-J. Ateudjieu**).**

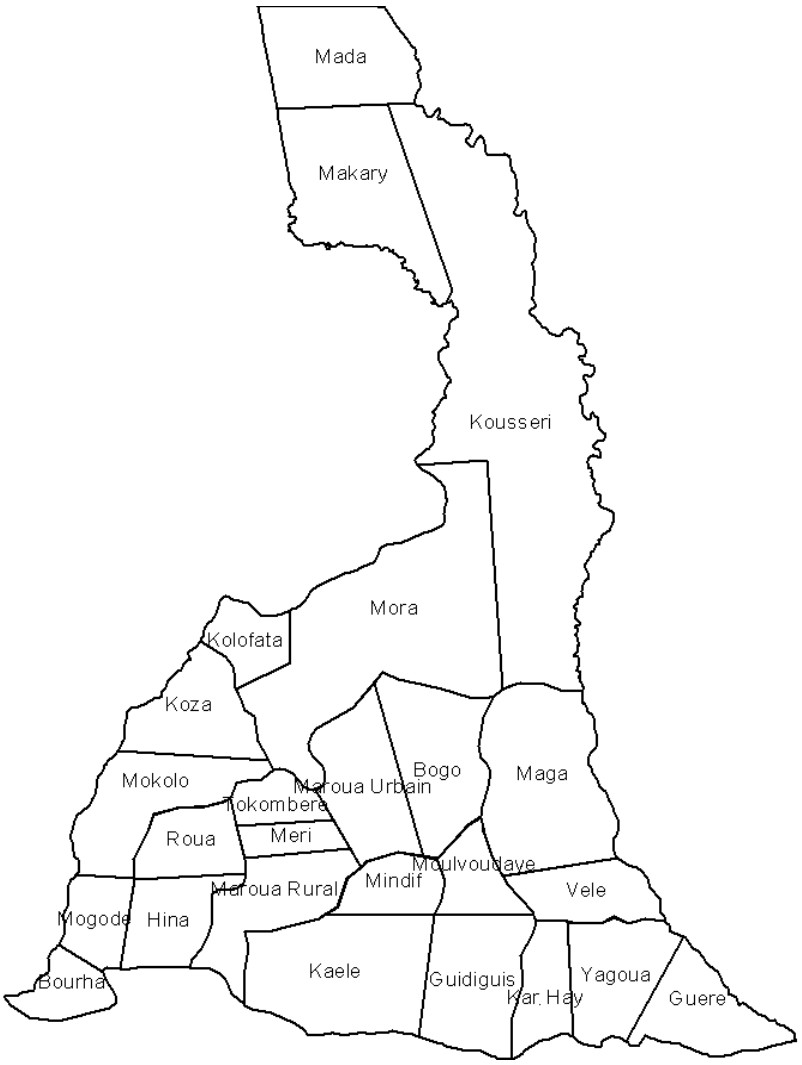

Figure 1.3: "Image of two of the circular chromosomes found within *Vibrio cholerae"*. The image shows different sections of the chromosome that aid in toxicity. Each chromosome has different sections."(86)

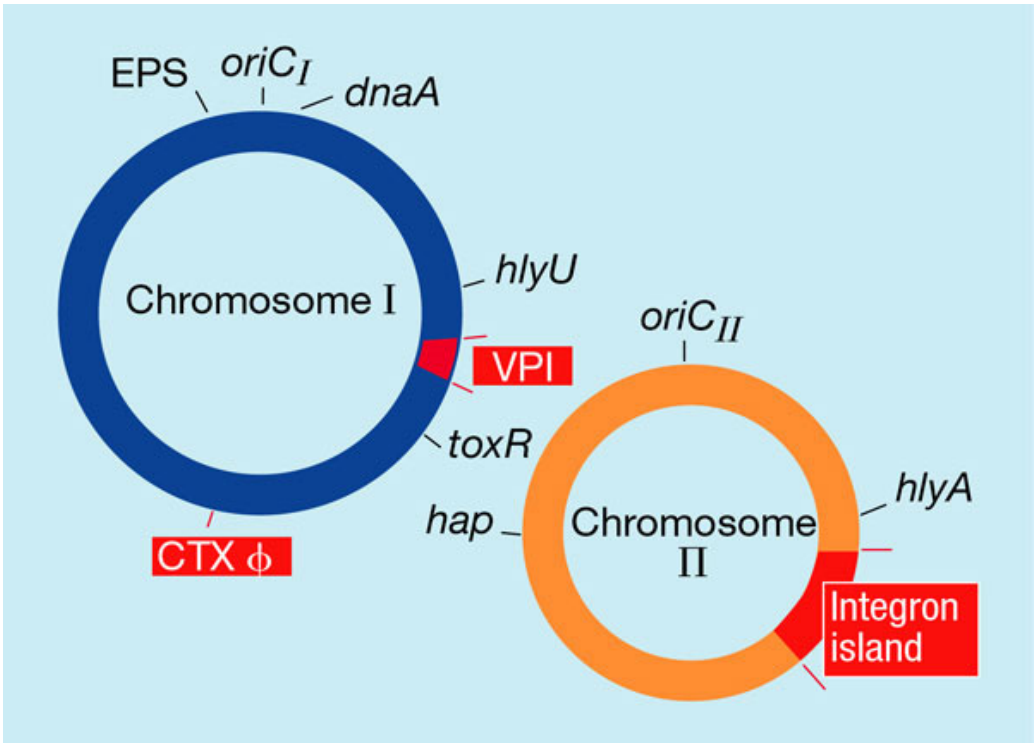

Figure 1.4: Vibrio cholerae (O group 1 antigen) (87).

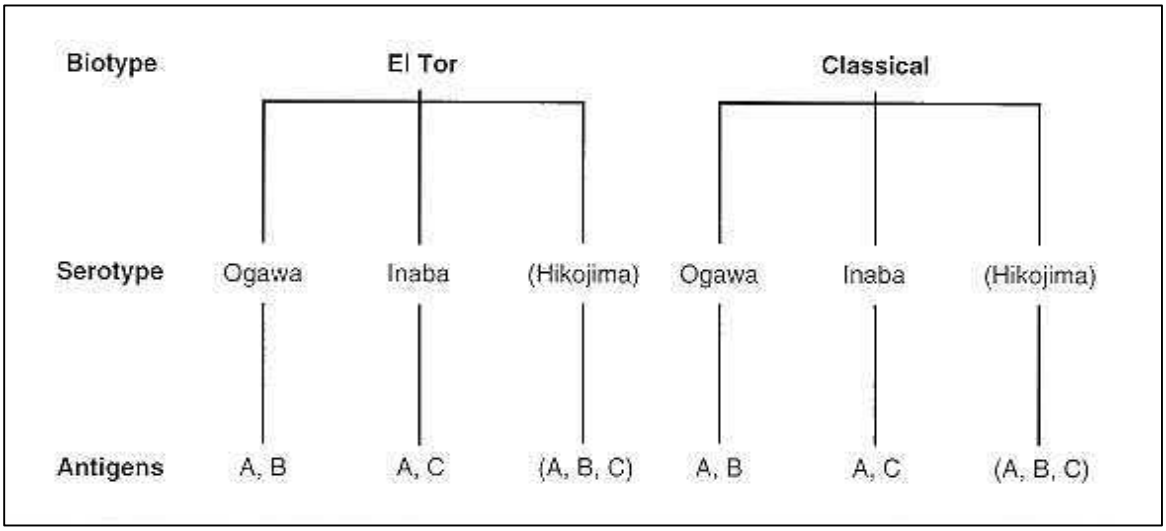

Figure 1.5: Crystal VC dipstick: Components and Schematic Diagram
(a) Cross-section of the Crystal VC dipstick showing its assembled components. (b) Schematic diagram of the immunochromatographic principle for the dipstick assay (88)
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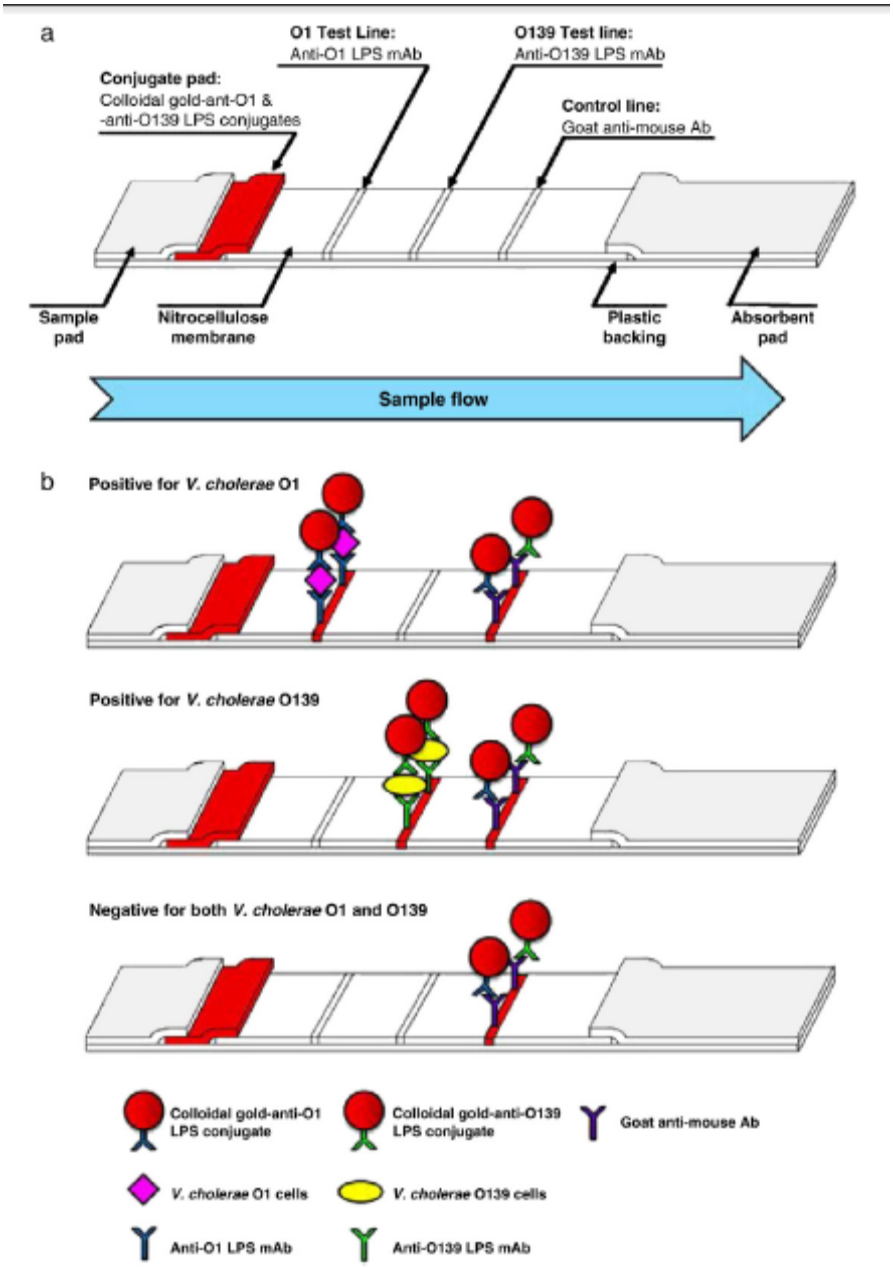

Figure 1.6: Life cycle of *Vibrio Cholerae* (15)

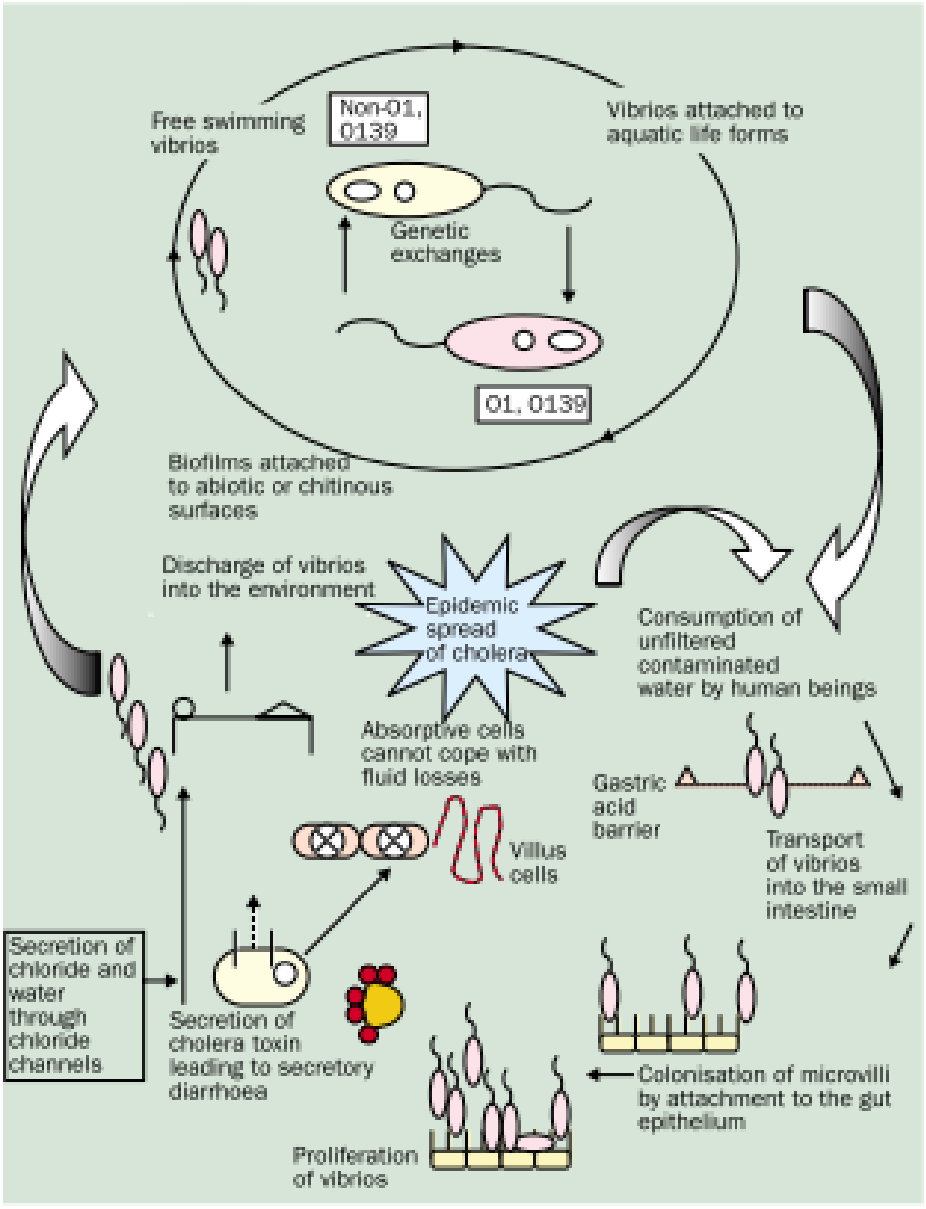

Figure 4: **Life cycle of *V cholerae* involves both environmental and human segments, which sometimes intersect**

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Chapter Two: Paper I

Clinical and Environmental Surveillance for *Vibrio Cholerae* in Resource Constrained Areas: Application during a one year surveillance in the Far North Region of Cameroon

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2.1 Abstract

Background

Biological Confirmation of the presence of *Vibrio cholerae* in both stool and water samples is often constrained due to resource and labor intensive gold-standards methods. To develop low cost, simple and sustainable surveillance techniques, we modified previously published specimen sampling and enrichment techniques and applied the use of modified dipstick testing in conjunction with the use of filter paper for specimen preservation during epidemiological and environmental surveillance in the far North of Cameroon from August 2013 – October 2014. This methods allows for increased specificity and rapid diagnosis in a field setting, simplified DNA storage for PCR analysis of specimens, reduced need for reagent-intensive microbiological testing, elimination of cold-chain storage, and simplified transfer to reference laboratories.

Methods

Clinical and environmental surveillance was conducted in 7 health districts in the far North of Cameroon between August 2013 and October 2014. The Clinical specimens were screened for V. cholerae 01 and 0139 using an enhanced dipstick method in which specimens are tested Crystal VC dipstick after 6h of enrichment in APW broth. The enriched stool sample is also preserved on filter paper for molecular processing. Environmental specimen are filtered through medical gauze and then incubated in a 50mL conical tube for 24 hours (range 22-26hours) at room temperature. After incubation, the enriched specimen is tested with Crystal VC dipsticks for *Vibrio cholerae* 01 and 0139. The enriched specimen is also preserved on filter paper. Filter paper specimens are first cut using sterile scissors, and then washed 2X's with sterile 1X PBS. DNA is extracted in

a 2% Chelex-100 solution, incubated at 100°C for 8 minutes, and centrifuge to remove supernatant and store at -20°C. DNA samples are tested using multiplex PCR for Vibrio spp. Including *Vibrio cholerae*, *Vibrio vulnificus* and *Vibrio parahemolyticus*. Those positive for *Vibrio cholerae* were further tested differentiate toxigenic and non-toxigenic *V. cholerae*; and to differentiate serogroups 01 and 0139.

Results

949 patients were enrolled in the study among all 7 study sites, of which 28 were *V. cholerae* 01 positive and 2 were *V. cholerae* non01 positive. Cholera was not detected in any of the study surveillance facilities until October 2014 when an outbreak was detected in Darak and Blangoua Health Facilities. Of the 28 patients enrolled in the study with clinically confirmed cholera and two additional non01 Cholera patients, nearly 40% (11) were children under 5 years of age, and nearly 75% were men. The modified dipstick methodology demonstrated increased sensitivity (99.8%). 1,012 water samples were obtained from the 45 environmental sites surrounding the 7 Health Facilities, of which 244 were *V. cholerae* non-01 positive and 0 where *V. cholerae* 01 positive. An environmental reservoir for cholera was not identified, however, there was a significantly increased risk of vibrio cholerae detection near Naga and Darak Health facilities.

Conclusions

Simplified laboratory and epidemiological methodologies can improve the feasibility of cholera surveillance in rural and resource constrained areas. The application of basic technologies such as the modified dipstick, the use of simplified gauze filtration for environmental sample collection, and the use filter paper for sample preservation enabled early case identification with reduced logistics and supply cost while reporting minimal

false positive results. The first year of clinical and environmental surveillance did not identify a reservoir for *V. cholerae* in the Far North of Cameroon; however, the simplified surveillance methods enabled early case detection and rapid response implementation.

2.2 Background

Annually only 1% or 100,000 of the estimated cases and ~2000 deaths due to *vibrio cholerae* infection are reported (1). The World Health Organization (WHO) recognizes that the actual burden is closer to 1.4-4.3 million cases and 28,000 – 142,000 deaths per year (2). It is well accepted that this is a vast underestimation of the total disease burden. This reduced reporting is largely affected by the difficulties in diagnosing cholera, which includes the difficulties and expense associated with the transportation of samples to a lab with the capabilities to confirm cholera infection. Of the 47 countries which reported cholera cases and deaths to the WHO in 2013, 22 of these countries were in Africa. In spite of reporting the largest cholera disease burden in the world, there continues to be a poor understanding of the disease patterns in much of Africa and likely further under-reporting of the disease. This is due to the challenge of proper disease surveillance and laboratory capacity requirements especially in remote/rural areas.

While population-based surveillance will provide the most accurate information for disease burden, it is too resource intensive for most cholera endemic settings. Sentinel surveillance is often employed in resource limited settings to monitor a disease of interest at a single or specific sites. While disease burden estimates are difficult from this type of surveillance, this method requires fewer resources while identifying when the disease of interest is present (3). However, even sentinel surveillance is difficult to sustain in low resource settings given other health and political priorities and the lack of funding to support this concerted effort to track a single disease. Since 1997, fortnightly environmental and clinical surveillance has been conducted in different locations in Bangladesh. These efforts have shown that this reduced surveillance methodology can aid

in understanding true disease burden, while providing data to better understand the disease, seasonality and transmission patterns(4).

Diagnostic confirmation of vibrio cholerae infection is challenging in rural and resource limited settings. The gold standard and accepted method of *V. cholerae* diagnosis is through culture confirmation, requiring well-equipped laboratories and trained laboratory personnel. This method is often too time consuming taking 2-3 days for confirmation, resource intensive and/or too expensive in these settings. As a result, cholera is often only diagnosed as suspected cases using the clinical definition of having three or more loose or liquid stools in the 24-hours with sudden onset in persons $\geq$ 5 years of age prior to presenting (5). More often, cholera is not diagnosed, and the patient is treated for diarrhea without identifying the etiologic agent. Commercially available dipstick tests, such as Crystal VC^{TM} allow for rapid diagnosis. Previous studies have reported low specificity when using the kits with direct testing of stool samples (6-8); however, a modified approach to using the dipstick in both stool (9) and environmental specimens (10) have recently been demonstrated to successfully improve the sensitivity of the dipstick in an urban field setting and a laboratory setting. In addition, the preservation, storage and transportation of positive isolates requires logistical and laboratory support that is often far beyond the scope of a rural health facility. We report the novel use of filter paper technology for preservation of specimens for DNA extraction and molecular processing. While culture methods are often considered to be the gold standard, they are not 100% sensitive. For our study, we considered a positive PCR to be a reliable gold-standard, however, there still could be true positives that are not detected by any of the methods.

In this study we demonstrate the successful implementation of modified sentinel surveillance using low-cost and rapid lab diagnostics in a low-resource setting. The methodology allows for concurrent environmental and clinical surveillance in an effort to determine hot spots for cholera activity and cholera transmission patterns while providing tools for early detection of cholera. We hypothesized that there is a reservoir for cholera in Lake Chad and that cultureable *V cholerae* may be detected prior to the onset of an outbreak which will allow for rapid response to cases and early intervention in outbreaks of the disease.

2.3 Methods

2.3.1 Study design

Cameroon has a population of approximately 20 million people, divided into 10 provinces with extremely varied geographies and several climate zones (25). The Far North region of Cameroon (FNC) is located in the Sahel desert and has a rural population. In Cameroon, less than half of the rural population have access to improved drinking water (11). The incidence of cholera disease is becoming increasingly common in Cameroon, particularly in the FNC where there were 9 outbreaks between 1996 - 2013; with the cholera outbreak in 2010 being the most serious outbreak in decades with almost 10,000 cases and 599 deaths (a 6.37% CFR) reported just 238 days after the outbreak began (12). These numbers are likely an underestimate of the true burden of disease due to lack of surveillance and laboratory capacity.

Diarrhea surveillance was established at 7 seven local health facilities (LHF) in the FNC, in and around Lake Chad, including: Kousseri (Kousseri Health District), Mada

(Mada health District), Ngouma (Makary Health District), and Maltam (Goulfey Health District), Blangoua District Medical Center (DMC), Darak DMC, and Naga (Naga Health District). These sites were selected as being geographically representative of the Cameroonian health facilities near Lake Chad. The selected sentinel sites implemented a reduced-sampling methodology in which each of the seven sentinel site district health facilities enrolled suspected cholera cases of any age into the study for a 3-day period every 15 days; during this time a trained research assistant is on site at the facility to monitor activities. Additionally, all other days of the month, any patient ≥ 5 years of age presenting with diarrhea is identified and asked to participate in the study. Concurrently, environmental sampling began in and around the seven LHF's. Three to six environmental sites were identified within the health district, and water samples were collected 1 day out of every 15 days. Study enrollment began August, 2013.

2.3.2 Laboratory Methods

Clinical Surveillance

Fecal specimens or rectal swabs from the consenting diarrhea subjects were collected using a stool cup (or from small children, it may also be collected from the diaper). These specimens, obtained from the monitoring sites and the sentinel sites, were screened for *V. cholerae* O1 and O139 using an enhanced dipstick method (Crystal VC, *Span Diagnostics* Ltd. 173-B, New Industrial Estate, Road No. 6-G, Udhna, Surat - 394 210, INDIA) in which the specimen is tested via dipstick after incubation for 6-8 hours in alkaline peptone water (APW). APW enriched samples which tested positive, as well as a 10% sample of negative clinical samples were inoculated into Cary-Blair transport media

for storage until transport for microbiological confirmation in the central reference laboratory in the Kousseri Health Facility (Figure 2.1).

Environmental surveillance

Surface water samples were collected from 3-6 sites near each of the sentinel health facilities (total of 42 sites) to be tested for the presence of *V cholerae* O1 and O139. Two to three liters of the surface water sample was collected in a plastic jar and then filtered through sterile gauze. The gauze is then incubated in APW for 24 hours (± 2 hours) and subsequently tested using the dipstick. APW positive samples and a 10% sample of negative clinical samples were inoculated into Cary-Blair transport media for storage until transport for microbiological confirmation in the central reference laboratory in the Kousseri Health Facility (Figure 2.2).

Microbiology at the Central Laboratory

All positive specimens and 10% of negative specimens were sent at routine intervals to the central laboratory for confirmation. The specimens were streaked directly onto thiosulfate citrate bile salt sucrose (TCBS) agar and incubated for 24 hours at 37°C. Immediately after inoculating the first TCBS plate, a pre-labeled APW vial was inoculated with the specimen and incubated for 6 hours at room temperature. After 6 hour incubation, a second TCBS plate was inoculated with the enriched specimen and incubated for 24 hours at 37°C. After the 24 hour incubation, any cholera-like colonies were selected with a sterile loop, re-suspended in 1-2 drops of PBS and tested via dipstick. All dipstick positive cultures as well as any cultures considered cholera-suspect, demonstrating the morphology of a cholera colony, were preserved in nutrient agar for further testing.

To evaluate the use of simplified specimen preservation and sample shipping methods, clinical and environmental samples from the enriched APW were preserved on Whatman 903 filter paper to be tested for vibrios using Polymerase Chain Reaction (PCR) methods. One to two drops of the enriched APW were preserved on Whatman filter paper and allowed to air dry; filter paper specimens were stored in individual plastic bags at room temperature until they were sent for extraction and PCR processing.

Molecular Biology and Microbiology at the Johns Hopkins Laboratory

DNA Extraction

Filter paper specimens were sent at regular intervals for processing in the US. Individual DNA extractions were performed by using methods similar to those previously published (13). Sterile scissors were used to cut filter paper circles for each dried filter paper specimen. Between samples, the scissors were bleached and rinsed to ensure there was no cross contamination when processing the next dried filter paper specimen. Each cut dried filter paper specimen was placed into a pre-labeled tube. One milliliter (mL) of sterile 1X phosphate buffered saline (PBS) was added to each sample tube and incubated for 10 minutes at room temperature. The samples were then centrifuged (14,000 x g for 2 min) and the supernatant was discarded. One mL of sterile 1X PBS was then added to each sample, and then immediately centrifuged (14,000 x g for 2 min) and the supernatant discarded. Finally, 150ul of a 2% (wt. /vol.) Chelex-100 solution (Bio-Rad, catalog no.1422832) was added to each sample. The samples were placed in a heating block at 100°C for 8 minutes. The samples were centrifuged (14,000 x g for 2min) and the

supernatant was removed to a new micro-centrifuge tube and either stored at -20°C or used immediately in a PCR amplification reaction.

PCR

Multiplex PCR reactions to identify 3 Vibrio species, to differentiate toxigenic and non-toxigenic *V. cholerae*; and to differentiate serogroups 01 and 0139 were conducted in a systematic order in conjunction with a 16S bacterial PCR along with Nanodrop measurements to confirm extracted DNA quality. Oligonucleotide primers and their respective amplicon sizes are listed in Table 2.1.

A multiplex PCR amplification was first performed to determine the presence of vibrio species in the extracted DNA sample by targeting the toxR genes of *V. parahemolyticus, V. cholerae*, and *V. vulnificus.* As previously described, the universal forward primer *UtoxF* was used in combination with species specific primers for *VvtoxR, VptoxR* and *VctoxR*, respectively (14). The multiplex reaction was run in 50 µL reactions containing 5 µL of extracted DNA, 2x Terra PCR Direct Buffer (with Mg2+, dNTP), 1.25 U/ µL Terra PCR Direct Polymerase Mix (Clontech Laboratories, Inc., Mountain View, California, USA), 30 pmol of the UtoxF primer, and 20pmol of each reverse primer. PCR conditions were optimized at initial denaturation of 4 minutes at 95°C, followed by 30 cycles each with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step of 72°C for 7 minutes (Bio-Rad S1000 Thermal Cycler, Hercules, Ca, USA). The amplified PCR product was analyzed by gel electrophoresis on a 1.5% agarose gel and visualized under UV light with ethidium bromide. The products for *V. parahemolyticus, V. cholerae*, and *V. vulnificus* were 297, 640, and 435 base-pairs (bp), respectively.

If *V. cholerae* was identified in the sample, a multiplex was then used to differentiate non-toxigenic and toxigenic *V. cholerae*. The multiplex uses primers targeting a gene encoding an outer membrane protein (OmpW) that is a unique gene conserved in the *V. cholerae* sequence as well as primers targeting the cholera toxin A (ctxA) gene. As described by Nandi et al (15) the multiplex reaction was run in 25 µL reactions containing 5 µL of extracted DNA, 2x Terra PCR Direct Buffer (with Mg2+, dNTP), 0.78 U/ µL Terra PCR Direct Polymerase Mix (Clontech Laboratories, Inc., Mountain View, California, USA), 15pmol of OmpW primers, and 6.2 pmol of ctxA primers. PCR conditions were optimized at initial denaturation of 5 minutes at 94°C, followed by 30 cycles each with denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step of 72°C for 7 minutes. The amplified PCR product was analyzed by gel electrophoresis on a 1.5% agarose gel and visualized under UV light with ethidium bromide. The products for ompW and ctxA were 588 and 301 base pairs, respectively.

All *V. cholerae* positive specimens were tested to determine if they belonged to serogroup 01 or 0139; regardless of their toxigenic nature. The multiplex uses primers targeting unique regions in the *rfb* gene specific for the 01 and 0139 serogroups. Following methods described by Hoshino et al (16) the multiplex reaction was run in 30 µL reactions containing 5 µL of extracted DNA, 2x Terra PCR Direct Buffer (with Mg2+, dNTP), 0.93 U/ µL Terra PCR Direct Polymerase Mix (Clontech Laboratories, Inc., Mountain View, California, USA), and 2µM of 01 and 0139 primers. PCR conditions were optimized at initial denaturation of 5 minutes at 94°C, followed by 30 cycles each with denaturation at 94°C for 1 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute,

with a final extension step of 72°C for 7 minutes. The amplified PCR product was analyzed by gel electrophoresis on a 2% agarose gel and visualized under UV light with ethidium bromide. The products for 01 and 0139 were 192 and 449 base pairs, respectively.

All samples that test negative for any vibrio species are then tested by 16S rDNA PCR to confirm DNA quality. The primers included a 6968-GC primer which amplifies the variable regions 6 and 8 (V6/V8) and primer L1401 to selectively amplify 16S rDNA genes. The methods, as described by Hasan et al consist of a 25ul reaction containing 5 µL of extracted DNA, 2x Terra PCR Direct Buffer (with Mg2+, dNTP), 1.25 U/ µL Terra PCR Direct Polymerase Mix (Clontech Laboratories, Inc., Mountain View, California, USA), 20 µM of 6968-GC and L1401 primers. PCR conditions were optimized at initial denaturation of 2 minutes at 94°C, followed by 35 cycles each with denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 68°C for 1 minute, with a final extension step of 68°C for 7 minutes. The amplified PCR product was analyzed by gel electrophoresis on a 1.5% agarose gel and visualized under UV light with ethidium bromide to reveal the 457 bp amplicon (17).

2.3.3 Statistical Methods

Clinical and Environmental data collected through October 2014 were used for the analyses in this report. Descriptive Statistics were used to plot the number of confirmed cholera cases and diarrhea comparing intensive versus routine surveillance for each study area over the first year of the study.

The environmental surveillance data included the type of water source (pond, river, ditch, well, sewage drain, or Lake) and the date of collection (season). The occurrence of

vibrios in the environment were compared over time by season, facility and water source. Loess non-parametric regression methods were used to fit a smooth function of season and facility to the presence of cholera (non01 or 01) in the environmental samples. We estimated the significance of *Vibrio cholerae* 01 detection over the first year of the study, taking into account the differing water sources, follow-up visits, and within-facility clustering of detection. We used generalized estimating equations (GEE) with a log link function and an ar1 correlation matrix to account for clustering at the facility level. Statistical analyses were conducted with Stata 13 (18).

2.4 Results

2.4.1 Clinical Surveillance

From August 2013 through October 2014, a total of 949 patients were enrolled in the study among all 7 study sites. 575 patients were enrolled in the study during intensive surveillance days, 374 patients were enrolled during routine surveillance days (Figure 2.3). The figure shows that most cases of acute diarrhea presenting both during the intensive surveillance every 15 days and the routine surveillance are not caused by *V. cholerae*. Though cholera was not common, severe diarrheal disease was common in Blangoua Health facilities (Figure 2.4). Cholera was not detected in these health facilities until October 2014 when a case was detected in Darak, Cameroon. The outbreak continued in Darak through October, with additional cases being detected in nearby Blangoua, Cameroon. In total, 28 clinically confirmed cholera were enrolled during routine surveillance days. Of those presenting with diarrhea on intensive days, most of the patients were children (>60%) less than 5 years of age.

A cholera outbreak was identified in Darak in early October, followed by 2 clinical cases being detected in Blangoua in late October. Of the 28 patients enrolled in the study with confirmed clinical cholera plus two non01 Cholera patients, nearly 40% (11) were children under 5 years of age, and nearly 75% were men (Table 2.3). The sensitivity and specificity of the modified protocol for the Crystal VC dipstick is presented in Table 2.2.

2.4.2 Environmental Surveillance

Between August 27, 2013 and October 31, 2014; 1,012 water samples were obtained from the environmental sites for the 7 Health Facilities, of which 244 were *V. cholerae* non-01 positive and 0 where *V. cholerae* 01 positive. The four types of water sources sampled included rivers, wells, sewage drains and Lake Chad. Binomial regression analysis showed an increased risk of vibrios in April, May and July as compared to the risk in January (Figure 2.6). At the facility level, there was a significantly increased risk of vibrio detection at sites near Naga and Darak as compared to Kousseri (Figure 2.7). Finally, as compared to vibrio detection in rivers, there is no significantly increased risk of detection at sites along Lake Chad, however a significantly increased risk of vibrio detection was seen in wells and sewage drains.

2.5 Discussion

Through this study we found that the simplified laboratory and epidemiological surveillance methodologies enabled rapid identification of cases during an outbreak while significantly reducing any false positive test results. This study is also the first to demonstrate the use of the modified Crystal VC dipstick method in a remote setting. The

results of the dipstick test were confirmed by a combination of culture and PCR for 1141 clinical and environmental samples, demonstrating a specificity of 99.8%. This is significantly higher than the specificity levels reported for direct use of the dipstick in stool samples (49-79%) (6-8) and corresponds to similarly improved rates of specificity found when used by George et al when used in a hospital setting in Bangladesh (9).

This study demonstrates the novel use of filter paper for environmental and stool sample preservation for molecular screening. This method proved to be a low-cost and low-maintenance diagnostic for the field setting, eliminating the need for culture confirmation and the intensive laboratory reagents and level of trained laboratory personnel needed to conduct the microbiological testing. The filter paper preservation is a simple blotting method that can be performed by non-lab personnel in a field setting. The filter paper does not need a cold chain for preservation and is not considered a biohazardous material, easing transport issues. The conventional culture techniques were conducted on all positive samples and 10% of negatives in this study; and the results were identical to PCR findings.

This study is the first to investigate a simplified epidemiological and laboratory surveillance methodology for use in remote and rural settings, and to successfully implement a sustained clinical and environmental surveillance of cholera in the Far North of Cameroon, particularly during a period of insecurity. While it was modeled after a similar methodology used in Bangladesh (4), the method in Cameroon was further simplified with the use of a modified rapid dipstick test, the use of a low cost gauze filtration device for environmental sampling, and the demonstration of dried, enriched specimens on filter paper for PCR confirmation of initial results. We demonstrated that

not only was epidemiological surveillance possible with 3 days of intensive surveillance every 15 days, but that incorporation of this surveillance into routine practices was possible. After more than one year of surveillance, the first cholera cases in Blangoua health district in 2014 were identified during our surveillance activities and the cases were confirmed using our laboratory methodologies in a rapid manner. This is important not only for the identification of a surveillance methodology that is feasible in remote and vulnerable settings, but also to demonstrate that this method can result in early outbreak detection, enabling rapid response and intervention to prevent further spread of the disease.

The surveillance results for the first year of this study did not confirm our initial hypothesis that there is a reservoir for cholera in Lake Chad. Nor did the regression analysis reveal a significant seasonal trend in vibrio presence in the environmental sites. However, this report is based only on the first year of surveillance. Given that outbreaks in the study area were only in the beginning stages at the conclusion of this first report, the surveillance data for year 2 of the study will provide an interesting comparison to year 1.

There are several limitations to this study, the most important being that the study site is in a vulnerable area that continues to struggle with safety and security issues as a result of a terrorist group. Safety issues led to loss of data in some study areas, particularly Darak which is located in Lake Chad. The presence of non-toxigenic *V. cholerae* 01 in the environmental sources during the early months of 2014 in Darak may have been an early warning sign for the area; however, the team was unable to maintain regular surveillance in this area to enable a complete analysis of events leading to the toxigenic *V. cholerae* outbreak in the fall of 2014. Clinical cholera cases were confirmed in Darak health district in October 2014, followed shortly by clinical cases in Blangoua health district; however

this study report is for the period of study initiation in August 2013 through October 2014. There were no clinical cases identified in our study area prior to October 2014; therefore we have limited positive results to power a case-control study. This limited positive sample size also likely negatively affected the reported dipstick sensitivity. Finally, in this study we clinically confirmed only *V. cholerae*, and therefore, were unable to further characterize the cases of non-cholera diarrhea enrolled in the study.

In conclusion, the first year of this surveillance study demonstrates the successful use and implementation of low-cost, simplified epidemiological and laboratory methodologies for surveillance in remote, rural or vulnerable settings. This study demonstrates the use of a modified dipstick protocol can be implemented in a field setting with improved specificity. The application of basic technologies such as the use of gauze filtration rather than more expensive filtration methods decreases supply and logistics costs, and allows for important environmental surveillance to provide information about the burden of cholera in previously undescribed areas. The application of dried filter paper methodology to cholera DNA preservation is novel and demonstrates a simplified method for assessment for vibrio presence in stool and environment.

Figure 2.1 Procedure for detecting V *cholerae* O1 from a fecal specimen using dipstick method

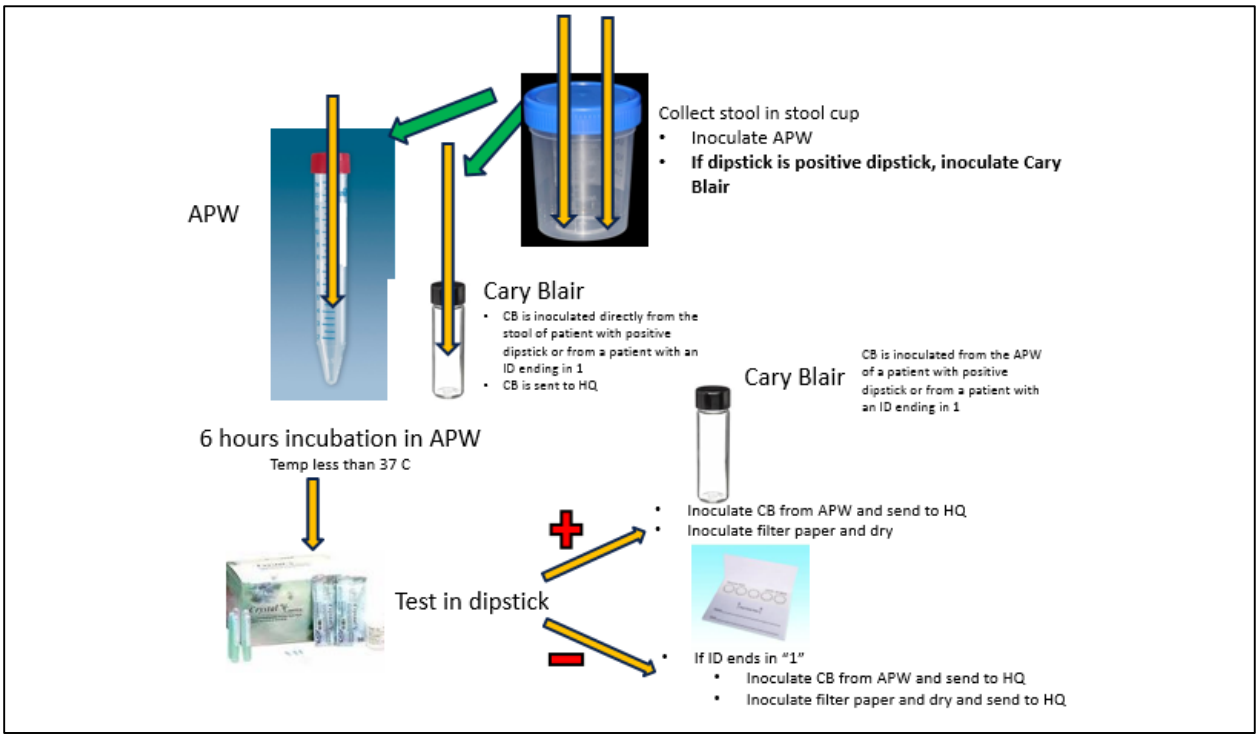


Figure 2.2: Procedure for detecting V *cholerae* O1 from environmental source using dipstick method

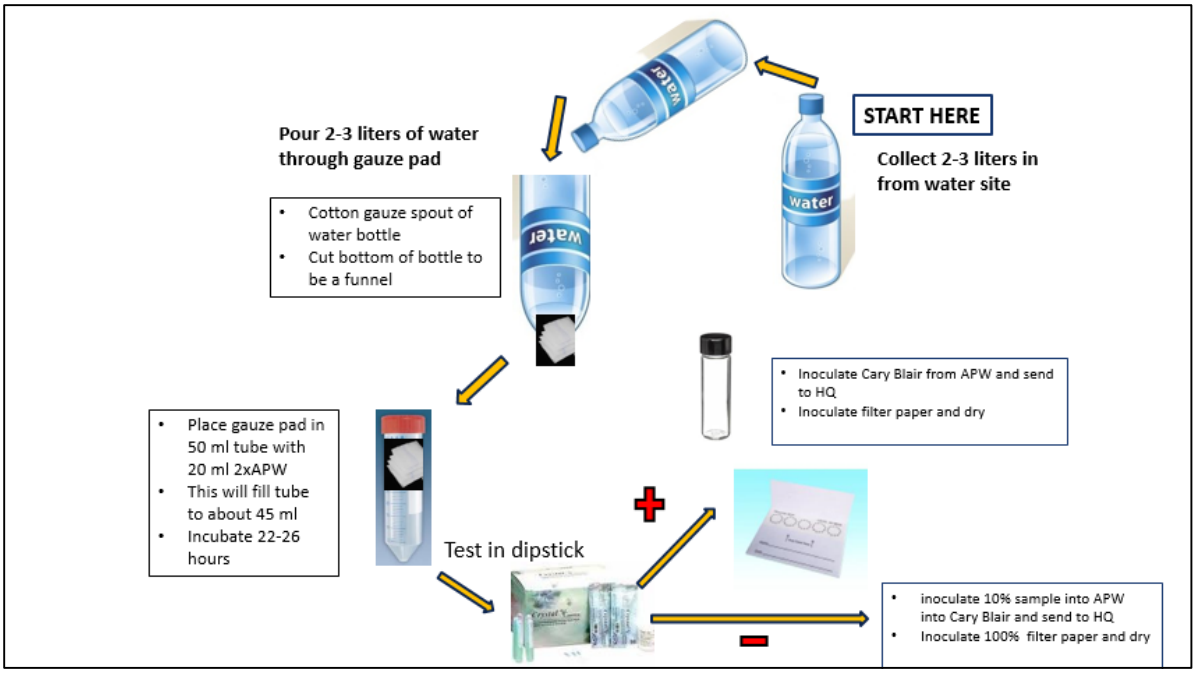


Table 2.1: Oligonucleotide Primers for PCR assays			
Primer Name	Sequence	Amplicon	Reference
UtoxF	GASTTTGTTTGGCGYGARCAAGGTT		(14)
vptoxR	GGTTCAACGATTGCGTCAGAAG	297bp	(14)
vctoxR	GGTTAGCAACGATGCGTAAG	640bp	(14)
vvtoxR	AACGGAACTTAGACTCCGAC	435bp	(14)
CtxA-F	CTCAGACGGGATTTGTTAGGCACG	302 bp	(15)
CtxA-R	TCTATCTCTGTAGCCCCTATTACG		(15)
ompW-F	CACCAAGAAGGTGACTTTATTGTG	588 bp	(15)
ompW-R	GAACTTATAACCACCCGCG		(15)
O1F2-1	GTTTCACTGAACAGATGGG	192bp	(16)
O1R2-2	GGTCATCTGTAAGTACAAC		(16)
O139F2	AGCCTCTTTATTACGGGTGG	449bp	(16)
O139R2	GTCAAACCCGATCGTAAAGG		(16)
6968 GC (V6/V8)	5’ – AA CGC GAA CCT TAC – 3’	457bp	(17)
L1401	3’ – GCG TGT GTA CAA GAC CC – 5’		

Figure 2.3: Monthly enrollment of Diarrhea and Cholera Cases

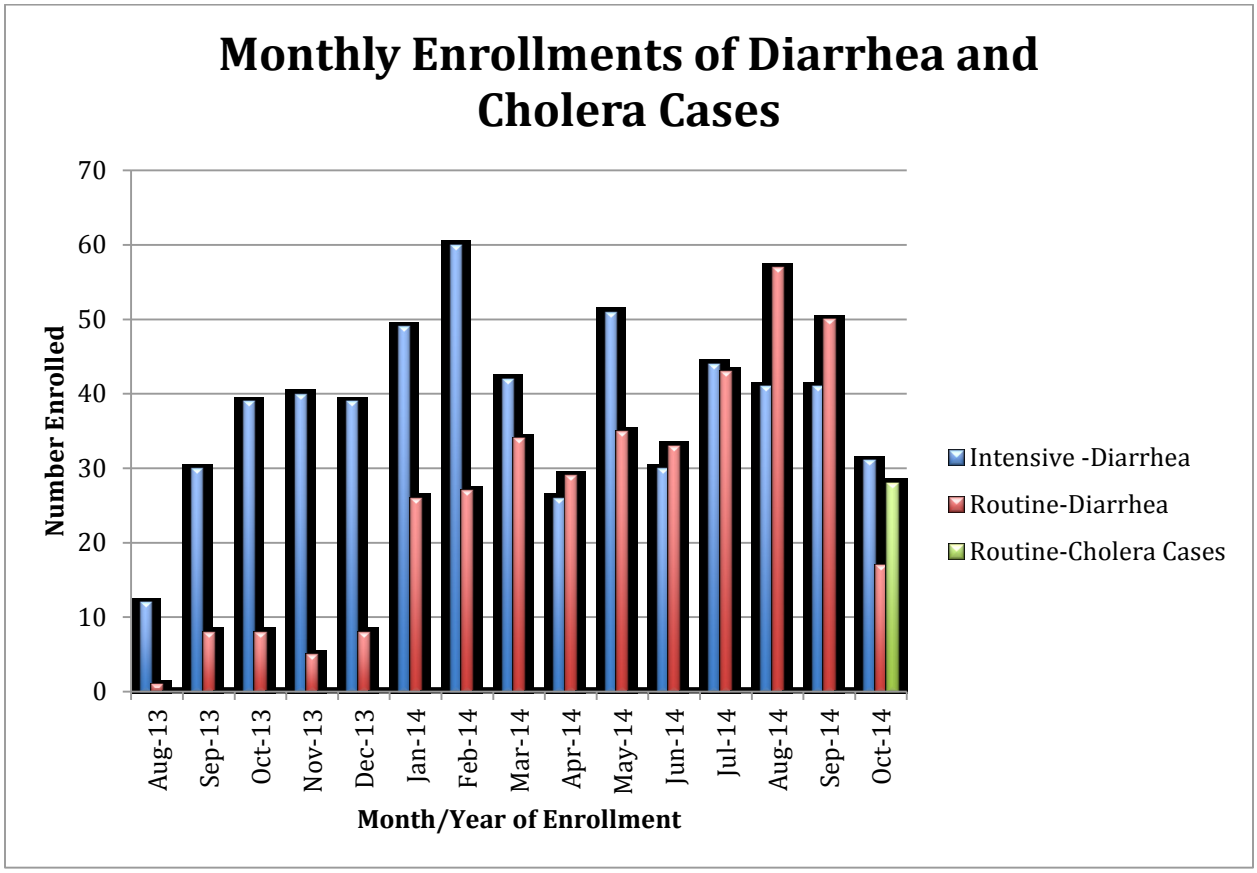


Table 2.2: Positive and Negative Predictive Values, Sensitivity and Specificity of Crystal VC dipstick as compared to the Gold Standard of PCR

Modified Dipstick Method	Clinical + Environmental Samples tested for Cholera		Total
	PCR Positive	PCR Negative	
Dipstick Positive	26	2	28
Dipstick Negative	2	1111	1113
Total	28	1113	1141
		Estimate	95% CI
Positive Predictive Value		92.9%	(76.5-99.1)
Negative Predictive Value		99.8%	(99.4-100)
Sensitivity		92.9%	(76.5-99.1)
Specificity		99.8%	(99.4-100)

Figure 2.4: Diarrhea with severe dehydration/Cholera by age group; according to Facility

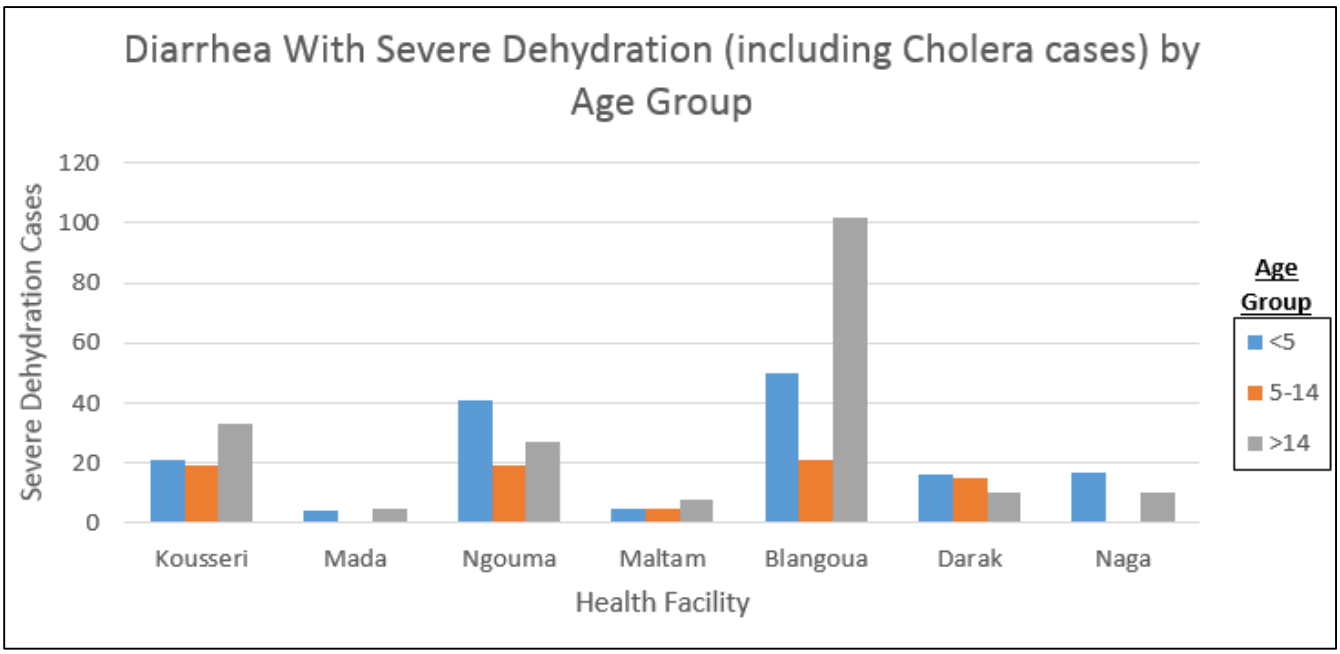


Table 2.3: Clinical Surveillance PCR Results: Dipstick Positive + 10% of negatives

Cholera Surveillance Stool sample PCR results: Dipstick Positives + 10% negatives					
Age group	No Cholera		Cholera		Total
	Male	Female	Male	Female	
<5	20	18	8	3	49
5-14	7	7	9	2	25
>14	20	27	5	3	55
Total	47	52	22	8	129

Figure 2.5 Multiplex PCR differentiating toxigenic *V. cholerae* strains of environmental samples from Darak Region

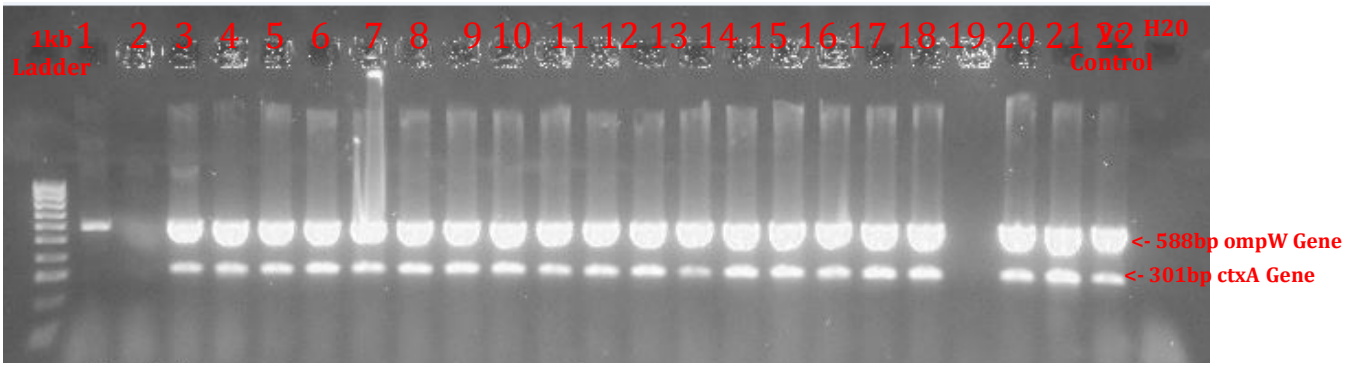
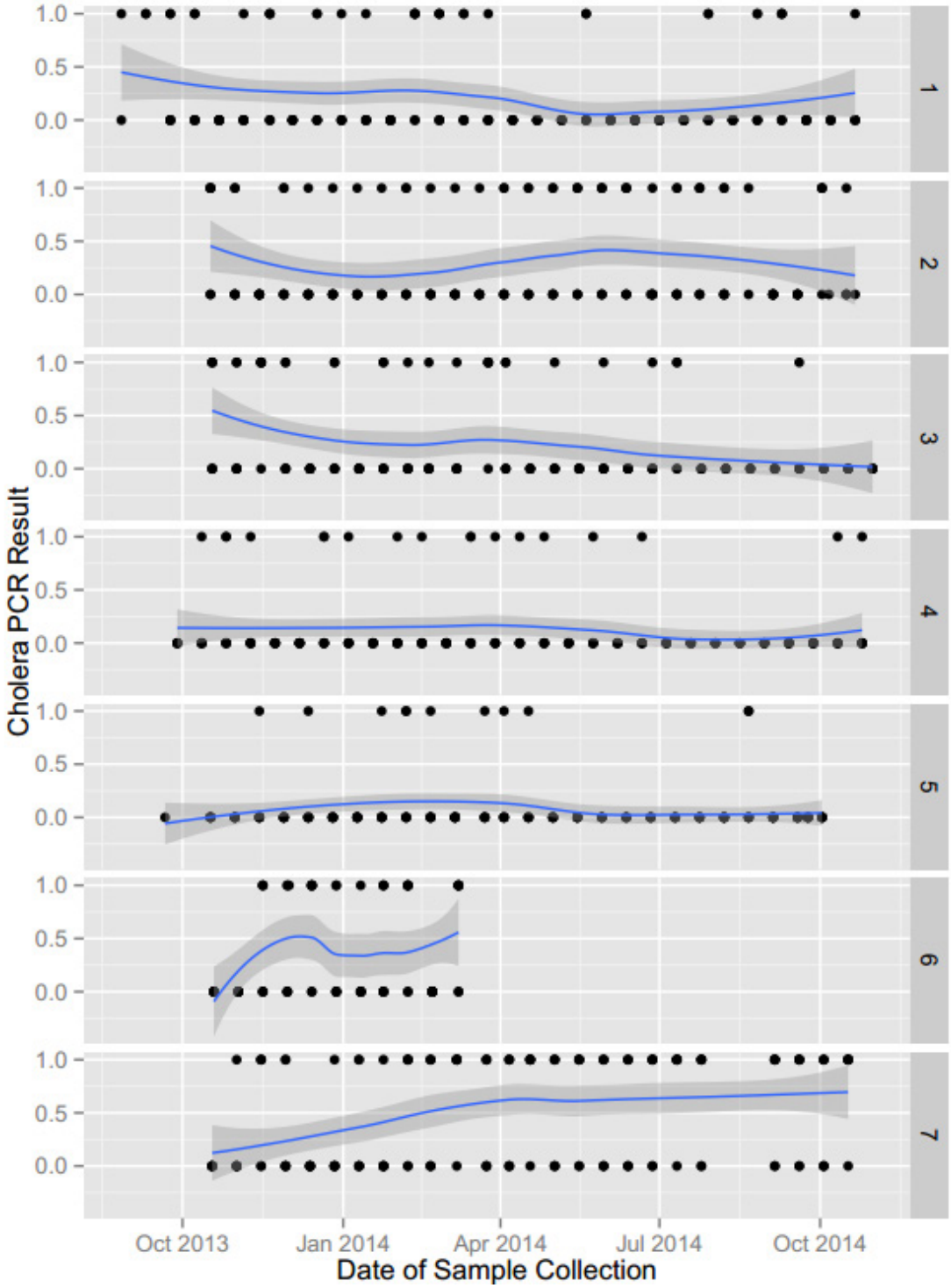


Figure 2.5: PCR of environmental samples from the Darak region; testing for the ompW gene (specific for *Vibrio cholerae*) and for the ctxA gene (testing for cholera toxin gene). Only the control strains were positive for ctxA, demonstrating that the specimens from Darak are non-toxigenic *V. cholerae* strains.

Figure 2.6: Generalized Estimating Equation Results: Vibrio non-01 detection

Factors	Odds Ratio (95% Confidence Interval)	P Value
Month: (Jan)	REF	
March	2.5 (1.9, 15.6)	0.001
April	4.8 (1.5, 14.7)	0.007
May	2.6 (0.9, 7.6)	0.091
June	1.9 (0.6, 6.2)	0.307
July	3.9 (1.2, 12.1)	0.021
Water Source (River)	REF	
Well	3.0 (1.2, 7.3)	0.016
Sewage Drain	10.4 (2.8, 38.1)	<0.001
Lake Chad	0.4 (0.1, 2.5)	0.298
Facility (Kousseri)	REF	
Blangoua	1.2 (0.3, 5.4)	0.837
Darak	9.7 (2.4, 38.3)	0.001
Naga	14.7 (3.8, 57.6)	<0.001

Figure 2.7: Cholera presence in environmental specimens (non-toxigenic non01 Cholera or toxigenic Cholera) compared across seasons, by Facility



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Chapter Three: Paper II

Molecular Epidemiology of Vibrio Cholerae, Cameroon, 2014 using novel specimen preservation methodologies

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3.1 Abstract

Background

There has been limited research characterizing the molecular epidemiology of *Vibrio Cholerae* in Africa. Knowledge of the genetic diversity of toxigenic *V. cholerae* strains provides understanding of strain relationship, epidemic potential, and transmission patterns. The constraints in facilitating this understanding is not only the lack of regular cholera disease surveillance in Africa, but also the lack of laboratory capabilities to preserve, store and ship isolates in a timely manner. We evaluate the use of simplified sample preservation methods for molecular characterization using MLVA for differentiation of V*ibrio cholerae* genotypes.

Methods

A sentinel surveillance study was conducted to assess simplified laboratory and epidemiological surveillance methodologies to assess cholera disease burden in a remote setting in the Far North of Cameroon. A total of 1,000 clinical cases were enrolled at the district health facilities and 1,012 environmental samples were processed among 45 distinct water sites. We assessed the genetic relatedness among 66 *V. cholerae* isolates and crude clinical and environmental specimens from Cameroon at 5 loci containing variable tandem repeats. The samples were collected from two geographically distinct outbreaks in the FNC in June 2014 and October 2014. In addition, we compared the genetic relatedness of the Cameroon samples to isolates from outbreaks in the Philippines and Mozambique.

Results

Isolates from 57 individual patients were analyzed; of these samples 16 were analyzed in two forms: culture isolates and crude specimens. The sequence analysis validated that the

crude specimens preserved on filter paper allowed for successful genetic characterization as compared to the culture isolates. The specimens from Cameroon formed two distinct clonal clusters distinct to each outbreak in 2014, however, specimens from the two outbreaks were identical at 3 loci indicating a relationship between the strains. One isolate from Mozambique was related to the June 2014 Cameroon outbreak samples. Samples from the Philippines were distinct from African samples, and formed two distinct clonal clusters.

Conclusions

The study demonstrated that the use of simplified laboratory diagnostics in remote and low-resource settings allows for the rapid identification of *V. cholerae* 01, and further, simplified DNA preservation methods facilitate timely molecular characterization of outbreak samples. The implementation of these methodologies can allow timely understanding of disease transmission patterns and enable proper planning and intervention targeting to prevent further disease spread. The molecular characterization did not suggest significant genetic diversity among strains in the Far North of Cameroon, rather that the outbreaks in 2014 were related despite being geographically distinct. The genetic relatedness shown between strains from Cameroon and Mozambique highlights the need for continued molecular epidemiological research to better understand the transmission and dissemination of strains within Africa.

3.2 Background

The Vibrio genus includes several different species which are known to be pathogenic, including *Vibrio parahemolyticus, Vibrio vulnificus, and Vibrio alginolyticus*. But the most widely known of these species is *Vibrio cholerae*. The etiologic agent of cholera, *Vibrio cholerae*, has more than 200 serogroups, differentiated by the O-antigen on the lipopolysaccharide (LPS) of the bacteria's outer membrane (1). Of these 200 serogroups, only two that produce cholera toxin (CT) are known to cause epidemic disease, serogroups 01 and 0139 (2). Vibrio species, and even differentiating pathogenic from nonpathogenic *V. cholerae* can generally be differentiated using basic biochemical and serological techniques (1). However, more advanced molecular techniques are needed to differentiate between different pathogenic strains; which provides crucial information to understand whether distinct isolates cause outbreaks in different geographic areas or whether there are common isolates that spread through wide geographic areas.

As previously described by Kendall et al, there are a number of molecular methods that have been established for differentiating between *V. cholerae* strains. These include rRNA restriction fragment length polymorphism (ribotyping), pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST). Due to the genetic similarity among clinical isolates, these methods have limited ability to differentiate the "near clonality" between pathogenic strains (3). Multi-locus variable-number tandem-repeat (VNTR) analysis (MLVA) examines short repeat DNA sequences that are repeated at a specific locus. The method uses the number of repeats at each specific locus to differentiate between isolates (4).

To date, many of the studies published using MLVA methods to characterize *V. cholerae* strains have focused on the endemic areas of south Asia (4, 5). A study examining environmental strains in comparison to clinical strains was performed in Bangladesh. The results from this research demonstrated that the vibrios were endemic in the aquatic environment in the study area of Bankerganj (6). Subsequent MLVA analysis on both clinical and environmental specimens demonstrated that the specimens collected from either small outbreak site, Bankerganj or Mathbaria, were distinct *V. cholerae* populations relative to the outbreak site. Additionally, they found that clinical or environmental isolates from a given time period were more likely to have a common sequence type (ST) than those collected in a subsequent month or time period. The sample size was small and produced only a few clinical and environmental isolates (i.e. 2 of 24 in Bankerganj, 1 of 16 in Mathbaria) in which the sequence types were the same. Thus, further research is warranted to assess the suggested benefits of using VNTR ST's to determine genetic relatedness during outbreaks, especially in geographic areas such as sub-Saharan Africa where the epidemiology likely differs from that of Bangladesh (7).

There has been limited research on the molecular characterization of cholera in Africa, and even less research in regards to understanding the molecular epidemiology of cholera in Africa. As the genetic diversity of toxigenic *V. cholerae* strains increases; it is increasingly important to understand their relationships and their epidemic potential (8). One of the most recent studies published in 2012 characterized strains isolated in Kenya. The study reported MLVA characterization of clinical isolates from outbreaks beginning in January of 2009. The demonstration of multiple distinct lineages that were also temporally and geographically independent supports the hypothesis that these outbreaks

were the result of endemic *V. cholerae* rather than imported cases or those spread by travelers (9). The continued use of MLVA for differentiation of clinical cholera isolates as well as any potential environmental isolates may provide further evidence of endemic foci.

Reports identifying the El Tor variant strain expressing the classical enterotoxin as the predominant strain in the world, including in Africa, were most recently confirmed by a ten year study in Zambia. The study emphasized the importance of further study and characterization of these altered strains to monitor their evolution as there has been a continuous change in the pattern of strains in potentially epidemic areas over the past decade (8). The proposed methods contribute new and timely information about the genetic diversity of cholera in Africa and elsewhere worldwide, using simple and efficient laboratory methods. These methods can provide crucial information to understand whether these outbreaks arise from the same strain, suggesting cholera spread; or whether the outbreaks represent unique strains and individually arising outbreaks.

In this study, we compare clinical samples obtained from two recent but geographically distinct outbreaks in Cameroon in which the samples were collected using simplified field surveillance methods. Field collected samples as well as culture isolates were preserved on Whatman filter paper, then sent for MLVA characterization. In addition, we compared the Cameroon strains to those collected using conventional methods from Mozambique in recent years as well as isolates from a recent 2014 outbreak in the Philippines. The results demonstrate that using the simplified methods, MLVA analysis and pathogenic vibrio strain differentiation is possible using crude DNA extracts from filter paper preserved specimens. We also demonstrated that the two outbreaks in 2014 in Cameroon, distinct in

time and geography, are related. Interestingly, we found that an isolate from Mozambique belongs to the same clonal cluster as specimens from the Cameroon outbreak in Bourrha in June 2014. The specimens from the Philippines were distinct from the African specimens; the 2014 Philippines were completely distinct from the 2012 and 2013 isolates, forming 2 different clonal complexes.

3.3 Methods

3.3.1 V. cholerae strains

A total of 92 *V. Cholerae* isolates and crude specimens were included in this study. 21 *V. cholerae* isolates from Bourrha Health District, Hina Health District and Mogode Health district in the Far North of Cameroon were collected during a cholera outbreak in June 2014, 18 *V. cholerae* crude specimens preserved on filter paper plus their corresponding culture isolates from Darak, Cameroon collected during a cholera outbreak in October 2014 plus 3 additional isolates from the Darak outbreak, 2 *V. cholerae* crude specimens preserved on filter paper plus their corresponding culture isolates from clinical surveillance conducted in Blangoua, Cameroon in October 2014, and 2 environmental crude specimens isolated during environmental surveillance in Naga, Cameroon in mid-September and mid-October 2014. In addition, 15 isolates from the Philippines (4 from an outbreak in December, 2012 in Lopez, Quezon; 6 from outbreaks in 2013:3 from Sinawal, General Santos City in April 2013 and 3 from T'boli, S. Cotabato in May 2013; and 5 from the 2014 outbreak in Davao del Sur) and 4 isolates from Manhica, Mozambique January 2008 outbreak and 1 isolate from Manhica, Mozambique February 2009.

3.3.2 Crude clinical and environmental samples

Clinical and environmental surveillance was initiated in the Far North of Cameroon (FNC) in August 2013. Surveillance was established at 7 seven local health facilities (LHF) in the FNC, in and around Lake Chad, including: Kousseri, Mada, Ngouma, Maltam, Blangoua, Darak, and Naga (Figure 3.1). In June of 2014, the surveillance team was notified of an outbreak in the Bourrha Health District outside of the study surveillance area; the team was deployed to assist applying the study's simplified field diagnostics to provide rapid diagnosis and confirmation of cases. During the time the surveillance team was aiding the health district, 21 cases were confirmed and 21 isolates were preserved for molecular characterization. In October 2014, a clinical outbreak was detected in the Darak study area; from October 19 – October 25, 2014, 21 clinical cases were confirmed using simplified lab diagnostics. The original field specimen and a culture isolate of each sample were preserved on filter paper for further molecular characterization. Shortly after the onset of the outbreak in Darak, two clinical cases were identified during study surveillance activities at the Blangoua Health Facility. Similarly, these two clinical cases were confirmed using simplified laboratory diagnostics and the field specimen plus a culture isolate were preserved on filter paper for comparison to the previous outbreak samples. 2 environmental samples collected during this time period at the Naga field sites also tested positive for *V. cholerae* and were preserved for molecular testing.

3.3.3 Laboratory Methods

Clinical Surveillance

Fecal specimens or rectal swabs from the consenting diarrhea subjects were collected using a stool cup (or from small children, it may also be collected from the diaper. These specimens, obtained from the monitoring sites and the sentinel sites, were screened

for *V. cholerae* O1 and O139 using an enhanced dipstick method (Crystal VC, *Span Diagnostics* Ltd. 173-B, New Industrial Estate, Road No. 6-G, Udhna, Surat - 394 210, INDIA) in which the specimen is tested via dipstick after incubation for 6-8 hours in alkaline peptone water (APW). APW enriched samples which tested positive, as well as a 10% sample of negative clinical samples were inoculated into Cary-Blair transport media for storage until transport for microbiological confirmation in the central reference laboratory in the Kousseri Health Facility. To evaluate the use of simplified specimen preservation and sample shipping methods, the enriched APW specimen for each sample was also preserved on Whatman 903 filter paper to be tested for vibrio's using molecular methods. 1-2 drops of the enriched APW was aliquoted to the Whatman filter paper and allowed to air dry; filter paper specimens were stored in individual plastic bags at room temperature until they were sent for extraction and PCR processing.

Environmental surveillance

Surface water samples were collected from 3-6 sites near each of the sentinel health facilities (total of 45 sites) to be tested for the presence of *V cholerae* O1 and O139. 2-3 Liters of the surface water sample was collected in a plastic jar and then filtered through sterile gauze. The gauze is then incubated in APW for 24 hours ($\pm$ 2hours) and subsequently tested using the dipstick. APW positive samples and a 10% sample of negative clinical samples were inoculated into Cary-Blair transport media for storage until transport for microbiological confirmation in the central reference laboratory in the Kousseri Health Facility. Similarly to clinical samples, the enriched APW specimen for all samples were preserved on Whatman filter paper for preservation for future molecular testing. 1-2 drops of the enriched APW was aliquoted onto the Whatman filter paper and

allowed to air dry; filter paper specimens were stored in individual plastic bags at room temperature until they were sent for extraction and PCR processing.

Microbiology at the Central Laboratory

Positive clinical and environmental specimens were streaked directly onto thiosulfate citrate bile salt sucrose (TCBS) agar and incubated for 24 hours at 37°C. Immediately after inoculating the first TCBS plate, a pre-labeled APW vial will be inoculated with the specimen and incubated for 6 hours at room temperature. After 6 hour incubation, a second TCBS plate is inoculated with the enriched specimen and incubated for 24hours at 37°C. After the 24hour incubation, any cholera-like colonies were selected with a sterile loop, re-suspended in 1-2 drops of PBS and tested via dipstick. All dipstick positive cultures as well as any cultures considered cholera-suspect, demonstrating the morphology of a cholera colony, were preserved in nutrient agar for further testing.

Microbiology at the Central Laboratory

All positive specimens were sent to the central laboratory in Kousseri, Cameroon for confirmation. The specimens were streaked directly onto thiosulfate citrate bile salt sucrose (TCBS) agar and incubated for 24 hours at 37°C. Immediately after inoculating the first TCBS plate, a pre-labeled APW vial will be inoculated with the specimen and incubated for 6 hours at room temperature. After 6 hour incubation, a second TCBS plate is inoculated with the enriched specimen and incubated for 24hours at 37°C. After the 24hour incubation, any cholera-like colonies were selected with a sterile loop, re-suspended in 1-2 drops of PBS and tested via dipstick. All dipstick positive cultures as well as any cultures considered cholera-suspect, demonstrating the morphology of a

cholera colony, were preserved in nutrient agar as well as blotted (using PBS to dissolve colony) onto Whatman Filter paper for further testing.

Molecular Characterization

DNA Extraction

DNA extractions of filter paper specimens were performed by using methods similar to those previously published (10). Sterile scissors were used to cut filter paper circles for each dried filter paper specimen. Between samples, the scissors were bleached and rinsed to ensure there was no cross contamination when processing the next dried filter paper specimen. Each cut dried filter paper specimen was placed into a pre-labeled tube. 1 milliliter (mL) of sterile 1X phosphate buffered saline (PBS) was added to each sample tube and incubated for 10 minutes at room temperature. The samples were then centrifuged (14,000 x g for 2 min) and the supernatant was discarded. 1 mL of sterile 1X PBS was then added to each sample, and then immediately centrifuged (14,000 x g for 2 min) and the supernatant discarded. Finally, 150ul of a 2% (wt. /vol.) Chelex-100 solution (Bio-Rad, catalog no.1422832) was added to each sample. The samples were placed in a heating block at 100°C for 8 minutes. The samples were centrifuged (14,000 x g for 2min) and the supernatant was removed to a new microcentrifuge tube and either stored at -20°C or used immediately in a PCR amplification reaction.

Toxigenic *V. cholerae* 01 was confirmed for all samples sent for sequencing using a multiplex targeting a gene encoding an outer membrane protein (OmpW) that is a unique gene conserved in the *V. cholerae* sequence as well as primers targeting the cholera toxin A (ctxA) gene. As described by Nandi et al (11) the multiplex reaction was run in 25 µL reactions containing 5 µL of extracted DNA, 2x Terra PCR Direct Buffer (with Mg2+,

dNTP), 0.78 U/ µL Terra PCR Direct Polymerase Mix (Clontech Laboratories, Inc., Mountain View, California, USA) 15pmol of OmpW primers, and 6.2 pmol of ctxA primers. PCR conditions were optimized at initial denaturation of 5 minutes at 94°C, followed by 30 cycles each with denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step of 72°C for 7 minutes. The amplified PCR product was analyzed by gel electrophoresis on a 1.5% agarose gel and visualized under UV light with ethidium bromide. The products for ompW and ctxA were 588 and 301 base pairs, respectively (Figure 3.2).

Subsequently, all specimens were tested to determine if they belonged to serogroup 01 or 0139 with a multiplex targeting unique regions in the *rfb* gene specific for the 01 and 0139 serogroups. Following methods described by Hoshino et al (12) the multiplex reaction was run in 30 µL reactions containing 5 µL of extracted DNA, 2x Terra PCR Direct Buffer (with Mg2+, dNTP), 0.93 U/ µL Terra PCR Direct Polymerase Mix (Clontech Laboratories, Inc., Mountain View, California, USA) 2µM of 01 and 0139 primers. PCR conditions were optimized at initial denaturation of 5 minutes at 94°C, followed by 30 cycles each with denaturation at 94°C for 1 minutes, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension step of 72°C for 7 minutes. The amplified PCR product was analyzed by gel electrophoresis on a 2% agarose gel and visualized under UV light with ethidium bromide. The products for 01 and 0139 were 192 and 449 base pairs, respectively (Figure 3.3).

MLVA analysis

The *V. cholerae* 01 positive samples were then genotyped at the five previously defined loci: VC0147, VC0436-7 (intergenic), VC1650, VCA0171 and VCA0283 (3).

Each locus was amplified using VNTR-specific primers (Table 3.1) and PCR conditions described previously (3, 5). Briefly, VC0147 and VCA0171 PCR products were amplified in 1 combined reaction while VC0283, VC0437, and VC1650 PCR products were a second separate reaction. Both reactions were comprised of a total reaction of 30 µL containing 3 µL of 10X PCR Buffer, 1.2 mM dNTPs, 50 mM MgCl2, 0.2 µL of 5 U Taq Polymerase, and 1 µM of each forward and reverse primers. PCR conditions for VC0147 and VCA0171 primers were optimized at initial denaturation of 5 minutes at 95°C, followed by 35 cycles each with denaturation at 95°C for 1 minute, annealing at 58°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension step of 72°C for 4 minutes. PCR conditions for VC0283, VC0437, and VC1650 primers were optimized at initial denaturation of 5 minutes at 95°C, followed by 35 cycles each with denaturation at 95°C for 1 minute, annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute, with a final extension step of 72°C for 4 minutes. The presence of amplified products was confirmed by gel electrophoresis; the amplified products were then purified using Millipore Manu filter plates (Merck, Darmstadt, Germany). The purified products were sequenced using a 3730xl Automatic Sequencer and the size was determined using internal lane standards with the GeneScan program (all from Applied Biosystems, ABI, Life Technologies Grand Island, NY) in combination with the formulae in Table 3.1. Alleles were determined by the number of repeats at each locus, and the number of repeats were listed in order of the five VNTR Loci to generate an isolate genotype: VC0147, VC0437, VC1650, VCA0171, and VCA0283 (Table 3.2). Therefore the genotype 6 4 6 17 20 indicates 6 repeats at the locus VC0147, 4 at the promoter of VC0437, etc. (3). Genetic relatedness of the strains was determined using eBURSTv3 (http://eburst.mlst.net).

3.4 Results

DNA was successfully analyzed from 82 of 89 initial specimens. Of these 82 specimens, 17 were analyzed in two forms: culture isolate and APW enriched stool sample (crude specimen). The isolates sequence types were identical to the crude sample sequence types in 15 of 16 patients. One isolate was 01 dipstick negative in contrast to the crude specimen which was 01 dipstick positive. PCR verified this isolate was a non-toxigenic V. cholerae non 01 isolate. This specimen was sent in error, as the colony morphology may not differ from that of a toxigenic V. cholerae 01, signifying that this was a mixed culture and therefore would not match the 01 crude specimen. Since the correct V. cholerae 01 isolate was not sent as a 01 positive to be compared for sequencing it is not included in the final comparison. One isolate differed from its crude specimen genotype at the 4th locus, suggesting that the person was infected with multiple strains. In addition, 2 environmental samples filtered and then enriched in APW (crude specimen) were also analyzed. DNA for MLVA genotype analysis except for the 5 isolates from Manhica, Mozambique were preserved and shipped for sequencing using Whatman filter paper and extracted according to the novel methods presented here. When all five loci were considered, there were 28 distinct genotypes among the 82 specimens analyzed (Table 3.2). The number of distinct alleles among the isolates at loci VC0147, VC0437, VC1650, VCA0171, and VCA0283 were 8, 2, 7, 13 and 9, respectively. Genotypes were defined as a clonal complex, when the genotypes were related to each other by an allelic change at a single locus. Five clonal complexes and 7 singletons were identified when the genotypes were analyzed using eBURST.

The Cameroon isolates belonged to two main clonal complexes, 1 and 3 (Figure 3.4). Clonal complex 1 contained 7 different genotypes from 20 isolates from the same outbreak in the Bourrha district in June 2014. The center of the clonal complex is known as the "founder" and is defined as the genotype with the largest number of single-locus variants (SLVs) (13). In clonal complex 1, the founder genotype (9-4-6-17-20) was present in 11 Bourrha clinical isolates. The 5 genotypes diverging from the founder were clinical isolates from the Bourrha outbreak (9-4-6-17-20; 9-4-6-18-20; 9-4-6-17-19; 9-4-6-17-21; and 9-4-6-17-23). Of these 5 genotypes, the last differentiated further with genotype 9-4-6-18-23 from a Mozambique clinical sample from an outbreak in February of 2009.

Clonal complex 3 contains 4 genotypes from 26 isolates and 18 enriched stool samples. The founder genotyped (9-4-6-16-25) was observed in 28 isolates and enriched stool specimens predominantly from the outbreak on the island of Darak in October 2014; with one clinical sample (both isolate and enriched stool specimen) from the Blangoua Health District in October 2014. 3 genotypes radiated from the founder, the first two genotypes 9-4-6-14-25 and 9-4-6-12-25 were from isolates and stool samples from the Darak Outbreak and the third 9-4-6-15-25 was from an isolate and stool sample from the Blangoua Health District. While this clonal complex is technically distinct from clonal complex 1 as by definition they differ at more than 1 loci; Clonal complex 1 and 3 are identical at the first 3 loci (9-4-6-X-X), demonstrating a clonal relationship between the two complexes.

The Philippine isolates comprised clonal complexes 2 and 4. The isolates from the outbreaks in Lopez, Quezon in 2012 and T'boli South Cotabato in 2013 clustered in clonal complex 2 comprised of 4 genotypes (11-9-10-17-20; 11-9-10-17-21; 12-9-10-17-21; 12-

9-10-17-22). While the isolates from the outbreak in July 2014 in Davao del Sur comprised clonal complex four which has 3 genotypes (12-9-8-22-27; 12-9-9-22-27; 12-9-9-23-27).

Of the five specimens from Mozambique included in this analysis; 3 isolates clustered together into 2 genotypes in clonal complex 5, 1 isolate was a singleton, and 1, as previously mentioned, was a genotype in clonal complex 1.

There were 6 additional singletons, including 3 singletons from Philippine isolates; 1 from the outbreak in Lopez, Quezon in 2012, and 2 singletons from 2013 in Sinawal, General Santos City in April and May 2013, respectively. 2 environmental specimens isolated from Naga Health District in September and October yielded 2 distinct singletons; both of which were identified by dipstick and PCR to be a non01 toxigenic *Vibrio cholerae*. Variation between the culture isolate from a single stool sample and the crude stool specimen yielded 2 genotypes (9-4-6-12-25 and 9-4-6-16-25), the variants are related variants, as they differ at only 1 loci of the small chromosome but the former classifies as a singleton.

3.5 Discussion

The results of this study show foremost that using the simplified laboratory diagnostics in vulnerable and remote field settings enables the rapid identification in the field of Vibrio cholerae 01 (and 0139) positive clinical and environmental samples. Further, the use of the simplified DNA preservation method allows for sample storage and transport in difficult settings. As a result of the implementation of these techniques, we were able to characterize an on-going outbreak molecularly. These rapid results can provide key stakeholders in the country information regarding disease transmission patterns to allow more proactive planning regarding interventions to prevent further spread.

The comparison of 16 culture confirmed *Vibrio cholerae* 01 isolates to the mixed stool cultures preserved on filter paper successfully demonstrated that there is no difference in the genotypic results regardless of the method in which the sample is preserved for molecular characterization. In settings in which classical microbiological methodologies for culturing and identifying positive *V. cholerae* clones is not possible due, these simplified methods have shown to produce identical results as a low cost, low maintenance alternative to characterize Vibrio strains.

The results of the genetic analyses did not suggest significant genetic diversity within Cameroon; rather that the 2014 outbreaks in Cameroon beginning in Bourrha Health District in May 2014 and continuing through June 2014 are related to the currently on-going outbreaks in the Darak and Blangoua Health Districts. Given that the two outbreaks comprised distinct clonal complexes, the results suggest that the outbreak strain that has mutated. Due to the difference in 2 loci and the limited samples, it is not clear whether the related strains were spread by travelers in the region or whether this strain persists in the Far North region on a small scale, and conditions were favorable in 2014 for its spread. Interestingly, one isolate from Manhica, Mozambique in 2009 was identified as being related to the strains present in the Bourrha outbreak in Cameroon. Given that the Mozambique strain is older than the strain present in the 2014 outbreaks in Cameroon, it suggests further investigation is warranted into strains from previous Cameroon outbreaks. This may suggest that the strain was carried from Mozambique to Cameroon, where it found suitable conditions to persist until outbreak conditions provided an environment optimal for it to spread.

The outbreaks in 2014 in Philippines are clearly distinct from those in Cameroon and Mozambique. However, the identification of two clonal complexes demonstrates that the 2014 outbreak in Davao del Sur is distinct from the 2012 and 2013 outbreaks. The use of filter paper preservation of isolates was used to enable shipment of DNA only for timely molecular characterization of the 2014 strains in comparison to those from previous years.

There are limitations to this study, including the difficulty in obtaining data and samples consistently due to concerns about security and safety of the staff in the outbreak areas in the Far North of Cameroon in 2014. While our study team was able to assist with outbreak in Bourrha, Cameroon, the presence of an insurgent group prevented further epidemiological characterization of the outbreak. The team was able to train the local health staff in our simplified diagnostics techniques for confirming cholera, however, our ability to collect specimens and their associated epidemiological information was limited as it was not safe for the team to work in the area for long periods. The same situation applies to the island of Darak in Lake Chad where an outbreak is still on-going. While this is a regular study site, surveillance has been difficult in 2014 with long interruptions due to safety issues. As a result of these conditions, environmental sampling was not possible during the outbreak to investigate a potential relationship between the clinical and environmental strains. However, our study is conducting environmental and clinical surveillance in surrounding Health Districts to monitor for potential disease spread. In addition, the comparison to Mozambique strains to those from Cameroon, while demonstrating a relationship in clonal complex 1, is limited, as this study area is not normally a part of our research activities and the strains available for molecular comparison

were limited. Further research is warranted to better understand the relationship among these strains.

In conclusion, this study demonstrates that simple and low-cost lab methods can be utilized in even the most vulnerable and resource limited settings and allow for molecular characterization of cholera outbreaks in a rapid and timely manner. The molecular data gathered in this study was promptly presented to the Ministry of Health of Cameroon to proactively plan interventions in the outbreak and surrounding areas in the Far North, to include a potential vaccination campaign and behavioral interventions. The ability to provide timely information to aide in outbreak response and intervention measures is critical to reduce disease burden and death.

In addition, we show clearly that the strains present in 2014 outbreaks in South-east Asia are distinct from those in Africa. Interestingly, we did show a relationship between strains present in the 2014 outbreaks in Cameroon and those isolated from Mozambique, two geographically distant nations in Africa. This finding and in addition to the fact that the strains in the two outbreaks in distinct areas of the Far North of Cameroon are similarly related warrants continued surveillance molecular characterization in these areas to elucidate more fully the relationship and disease transmission patterns.

Figure 3.1 Map of Field sites, Far North Cameroon

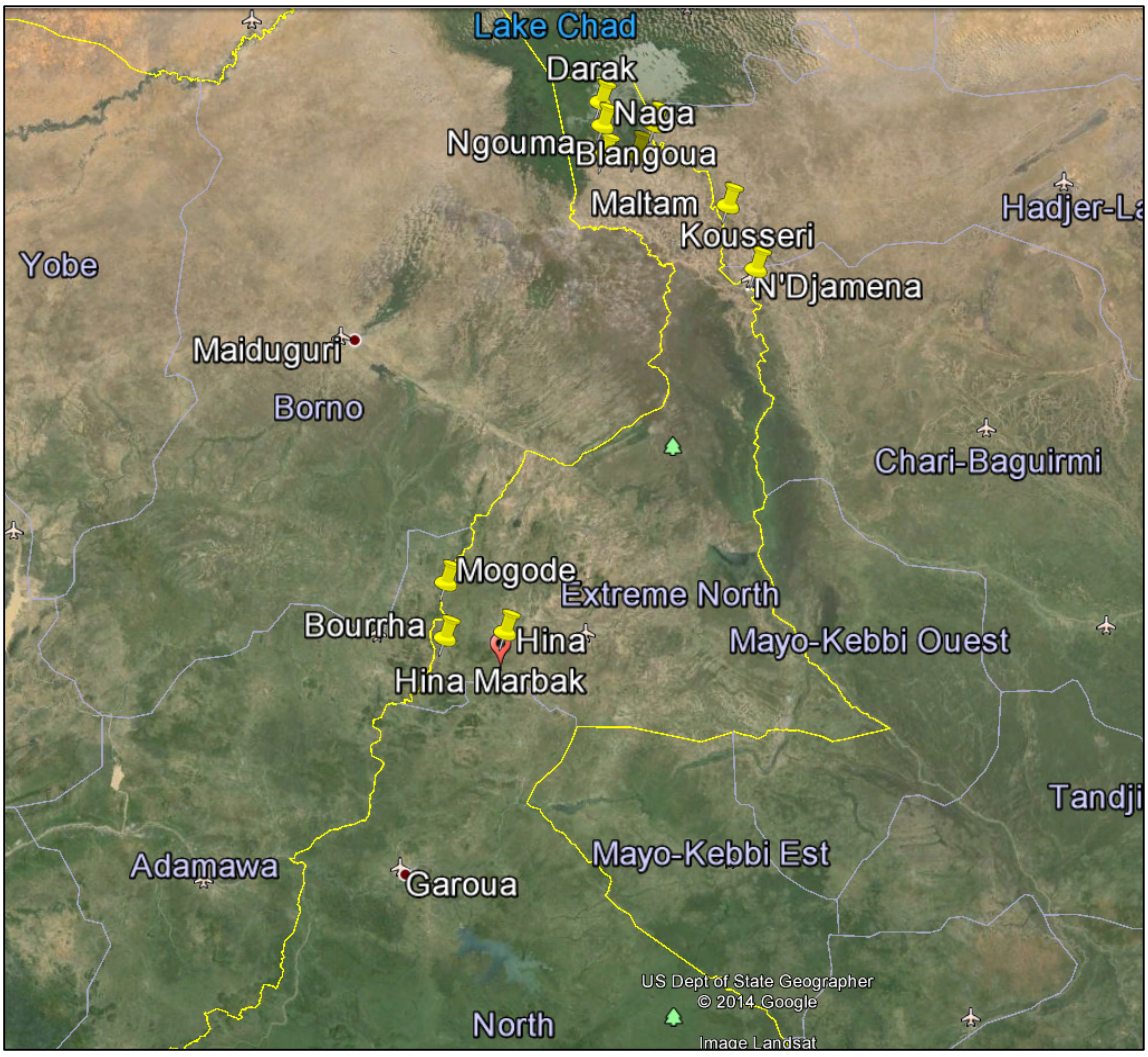


Figure 3.2: Detection of V. cholerae ompW and ctxA-specific genes

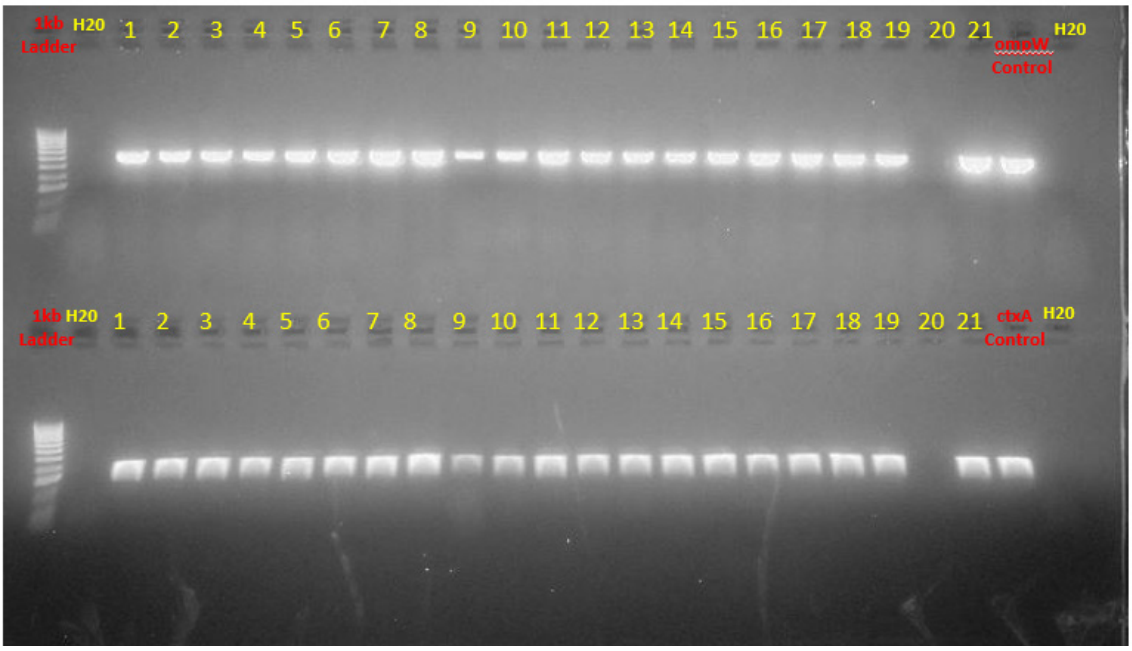


Figure 3.3 Detection of V. cholerae O1 *rfb*-specific genes

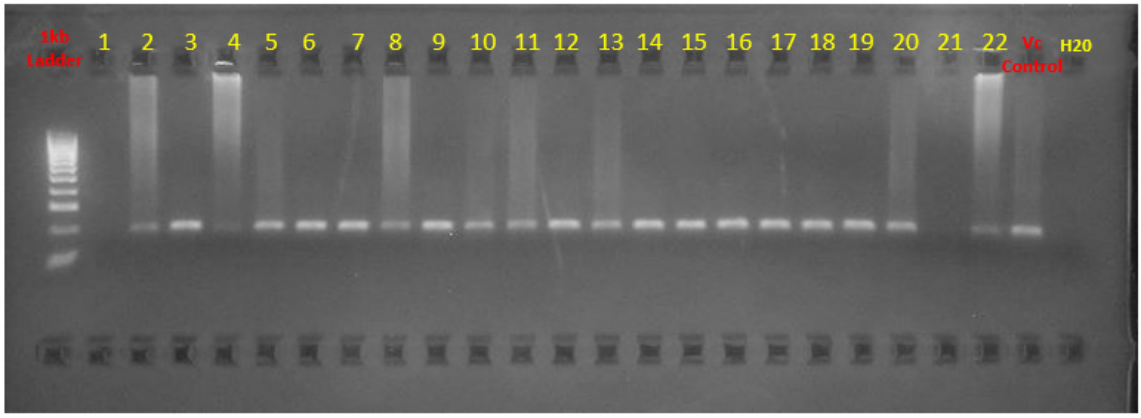


Table 3.1: VNTR Primers and Formulae for *V. cholerae* MLVA			
Primer Name	Sequence	Range	Formula
VC0147-F	TTGTCATGGCTTGGATTTGG	186 - 224	(x-150)/6
VC0147-R	TET-ACGTGCAGGTTCAACCGTG		
VC0437-F	CGTTAGCATCGAAACTGCTG	265 - 301	(x-245)/6
VC0437-R	TET-GTTGCCGCCATCACCAGCTTG		
VC1650-F	CTACCAAGCGGCGGTTAAGCTG	370 - 440	(x-306)/9
VC1650-R	TET-CCGCTAACTGAGTGACCGC		
VCA0171-F	GCTGAAGCCTTTCGCGATCC	316 - 442	(x-265)/6
VCA0171-R	FAM-AGGCGCCTGATGACGAATCC		
VCA0283-F	AGCCTCCTCAGAAGTTGAG	118 - 244	(x-95)/6
VCA0283-R	FAM-GGAGGTAGCTACGAATTCTAC		

Table 3.2: *V. cholerae* Isolate Genotypes and MLVA group

Original ID	Location, Year	VC0147	VC0437	VC1650	VCA0171	VCA0283	MLVA Group
15B_Cam	Bourrha, CMR; June 2014	6	4	6	17	20	1
300205 (VC Ogawa)_F/4	Mozambique Strains	7	4	2	13	14	Singleton
003B PHIL.	Sinawal,General Santos, PHL; April 2013	7	9	9	7	21	Singleton
300043 (VC Ogawa) _F/5	Mozambique Strains	8	4	6	18	21	5
300208 (VC Ogawa)_F/6	Mozambique Strains	8	4	6	18	21	5
300209 (VC Ogawa)_F/7	Mozambique Strains	8	4	6	19	21	5
5B_Cam	Bourrha, CMR; June 2014	9	4	6	17	19	1
12B_Cam	Bourrha, CMR; June 2014	9	4	6	17	19	1
6B_Cam	Bourrha, CMR; June 2014	9	4	6	17	19	1
25B_Cam	Bourrha, CMR; June 2014	9	4	6	18	20	1
14B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
21B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
7B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
20B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
23B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
22B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
11B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
24B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
26B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
29B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
28B_Cam	Bourrha, CMR; June 2014	9	4	6	17	20	1
4B_Cam	Bourrha, CMR; June 2014	9	4	6	17	21	1
302015 (VC Ogawa)_F/1	Mozambique Strains	9	4	6	18	23	1
27B_Cam	Bourrha, CMR; June 2014	9	4	6	17	23	1
014B PHIL.	Sinawal,General Santos, PHL; April 2013	11	4	1	13	14	Singleton
013B PHIL.	Lopez, Quezon, PHL; Dec2012	11	9	10	17	20	2
004B PHIL.	T'boli, South Cotabato, PHL;	11	9	10	17	21	2

	May 2013						
017B PHIL.	Lopez, Quezon, PHL; Dec 2012	11	9	10	14	24	Singleton
011B PHIL.	Davao del Sur, PHL; July 2014	12	9	8	22	27	4
005B PHIL.	Davao del Sur, PHL; July 2014	12	9	9	22	27	4
012B PHIL.	Davao del Sur, PHL; July 2014	12	9	9	23	27	4
010B PHIL.	Davao del Sur, PHL; July 2014	12	9	9	22	27	4
007B PHIL.	T'boli, South Cotabato, PHL; May 2013	12	9	10	17	21	2
009B PHIL.	T'boli, South Cotabato, PHL; May 2013	12	9	10	17	22	2
600070-DN	Darak,CMR; Oct 2014	8	4	7	10	25	Singleton
221442	Naga,CMR; Oct 2014	15	4	6	12	24	Singleton
600068-DP	Darak,CMR; Oct 2014	9	4	6	12	25	3
600059-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600052-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600052-DR	Darak,CMR; Oct 2014	9	4	6	14	25	3
600066-DR	Darak,CMR; Oct 2014	9	4	6	14	25	3
600064-DR	Darak,CMR; Oct 2014	9	4	6	14	25	3
600072-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600058-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600066-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600071-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600064-DP	Darak,CMR; Oct 2014	9	4	6	14	25	3
600059-DR	Darak,CMR; Oct 2014	9	4	6	14	25	3
600058-DR	Darak,CMR; Oct 2014	9	4	6	14	25	3
600071-DR	Darak,CMR; Oct 2014	9	4	6	14	25	3
500289-APW	Blangoua,CMR; Oct 2014	9	4	6	15	25	3
500289-culture	Blangoua,CMR; Oct 2014	9	4	6	15	25	3
600070-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3

600057-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600048-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
500291-culture	Blangoua,CMR; Oct 2014	9	4	6	16	25	3
600046-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600060-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600055-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600041-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600054-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600069-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600055-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600067-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600065-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600050-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600057-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600040-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600060-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600061-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600065-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
500291-APW	Blangoua,CMR; Oct 2014	9	4	6	16	25	3
600045-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600047-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600061-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600069-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600043-DP	Darak,CMR; Oct 2014	9	4	6	16	25	3
600053-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600067-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
600068-DR	Darak,CMR; Oct 2014	9	4	6	16	25	3
30B	Bourrha, CMR; June 2014	9	4	6	17	23	1
221438	Naga,CMR; Sept 2014	10	4	6	99	25	Singleton

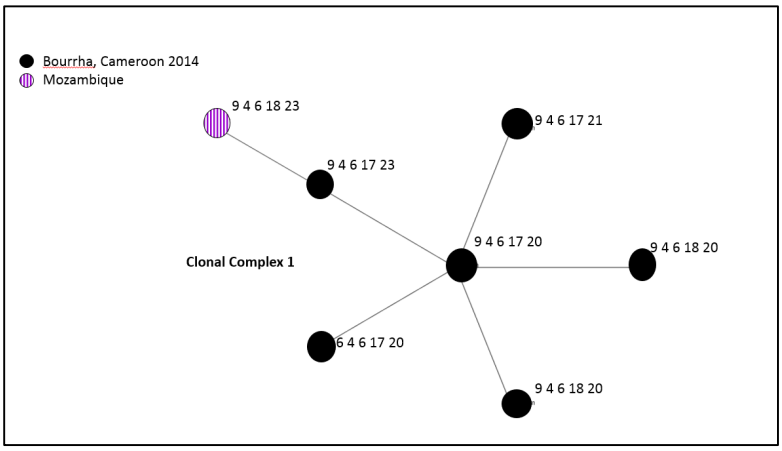
Table 3.3: Number and percentage of initial *V. cholerae* isolates differing at each loci

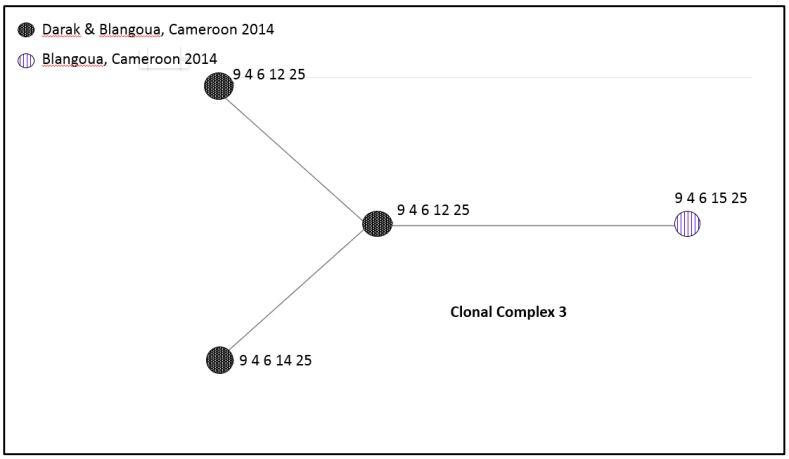
No. of isolates	No. (%) of isolates differing at at each loci				
	Large-chromosome loci			Small-chromosome loci	
	VC0147	VC0437	VC1650	VCA0171	VCA0283
Overall (82)	8 (9.75)	2 (2.4)	7 (8.5)	13 (15.6)	9 (11.0)
Cameroon (66)	5 (7.6)	1 (1.5)	2 (3.0)	8 (12.1)	6 (9.1)
Isolate matches crude specimen (16)**	0	0	0	1 (5.9)[¥]	0

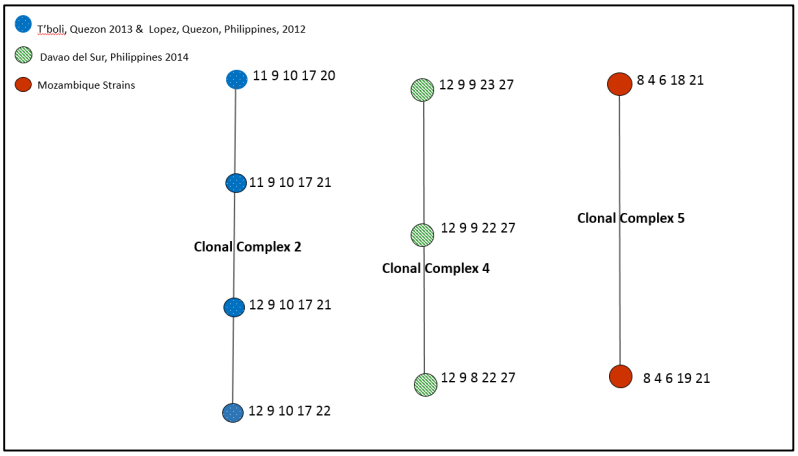
**Note: One isolate was 01 dipstick negative in contrast to the crude specimen which was 01 dipstick positive. PCR verified this isolate was a non-toxigenic *V. cholerae* non 01 isolate; the colony morphology may not differ from that of a toxigenic *V. cholerae* 01, signifying that this was a mixed culture and therefore would not match the 01 crude specimen. Since the correct *V. cholerae* 01 isolate was not sent as an 01 positive to be compared for sequencing it is not included here.

[¥] One isolate (600068) differed from its crude specimen genotype at the 4th locus, potenitally signalling that the person was infected with multiple strains.

Figure 3.4: Clonal Complexes







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Chapter Four: Paper III

Overview of a Rapid risk Assessment Tool (RAT) for describing geographic rapid risk of cholera burden in developing countries to assist in implementation of Oral Cholera Vaccine

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4.1 Abstract

Background

The World Health Organization supports use of oral cholera vaccine (OCV) in developing countries with endemic cholera. The current supply and production capabilities, however, for OCV are not sufficient to vaccinate entire nations. Therefore, cholera-endemic countries must make decisions about use of OCV in the highest risk areas. We present a rapid assessment tool that uses retrospective national, regional and sub-regional cholera surveillance data and geographic distribution of risk factors to identify high-risk areas of the country where OCV use can be targeted.

Methods and Results

The assessment begins by determining the cholera incidence rate at a provincial level. The incidence and mortality rates are adjusted for missed cases and deaths by extrapolating rates from recently published active case finding studies. If more localized rates are needed further assessment of cholera incidence and risk is conducted at the district level. If district level case and mortality data is available, the incidence and mortality rates are determined and adjusted for missed cases and deaths to differentiate districts with low, medium and high risk of cholera. If case and mortality data is not available or is insufficient to differentiate high risk areas at the sub-provincial level, risk can be determined through a weighted risk factor approach to identify risks of low, medium and high risk of cholera. Kenya was the case study for the development of the cholera RAT, evaluating surveillance data from 1997 through 2010. Using both detailed surveillance data at the district level as well as the weighted risk factor approach, several high risk areas were highlighted including Marsabit, Moyale, Turkana and Isiolo Districts.

Discussion

The pilot testing of cholera RAT in Kenya highlights the importance of the weighted risk factor approach; given current vaccine availability MoH's or other Public Health leaders interested in vaccination will have to use a targeted approach to most strategically use vaccines allotted. In Kenya, the RAT highlighted several key areas of risk, including the area of Turkana whose provincial level rates were not considered in the high risk category, highlighting the importance of working from a national level down to sub-province or even sub-district levels. The cholera RAT will continue to be refined as it is tested in further cholera endemic countries; however this study demonstrates the advantages of this simple tool using locally available data in conjunction with national cholera disease rates to better understand the cholera disease burden for cholera vaccine and intervention programs.

4.2 Introduction

The World Health Organization recommends use of oral cholera vaccine (OCV) in countries with endemic cholera, or in areas at risk for outbreaks of cholera (1). OCV use is recommended in conjunction with other cholera prevention and control strategies. Dukoral, a killed *V. cholerae* (01) vaccine with a non-toxic B subunit component was the first oral cholera vaccine to be prequalified by the World Health Organization (WHO). In studies conducted in Bangladesh and Peru in the 1990's, Dukoral was found to have high short-term protection (85%) and a two year combined protection of 60% (2). A modified, bivalent killed oral cholera vaccine, Shanchol, was subsequently prequalified by the WHO in 2011 (3). Shanchol contains both *V. cholerae* 01 and *V. cholerae* 0139 strains, and its safety and efficacy has been assessed in a large scale randomized control trial in Kolkata, India. It has been shown to provide 67% protective efficacy at two years of follow-up (4), 66% protective efficacy at three years of follow-up (5), and most recently, a sustained 5-year protective efficacy of 65% (2). In addition to the initial safety and efficacy studies conducted on the vaccines; studies have been conducted to assess the feasibility and effects of a large-scale vaccination campaign in multiple African settings.

Dukoral demonstrated protective efficacy in two large mass-vaccination campaigns in Mozambique (2004) (6) and in Zanzibar (2010), with the campaign in Zanzibar demonstrating both direct and indirect (herd) protection (7). In 2012, Shanchol was finally deployed for use in Haiti in the midst of fighting a cholera epidemic that was first detected in October 2010. The vaccination campaign in Haiti reported high coverage rates up to 92.7% in Bocozel and 2-dose completion rates >90% in both Grand Saline and Bocozel (8). In 2013, the first large-scale reactive vaccination campaign using Shanchol was

implemented in Guinea; with one-dose coverage of 92% in Boffa and 71% in Forecariah. 2-dose completion coverage was slightly lower at 68% in Boffa and 51% in Forecariah (9). Follow-up studies in both countries have demonstrated that large-scale vaccination campaigns are feasible and acceptable during outbreaks, reflected by high acceptance rate and high population coverage in both urban and rural settings (8, 10).

Despite its proven ability to reduce cholera risk, OCV is not widely used. One reason for this is that the supply of OCV is limited worldwide. In 2010, Waldor et al published a perspective piece on the need for an OCV stockpile, at the time of publication there were only 400,00 doses of Shanchol and Dukoral available for shipment (11). In 2013, the WHO began the initiative to create a stockpile of 2 million doses of OCV. In the same year, the GAVI alliance promised further support to increase the stockpile to 20 million doses by 2018 (12). Even as the stockpile is built and maintained, a recent estimate of the global burden of cholera estimated that 1.4 billion people are at risk for cholera in endemic countries (13), further highlighting the need for increased vaccine production. With the current limited supply of OCV, cholera-endemic countries interested in OCV use are likely unable to vaccinate their whole country. In deciding where to introduce OCV, ministries of health must identify regions of the country with the highest cholera risk over the next 3-5 years. In countries with sensitive surveillance for cholera over multiple years, historical incidence of cholera by region can be used to predict where cholera will most likely occur in the future. Few countries have such surveillance, particularly in Africa, which has become the continent accounting for the majority of reported cholera cases worldwide. In 2012, Sub-Saharan Africa reported 71% of cholera cases and 86% of cholera deaths, worldwide (14). In 2010, the African Cholera Surveillance Network (Africhol) was

created to begin population based cholera surveillance in 11 countries, collecting data on demography, symptoms and risk factors (14). Without longitudinal surveillance, countries can utilize a risk-based approach to identify areas most likely to experience cholera, such as history of past outbreaks, poor water and sanitation infrastructure and distance to nearest health facility (13).

We present a Cholera Rapid risk Assessment Tool (RAT) that uses locally available data for use by Ministries of Health, and other interested parties to use as a tool to aid in preparing a preventive strategy to the threat of cholera disease. While the primary focus of the tool is to target high-risk areas for OCV use, it can just as easily apply to other prevention strategies, such as safe water programs, as well as bolstering of treatment supplies and training. This method uses retrospective surveillance data to calculate incidence rates of cholera disease by geographic region, starting at the provincial level and working down to sub-provincial/sub-district administrative levels where possible. The tool includes an adjustment for severe cholera cases and deaths not seen in health facilities, which is important in many low income countries where healthcare utilization is low, even in the setting of severe disease such as cholera (15, 16). The tool also identifies if regions and districts have previously surpassed the presumptive threshold of 1 case per 1,000 persons as a limit for the cost-effectiveness of OCV introduction (17). Where incidence cannot be estimated or where more defined areas of risk are needed to target vaccination, district risk factors for cholera are employed to suggest those districts at the highest risk of cholera.

The cholera RAT is not a predictive model, in the sense that it is not intended to predict the exact timing, severity, or location of the next outbreak. This cholera RAT is intended

for use in countries with endemic cholera, in which cholera outbreaks occur on a recurring basis, either consistently (e.g. Bangladesh, Mozambique coast) or sporadically (e.g. much of Africa). The cholera RAT is not applicable to predicting cholera in a region that has never experienced cholera in recent years, such as Peru in 1991 or Haiti in 2010; however, many of the risk factors included do provide a guide as to what could happen if cholera was introduced into a high risk area. Rather, by assessing retrospective cholera disease burden data, the cholera RAT can suggest where cholera has historically occurred in a country and if rates have reached levels high enough for the ministry of health to consider interventions, such as OCV introduction.

4.3 Methods

4.3.1 Overview of Cholera Rapid risk Assessment

The Cholera Rapid risk Assessment Tool focuses initially on regional level data, beginning with a broad approach to collate data to determine cholera disease burden. Regional level data may be more readily accessible; however, there are circumstances where there may be no interest in the cholera disease burden at this level. One example may be that there may not be sufficient support, to include vaccine supply, to target an intervention on such a large scale. Another example may be that there is knowledge that a specific district or sub-region may be of interest for analysis. While the RAT is adaptable to specific settings, analysis of regional and then district level data, systematically, may highlight areas of risk that were not previously realized and further enhance cholera control efforts in the country. Data sources providing information needed for the cholera risk assessment may include WHO reports, Integrated Disease Surveillance and Response

(IDSR) reports in participating African countries, or disease-specific line lists collected at a regional, district or sub-district level detailing cases and deaths. Care should be taken to select the most complete reports with consultation and guidance from the Ministry of Health (MoH). Where possible, reports should be collated to provide the most complete case and death count. Due to the uncertain nature of cholera outbreaks in areas such as sub-Saharan Africa, we hope to capture a minimum of five years of data. Analyzing a minimum of five years provides information on whether the disease is endemic in the country/province/district based on WHO definitions of more than 3 outbreaks in five years (18), as well as providing information to compare periods or years when cholera was present as compared to those when there was no cholera; providing a better understanding of the risk in the area of investigation.

Figure 4.1 presents a flow diagram outlining the methodology encompassing the cholera RAT and its recommendations.

Step 1: *Collection of retrospective counts of cholera cases and deaths at Regional/Provincial level.* This is accomplished by working with Ministries of Health and Regional Health facilities to collate the aggregated case data by region or province.

Step 2. *Calculation of regional incidence rates of reported cholera cases*. Census data should be obtained to get the regional denominators for the years for which cholera data is available. Population projections might be needed for years in which census data is not available, and population growth rates are usually available from census data. The number of reported cholera cases is divided by the population number to get the rate of cholera cases by year and region. Incidence should be expressed per 1000 persons annually.

Step 3a and 3b: *Adjustment for cases/deaths not reported to MOH*. To account for cholera cases not captured by surveillance, the percentage of cholera cases (Step 3a) and deaths (Step 3b) that are not reported, including persons who did not seek care at a medical facility, should be estimated. If possible, local data on health utilization during cholera outbreaks should be used to estimate the percentage of cases not seeking care. In a recent publication, results from Health utilization surveys were used to adjust surveillance estimates of pneumonia, allowing for more accurate estimates of case burden (19). Care should be taken to use the same criteria and definition of cholera as used for reported cholera cases in health facilities, in order to ensure that cases of equal severity are counted rather than mild or asymptomatic cases. Existing health utilization data on syndromic diarrhea in a non-cholera setting (e.g. DHS) is not appropriate for cholera as it is a rare event and therefore it may not be frequent enough to be detected in a DHS survey. If health utilization data is available for cholera, the number of cases of cholera reported from surveillance should be divided by the percentage of all cholera cases that sought care at a health facility as ascertained from a health utilization survey. If health utilization data is not available, other data sources that should be considered include WHO reports, IDSR reports or separate line lists at regions and district level. Where possible, reports should be collated to provide the most complete count. The selection of the most complete reports should be made in consultation with the MoH.

Because health utilization data during cholera outbreaks will be rarely available locally, and it may be difficult to supply sufficient line-list or IDSR reports depending on the location; the base-case algorithm uses estimates from the literature. The suggested adjustment factors in the cholera RAT utilizes missed case and missed death rates reported

in an active case finding study conducted by Shikanga et al. During a period of civil unrest in 2008, western Kenya suffered a cholera outbreak. The Ministry of Health conducted a case finding study in the three districts reporting the highest number of cholera cases. The case finding included interviewing administrative persons and sub-chiefs of the various districts, interviews of any households reporting cases plus other households of reported interest. The study reported that there were 271 of 396 cholera cases (46%) not reported and that the active case finding found 30 missed deaths and an adjusted case fatality rates (CFR) of 24% as compared to the MoH reported 15 deaths and CFR of 5.5% (20). In the absence of more country specific data, the RAT will apply the rate of (46%) missed cases and (24%) missed deaths to adjust the total number for disease burden calculations.

Step 4. *Calculation of regional incidence rates of total cholera cases.* In this step the numerator is adjusted to account for the unreported cholera cases calculated in step 3. As in step 2, the incidence is calculated using the population denominator. At this point, the annual calculated incidence by region can be compared to a putative threshold for OCV vaccination of 1 case per 1000 persons. The threshold for vaccination is based on an analysis exploring the cost effectiveness of oral cholera vaccine implementation in endemic areas. Assuming costs representative of estimates for Bangladesh, the model found that if the vaccine cost is approximately 1 USD and the expected incidence is 1 per 1000 persons or greater, than the introduction of vaccination should be considered (17). The cut off of an incidence of 1/1000 is applied to the assessment in the cholera RAT analysis to identify areas of increased or high risk where authorities or public health specialist may want to focus attention as cholera intervention strategies are considered.

With the current level of availability of OCV, a regional population (e.g. Province) is likely too large to fully vaccinate, a further breakdown of the risk at smaller administrative levels may be warranted. If there is sufficient supply of OCV available and the MoH determines that vaccination is warranted at the regional level based on number, the cholera RAT investigation may stop at step 4; otherwise the investigation may proceed to step 5.

Step 5a Calculation *of sub-regional incidence rates of total cholera cases.* Step 5a involves assessment of the number of cholera cases at the sub-regional level where data is available for review. The procedure in this case is similar that that undertaken at the regional/provincial level in steps 1-4. Data at a sub-regional/district level is gathered by working with the respective district level Ministries of Health facilities to collate the aggregated case data by sub-region or district. The data source for sub-regional data might or might not be the same as for regional data.

Step 5b is the alternative and/or additional option to assess sub-regional risk of cholera if Step 5a was not possible due to lack of available cholera surveillance data or if the data is not sufficient to identify the desired target population. Risk is assessed by a cumulative score for the sub-region according to the presence of several key risk factors for cholera. The weighting system is based on the Delphi consensus method. The Delphi is based on a multi-stage iterative process in which a panel of experts anonymously provides responses to a structured questionnaire. The process is repeated for 2 or more rounds, after each round, the statistical analysis of the group's collective opinion is used to form the subsequent round of questionnaire. This process is repeated in order to achieve a consensus among the experts (21). A panel of 20 experts was asked to assign weights to cholera risk factors for use in the cholera RAT. 2 rounds of the survey were conducted with this panel of

experts, highlighting risk factors associated with high risk, medium risk and low risk of cholera. The risk factors are detailed in Table 4.2. The list includes various factors of importance when considering impending cholera risk. The risk factors are listed according to importance for consideration of cholera risk. The key risk factors to consider in an endemic setting are risk factors 1 through 9; these risk factors were rated the most important among our panel of experts in the Delphi survey with minimal discord in their scoring. The remaining risk factors of low to medium ranked importance should be considered if the data is readily available. The risk score assigned to each risk factor is based directly on the Delphi results.

The first factor to consider is the incidence rate calculated in steps 1 through 5. Disease burden is the most direct measure of risk that can be applied. Evidence of this risk is demonstrated in the disease burden of endemic countries such as Bangladesh and India with annual cholera outbreaks, as well as endemic African countries such as Zimbabwe and Mozambique, which have demonstrated, repeated outbreaks since cholera first appeared in Africa (6, 22). The highest risk is in areas with rates of disease greater than 2 per 1,000 persons, heightened risk where vaccination should be considered include areas with incidence rate of 1-2 per 1,000 persons. The risk weights assigned are based on whether the district has reached the previously described incidence rate threshold of 1 per 1000.

Risk Factor 2 is the occurrence of a cholera outbreak within the previous 5 years to include the number of outbreaks during this time. Populations with a history of cholera outbreak, particularly more than one outbreak, is a strong predictor of another outbreak (23). The data to determine previous cholera outbreaks is collected through the previous

steps in the RAT, including collation of MOH reports, WHO reports, line lists and IDSR reports, if available. Previous presence of a cholera outbreak is the strongest risk factor considered, therefore it is awarded the highest weight. The greatest risk would be in an area which is considered endemic, as previously stated, defined as having more than three outbreaks in the previous five years (18), Therefore the weighting assigned is tiered: a score of 0 for no previous outbreaks, a score of 2 for 1-2 outbreaks in the previous 5 years, and a score of 4 for 3 or more outbreaks in the previous 5 years (an endemic area).

Risk Factor 3 is the consideration of outbreaks in neighboring areas. Cholera has historically spread from country-to-country through travelers; the first six pandemics spread globally from the Indian subcontinent. In August of 1970, the seventh pandemic spread to West Africa in Guinea. Figure 4.2 shows the route of spread of the disease upon entry. The disease spread country-to-country from Guinea to Sierra Leone, Liberia, Cote d'Ivoire, Ghana, Togo, Benin, Nigeria and Cameroon successively between September 1970 and February 1971 (24). The same transmission patterns continue to be seen today, therefore neighboring countries as well as regions within a country must be considered when assessing cholera risk. The risk weight is assigned considering areas surrounding a district or sub-district. If there has been no cholera in the current year in the surrounding area then a risk score of zero is applied; if there has been cholera confirmed in neighboring areas in the past year that do not share a direct border the risk score is; and if there has been cholera confirmed in neighboring areas which share a common border then the risk score is 7.

Risk factor 4 is the distance to a health facility. A 2005 study in Vietnam demonstrated that persons living closer to a health facility reported more frequent medical events than

those living further away (25). Distance to health facilities likely plays a role in health care utilization, in both mild cholera cases and most especially in severe cholera cases, when patients may not have adequate time or transportation to reach care. The best data for use in the RAT assessment would be measured by surveying how far the population must travel to reach a health facility. In the event that such a comprehensive survey does not exist, or if GIS measurements of households and facilities are not available, a proxy for this measure is to assess the health facility density per district. This World Health Organization (WHO) health infrastructure indicator can be considered a risk factor (26). The WHO states the target for this indicator is to have 2 health facilities per 10,000 persons. A risk score of 2 is assigned to districts/sub-regions with less than 2 health facilities per 10,000 persons. A risk score of 1 is assigned to districts/sub-regions with 2 or more facilities per 10,000 persons.

Risk Factors 5 and 6 are access to safe water source and access to improved sanitation, respectively. Inadequate access to clean water and proper sanitation are also considered as potential indicators of cholera risk. Several studies have shown that access to improved water can significantly reduce the number of cholera cases (23, 27). Esrey et al showed that sanitation and hygiene reduced the risk of diarrhea morbidity and mortality, with diarrhea mortality reduced to the greatest extent by flush toilets than latrines (28). A 2010 meta-analysis evaluating the effect of water, sanitation and hygiene for the prevention of diarrhea proposed a 17% and 36% diarrhea risk reduction with improved water quality and excreta disposal, respectively (29). The risk can be quantified by accessing data on the percent of the population using protected water sources and improved sanitation. The data is collated at district level and weighted based on division into DHS subgroups of improved

and non-improved water sources and improved and unimproved sanitation (30). The current risk score of 7 is applied if 60% or more of the district's population uses water sources characterized as unimproved, if 30%-60% of the population use unimproved water sources the risk score is 4, and if less than 30% use unimproved water sources the risk score is 1. The weighting similarly applies for to the percent of the population using unimproved sanitation in the district.

The case-fatality rate is the 7th risk factor considered of high importance. Case fatality rates should be less than 1% when cholera is treated promptly; however the high mortality rates registered throughout Africa in the last decade highlights a lack of appropriate access to health care (20, 31). The case fatality rate is adjusted as described in step 3a/3b, and the average case fatality rate for the time period of 1999-2010 in Kenya is weighted based on the Delphi assigned weight of 9. If the CFR is <1%,the risk score assigned is 1, if CFR is between 1-2% then the risk score is 4, and if CFR is >=2% the risk score assigned is an 8.

Risk factor 8 is the use of oral rehydration solution (ORS). This risk factor is an important measure of health care access as well as knowledge of treatment of diarrheal disease and dehydration. The use of ORS is likely associated with mortality rates given its pivotal role in reducing CFRs. This was first clearly demonstrated in the early 1970's when a severe cholera epidemic broke out among Bangladeshi refugees in West Bengal, India. When intravenous fluids ran out, the use of ORS was employed by family to treat more than 3,000 patients, reducing the mortality rate from 30% to 3.6% (32). The method was shown to be a simplified, low cost method not only for health-care workers, but family members to treat diarrheal patients (33). The data for this risk factor can be found working with the Ministry of Health, as the percentage of mothers who use ORS to treat children

with diarrhea is a common measure used in DHS surveys. If more than 75% of people are administering ORS for diarrheal disease, the risk score is 0, if only 25-75% of people are administering ORS for diarrheal disease treatment the risk score is 4, and if <25% are administering ORS the risk score is 7.

Risk factor 9 accounts for the presence of vulnerable populations. Cholera is known to be a public health problem among displaced populations due to lack of or insufficient clean water and proper sanitation, among other factors. This risk factor accounts for the presence of refugee, internally displaced persons (IDPs), slums or tribal communities in the sub-region being evaluated. It also allows accountability for recent natural disasters, such as flooding or earthquakes, and for poor access to health care as a result of terrain or transportation issues. These vulnerable populations have been shown repeatedly to have a risk of cholera outbreaks, including IDPs and refugees in Kenya (34) and the on-going epidemic in Haiti (35) . The data for this risk factor can be found through working with the Ministry of Health. The risk score is 1 if one vulnerable group is present; .the risk score is 3 if 2 vulnerable groups are present and the risk score is 6 if 3 or more vulnerable groups are present in the district.

The remaining risk factors were weighted as medium to low level important for cholera risk. These risk factors include population density, proximity to large water bodies, socioeconomic status (SES) disparity or poverty, Under-five mortality rate, water-switching, cultural behaviors and environmental factors. The evidence for risk, risk weight factor and cut-offs for these risk factors are further detailed in table 4.2. If the data for these risk factors are available, they should be incorporated into the RAT for improved ascertainment of risk.

Population density and overcrowding have been identified as risk factors for cholera in many studies (36-38). This risk factor has been linked to the lack of sanitation in overcrowded and poverty stricken areas, or potentially because living in close proximity decreases the distance needed for transmission (37). This data can be found through census data, and the risk weight is assigned based on a weight of 6 incrementally assigned per quintile.

Several studies have found that proximity to estuaries (39, 40) as well as lake areas (41), have increased risk of cholera. The data for the risk due to proximity to large water bodies including lakes or estuaries can be gathered by working with the Ministry of Health for maps of the country including water bodies and district markings. The risk score is Score is 1 if there is less than 1 water body in the district, 3 if 1-2 water bodies and a risk score of 6 if there are more than 2 water bodies.

The risk factor for SES disparities takes into account the fact that cholera is often associated with poverty, where safe water and sanitation are lacking. Socioeconomic disparities are an important component of cholera risk as cholera has been shown to be more prevalent in low-income countries as compared to middle or high income countries (42). The risk associated with poverty and socioeconomic status (SES) may be measured in different ways depending on the data available in each country. The percent of the population living below a country's poverty line can be extracted from DHS surveys or census information. If less than 30% of people in the district are measured to live in poverty, the risk score is 0, poverty levels between 30-60% have a risk score of 1, and $\geq$ 60% has a risk score of 2.

Under-five mortality is the probability per 1,000 that a newborn baby will die before reaching age five, if subject to current age specific mortality rates. Under-five mortality rates are reported regionally/provincially for DHS surveys. In the event that a country has sub-regional data for under-five mortality rates, these should be applied. Otherwise, under-five mortality rates for the region are applied to each district within the region, with the understanding that under-five mortality is the result of a wide variety of inputs such as access to health care, poverty, water and sanitation, among other variables (43) and will provide a more comprehensive understanding of each district in conjunction with district level indicators for these other variables. A risk score of 1 applies to a district with a U5M of < 50 per 1,000; a risk score of 3 applies to a U5M of 50-100 per 1,000, and a risk score of 5 applies to a U5M of >100.

Health care infrastructure, accessibility and supplies are considered in the risk factor for local health facilities ability to set up a Cholera Treatment Center (CTC). This risk factor considers the foundation of knowledge and preparedness at a district level to support a cholera outbreak, including the ability to set up cholera treatment center and oral rehydration units as separate wards, the presence of supplies to treat cholera (ORS, ringer lactate and IV lines), and the presence of healthcare staff trained to support cholera patients. The weighting is based on incremental levels of knowledge and preparedness; Local health facility trained as CTC is a risk score of 0; if the local health facility has been given basic guidance but not regularly practiced with a plan to implement is a risk score of 3, and if the local health facility has been given no training the risk score is 5.

The risk factor accounting for a district being a high transit area is important due to the increased disease burden due to more dense population but also potential for the increased

circulation and transmission of cholera in the surrounding urban area (44, 45). This risk factor weighs whether a district has a large urban area, whether transport is readily available, including good roads, train or airplane, and whether there is a busy marketplace in the district or area. The risk score is weighted based on the incremental amounts of transit in the district; if 1 transit area is present the risk score is 1; if 2 transit areas are present the risk score is 3; and if 3 or more transit areas are present in the district the risk score is 5.

A rarely considered cholera risk factor that is rarely considered is a measure of "water-switching". Water Switching is based on the risk associated with an unstable water supply. In areas of conflict, natural disaster, poverty where there are not a regular water supply (among others) people may be forced to change water supplies to unsafe sources. Insufficient infrastructures for safe water is recognized as a major factor that contributes to cholera outbreak (46), and water switching is one way to measure this risk. Most countries will not have surveyed for this information, and in that even rainfall measures can be used as a proxy. The data can be collected from the national meteorology department. The scoring of this risk factor will depend on if there is a water switching survey available or if rainfall data is used. Rainfall data is dynamic as the survey data will be static. In the event that a survey has been conducted to assess water switching, risk score is based on level of risk to include how many times they had to switch, why the switch occurred and if the switch was to a more at-risk water source. In the event that water switching data is not available, rainfall can serve as a proxy (risk factor 18) with the idea that a significantly different (over or under-abundance of rain) would lead to increased cholera risk and conditions (46) that people might need to switch water sources.

Cultural behaviors such as hygienic behaviors, funeral practices, large social gatherings and literacy, are grouped into one risk factor. This blended risk factor is to allow for adjustment for these practices in a community that have been linked to increased cholera risk (47, 48). This data will be found at the district or sub-district level and may be more difficult to gather for a rapid assessment. The weight risk assigned is a total of 4, based on the number of these "at-risk" behaviors practiced regularly in the community at the district or sub-district level.

Studies have continued to demonstrate the relationship between elevated temperature and increased cholera risk (49, 50), given appropriate transmission conditions. The data for the risk associated with temperature can be obtained by working with the countries meteorological department. The risk score is based on average temperature in the previous two months; if the temperature is higher than the average the risk increase. We weight the risk based on statistically significant difference in temperature: the risk score is 0 if the p value is greater than 0.1, the risk score is 1 if the p-value for the increase in temperature is between 0.05-0.1, and the risk score is 2 if the p-value of the increase in temperature is less than 0.05.

In addition, if a country does not have data for specific risk factors in the RAT, this does not have to impede the analysis. The analysis can be done based on the risk factor data available. The total risk per district will be based on the risk points tallied per the total risk point potential for the considered risk factors. Districts or sub-regions are stratified into low, medium and high risk based on their risk score. Low risk areas are areas where no vaccination is warranted; assigned to areas with incidence rates less than less than 0.5 per 1000 or districts with risk scores in the lowest quintile. Medium cholera risk areas are

areas with incidence rates between 0.5 -1 per 1000 and/or risk scores between the 2nd and 4th quintile. In these areas the cholera risk is below the threshold for vaccination, however due to the presence of cholera previously or reasonable risk factors cholera interventions are recommended to include enhanced cholera surveillance, WASH interventions and cholera prevention education. High-risk areas are areas where the cholera incidence rate is greater than 1 per 1000 and/or the risk score is in the top quintile of risk score. In this highest quintile of risk, where available and feasible, the use of OCV in addition to other cholera interventions should be considered.

4.3.2 Study Site

Kenya has reported regular epidemics of cholera since shortly after the disease first appeared in Africa in 1971 (51). Cholera surveillance in Kenya is monitored regularly as part of the Integrated Disease Surveillance and Response (IDSR) program, which is managed by the Division of Disease Surveillance and Response within the Ministry of Health of Kenya. Weekly reports of reportable diseases, including line-lists of cases and deaths, are reported from district health facilities to the central MOH in Nairobi. The program was adopted to include cholera disease reporting in 2000 (51); Kenya has surveillance data that can be extrapolated for use in assessing the cholera RAT, with assumptions being made in the years just prior to adoption of the IDSR program as well as in the early years of its incorporation. The time frame included in the Kenya analysis is from 1997 through 2010, however, the 1997-1998 data was not available at the district level, only the provincial level. Therefore, we include information from 1997-98 as much as the available data allows and working with key informants to supply data where gaps

were identified. Kenya has modified their districts and county level designations multiple times in the period analyzed; since the majority of the data utilized in the analysis is from the period when there were 69 districts we present the results according to the districts that existed as of 2007 (52).

Step 5b and the subsequent pathway to risk assessment was also evaluated in the Kenya analysis to determine the value of the risk factor approach in targeting cholera risk at district level. To further build the tool we worked with the personnel at DDSR, as well as utilizing Kenya's open source data to extrapolate parameters for relevant cholera associated risk factors at the district level. The risk factor for previous cholera outbreak was tabulated using the line list data provided by DDSR. The information to assess the health facility density, as a proxy for distance to health facility was abstracted from the 2009 Kenya census data on health facilities per district and sub-district (53). Water and sanitation risk factor data was based on the percent of the population using unprotected water sources and unimproved sanitation by district level, derived from the 2009 Kenya census data and published on Kenya's open data site. Poverty/SES disparities risk was also abstracted from the Kenya 2009 census information utilizing poverty rates by district (53). Under five mortality rates and ORS use for Kenya are available in the 2009 DHS Survey, however this data is only available at the provincial level. Broad assumptions were made applying provincial level rates to each district.

4.4 Results

From 1999-2010, cholera was reported during at least one year in all seven provinces in Kenya (Figure 4.3a and b). When adjusting the rate for health utilization, in 1999 two provinces reached the threshold of 1 case per 1000 persons (Coast, Eastern), with

one province reaching the threshold in 2007 (Northeast) and 2009 (Eastern, Figure 4.3a and 4.3b). The total number of cholera cases during the entire period was greatest in the Eastern Province (n=9440), followed by Coastal Province (n=6400) and Rift Valley province (n=6338). The highest adjusted case rate in any province was 2.67 per 1000 persons in 1999. If the threshold were lowered to 0.5 cases per 1000, then five provinces would have met the threshold during the study period, though only Eastern and Northeastern Provinces would have reached this lower threshold in more than one year.

Figure 4.4a-h shows the annual rate of cholera for all districts in Kenya, divided by province. Although only three provinces reached the 1 per 1000 threshold, many more districts reached the threshold in at least one year – namely 24 of the total 69 districts (Table 1). Of these districts, seven districts reached the threshold at least two times. Although the district level data for 1999-2000 was estimated, it is likely that at least one district in Nyanza Province might have reached the threshold three times. The districts that reached the threshold more than once fall into two broad ecologic zones – the arid, sparsely populated regions of Northeastern Kenya (Isiolo, Tharaka, Mandera, Wajir) and the seasonally wet districts bordering Lake Victoria in the western part of the country (Kisumu, Nyando, Suba).

Applying the risk-based approach at the district level (Step 5b) revealed a range of risk scores from 15 in Taita Taveta District in the Coast Province (low-risk) to 54 in Moyale District in the Eastern Province (high risk) (Figure 4.5a). These results do align with the incidence rates, as Taita Taveta did not report a single case during the time period analyzed, classifying as low risk; while Moyale District had an extremely high incidence rate in 1999 (>11/1000). However as noted in the methods section, data was not available at the district

level for all risk factors, therefore the cumulative risk weight was based on the data available. Figure 4.5a illustrates the cumulative weighted cholera risk results by District in Kenya, demonstrating that within a Province, the risk by district varies. The risk weights were also compared by scoring based on the highest value among the districts. This score was weighted set as the highest risk weight, and the remaining scores for the district values were weighted in comparison to this score. These results are shown in Figure 4.5b. The two scoring systems highlighted similar high risk districts; if the top five percent for risk score are considered both scoring methods included Marsabit, Moyale and Turkana districts. There was one difference where the weighting based-on cut off weighted Suba district in the top 95%, weighting by scaling weighted Isiolo in the top 5%. The results of the risk-based approach falls in line with the prediction of cholera thresholds at 1 per 1000 if the highest risk areas are compared to the results in Table 4.1; all districts in the top 5% of risk surpassed the threshold of 1 per 1000 in one or more years. Based on the adjusted incidence rate results, the government of Kenya might want to consider the results in Table 4.1; in which 8 districts were highlighted as having surpassed the threshold of 1 per 1000 in more than one year. With the current vaccine shortages, the government is unlikely to have access to sufficient vaccines to target all of these districts. Considering at the risk-factor based approach, the highest risk districts are further stratified to potentially guide the government towards a more refined consideration for intervention

4.5 Discussion

The Kenya cholera risk assessment highlights that Government and/or Ministry of Health personnel of the country working with the RAT will have to determine how to target

vaccination and other cholera interventions. In Kenya, the provincial populations that surpassed the threshold of 1 per 1000 were between 1.3 million and 6.1 million persons. Current oral cholera vaccine production rates are limited with current production of only 2 million doses per year, for worldwide distribution (54). Recent initiatives to increase production and distribution of oral cholera vaccine will likely increase the availability of oral cholera vaccine, however for the current and near future, assessments with case rates above the threshold of 1 per 1000 with large base populations will have to target vaccination efforts to those at highest risk(12) .

The cholera RAT will continue to be evaluated in other endemic countries such as Uganda and Nepal. The beta assessment of the cholera RAT and its tools utilizing data supplied by the Ministry of Health of Kenya, among other sources, highlights areas where further development is needed. Given Kenya's history of surveillance and the fact that it has maintained historical records, the data from Kenya highlighted multiple districts or sub-regions with a high-risk or above threshold for cholera vaccination consideration. This illustrates that most countries may need to incorporate the use of risk factors for cholera in order to target cholera interventions to areas at greatest risk where cholera vaccination campaigns are financially and politically feasible.

The RAT analysis will be used to inform the MOH's decision to use OCV. In Kenya, several provinces were highlighted to have elevated risk (above the vaccination threshold of 1 case per 1000 persons) during the period of analysis, including the coastal province, the Eastern Province and the North Eastern Province. Continued data analysis revealed areas at high risk of cholera in specific districts in these provinces as well as others that at a provincial level may have been missed, including districts in the Rift Valley, which

at a provincial level did not surpass the threshold of 1 case per 1000 during the time period analyzed. Turkana district in the Rift Valley is an arid area known to be prone to cholera outbreaks (51) which surpassed the 1 case per 1000 during the time period, recording between 1-3 outbreaks and received a risk score of 43 (high risk). This highlights the importance of working down, from a national level to sub-region, and beyond if needed.

Advantages of the RAT include that it is a simple tool that utilizes local data in addition to nationally reported rates while engaging the Ministry of Health. In comparison to setting up a prospective surveillance study to determine the cholera risk and incidence at local levels, the RAT provides an estimate of this information in a very short period of time. Similarly to cholera disease risk and the implementation of oral cholera vaccination, a major hindrance in the development and deployment of a haemophilus influenza type b (Hib) vaccine was the lack of understanding of Hib disease rates in developing countries. A Rapid Assessment tool was developed to estimate the rates of Hib and pneumonia within 7- 10 days using retrospective local data, and has since been used in developing countries in sub-Saharan Africa, the Middle East and Asia (55). The process of gathering the data for the RAT engages local policy makers and the Ministry of Health. By working closely with key health and political personnel, the RAT can provide evidence necessary for policy makers to implement the use of OCV as an intervention for cholera outbreaks.

The RAT has several limitations. The successful application of the RAT depends on having some surveillance in place during recent history. If a country's surveillance is poor, then the RAT results will also be of poor quality. Cholera surveillance programs such as those being implemented by Africhol will continue to strengthen the RATs applicability. The RAT cannot predict when or where cholera will occur. In the future the RAT will

hopefully work in conjunction with prediction models currently being developed. The vaccination threshold of 1 cholera case per 1000 persons is an estimate based on cost-effectiveness of the vaccine. If the demand for the vaccine increases, increasing production and reducing the cost of the vaccine, this threshold could change. The current adjustment for missed cases and deaths is based on limited evidence, more health utilization surveys in cholera outbreak settings are needed to improve understanding of health care seeking in different settings.

In conclusion, the results of the cholera RAT must be considered based on the setting in which the assessment was conducted. The RAT is built to allow for assessment even when data availability is limited; however, the quality of the data available will influence the strength of the results. The cholera RAT will be strengthened as it is refined through continued use and application in cholera endemic countries. Until oral cholera vaccine becomes more widely available, understanding the disease burden in each country setting will continue to be an important aspect of cholera vaccination and intervention programs.

Figure 4.1: An Overview of the Methodology for cholera RAT development

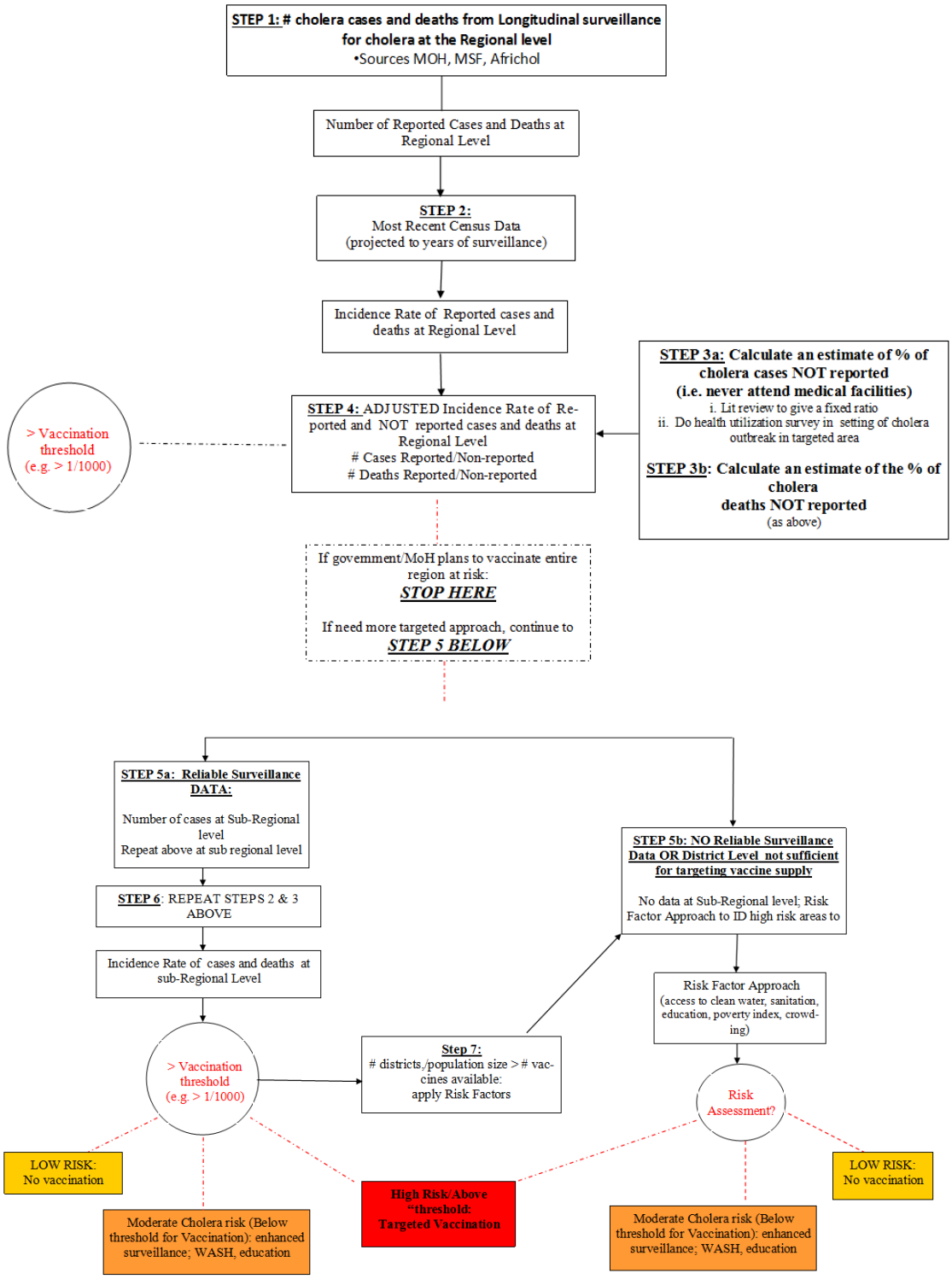

Figure 4.2: Entry of cholera in Africa: Routes of transmission (24)

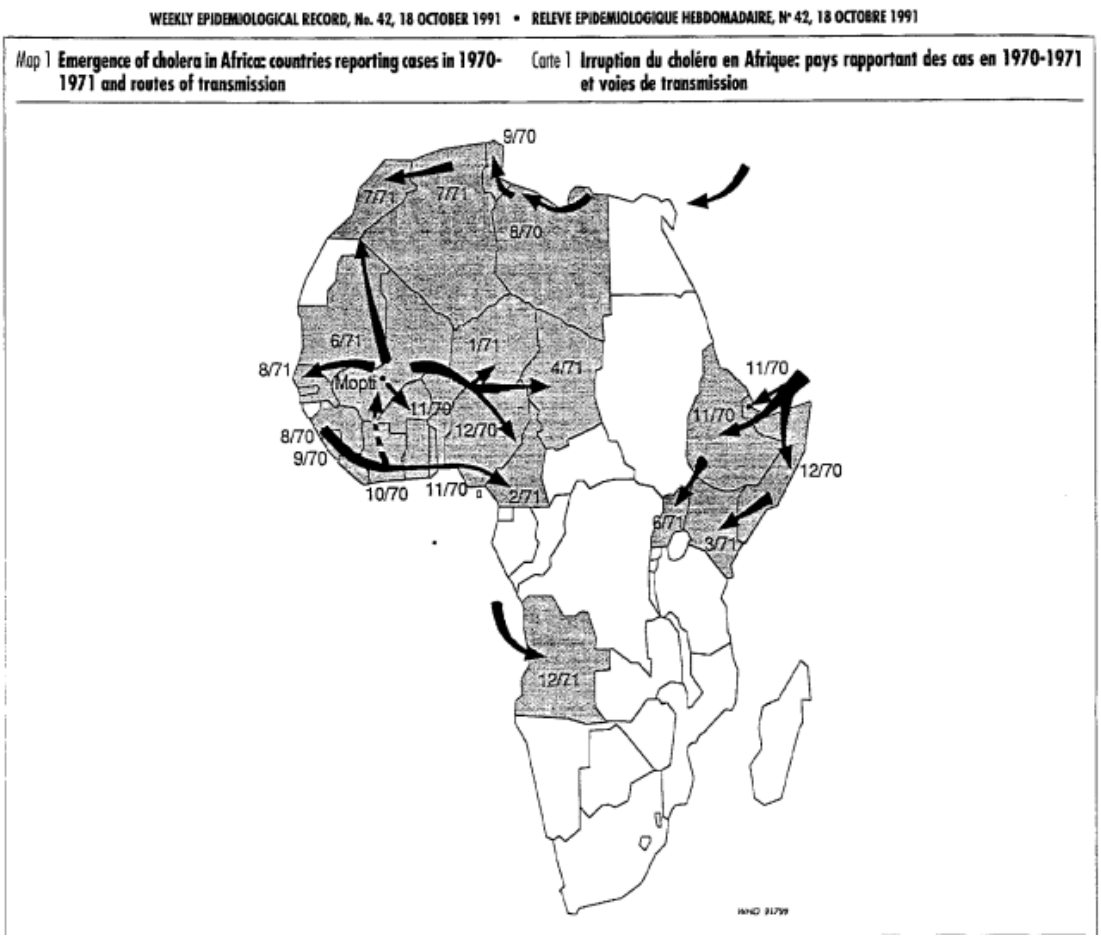

Figure 4.3a: Adjusted Cholera Case Rates by Province

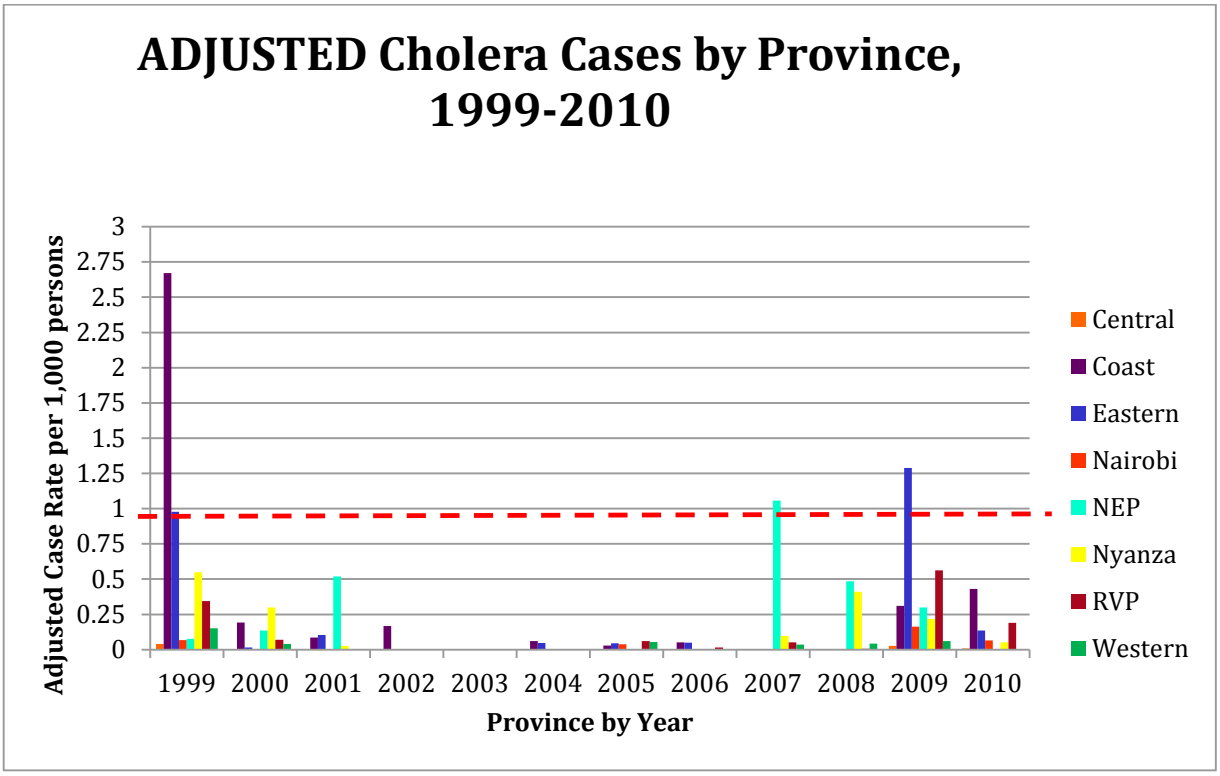

Figure 4.3b: Adjusted Cholera Case Rates grouped by Province, 1999-2010

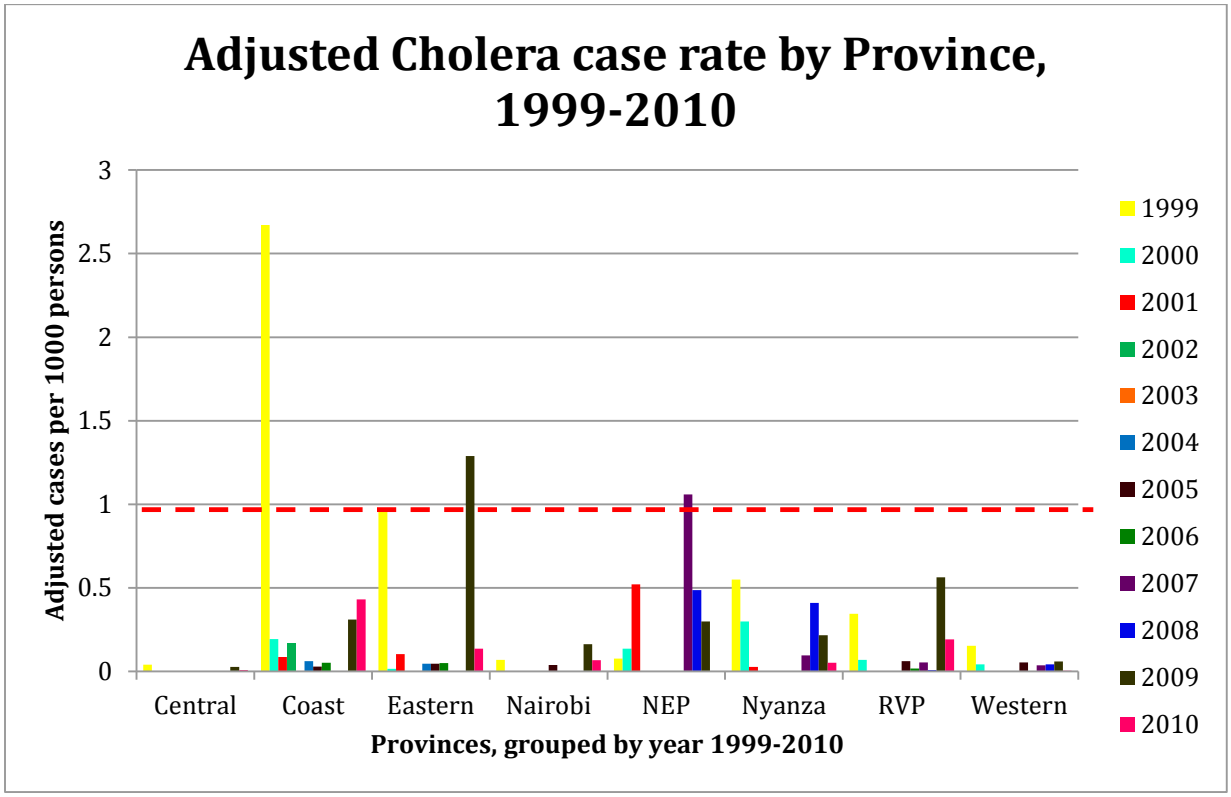

Figure 4.4a: Central Province Adjusted Case Rate

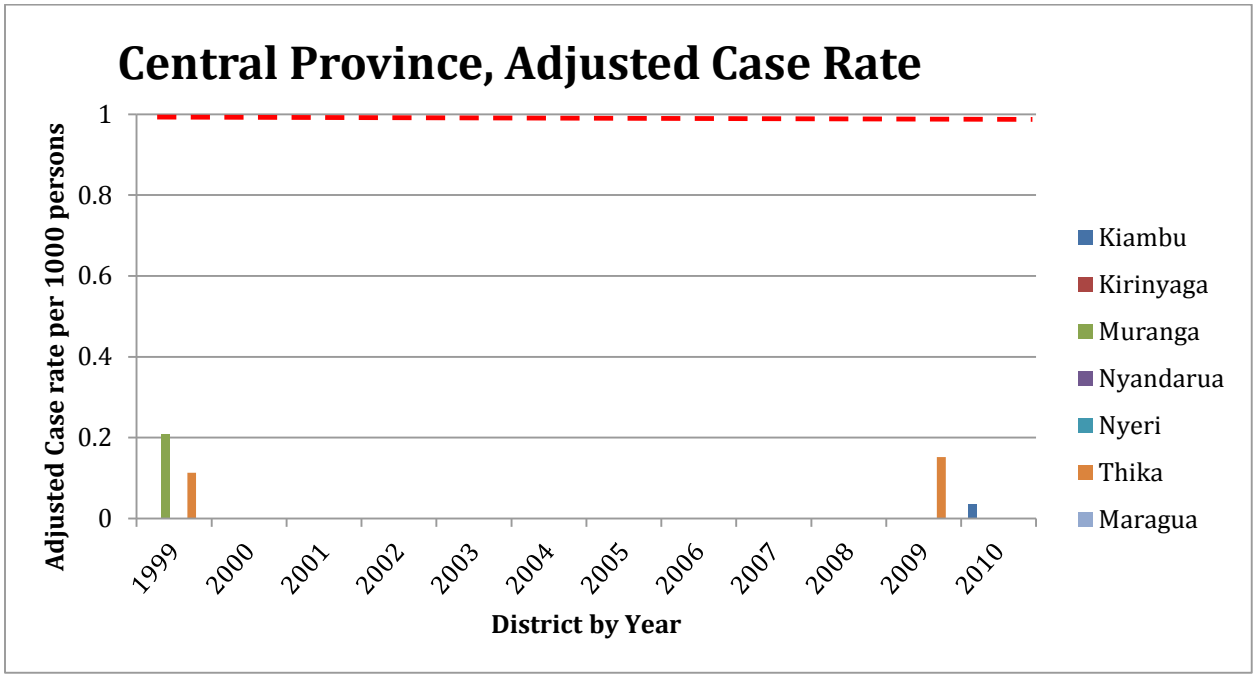

Figure 4.4b: Coast Province Adjusted Case Rate

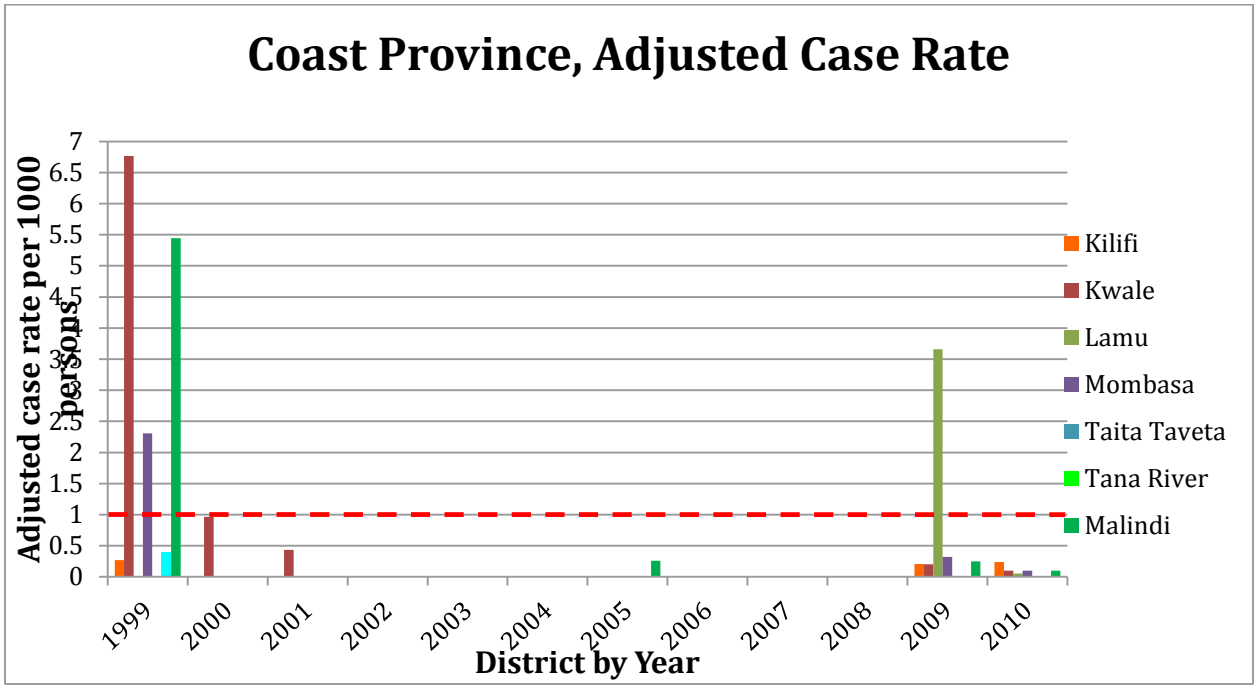

Figure 4.4c: Eastern Province Adjusted Case Rate

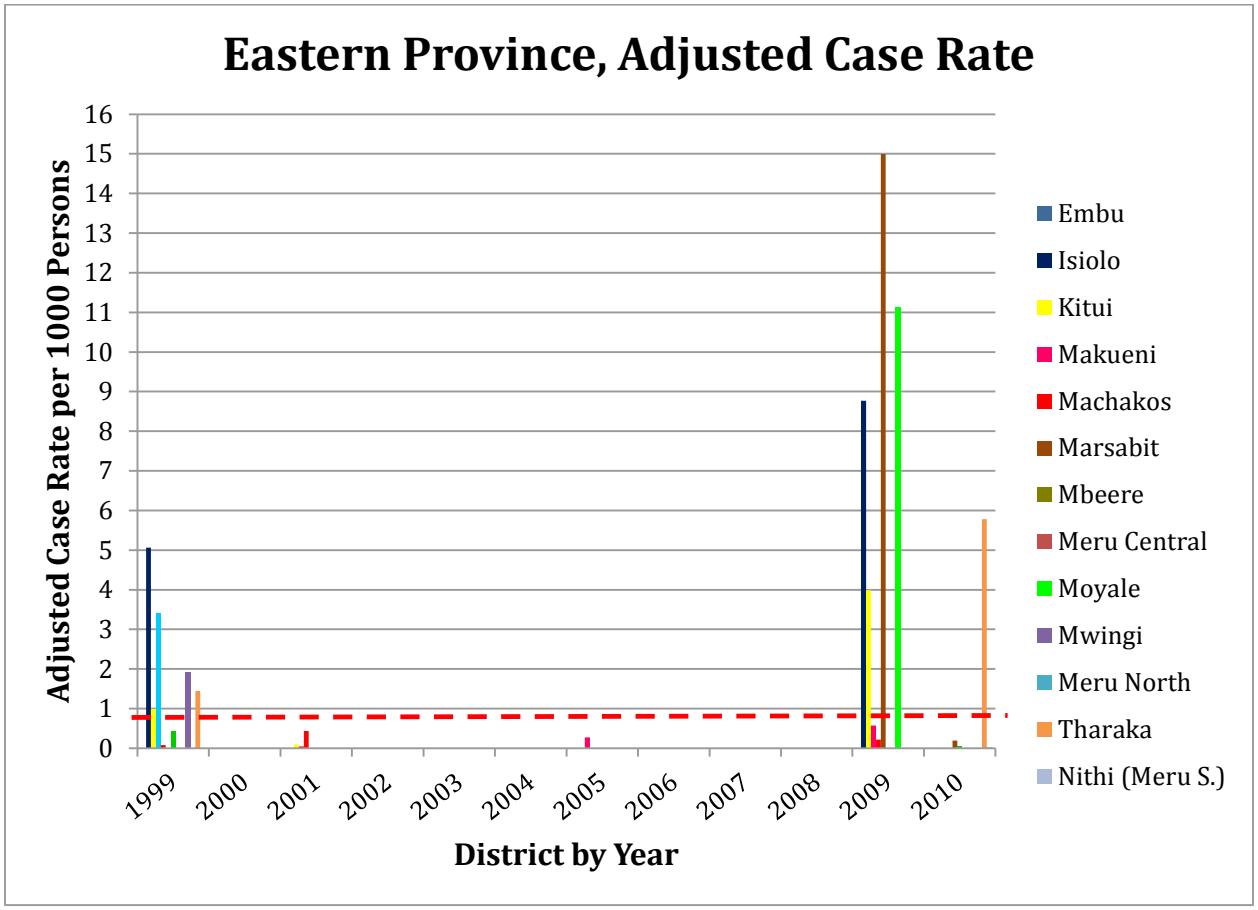

Figure 4.4d: Nairobi Province Adjusted Case Rate

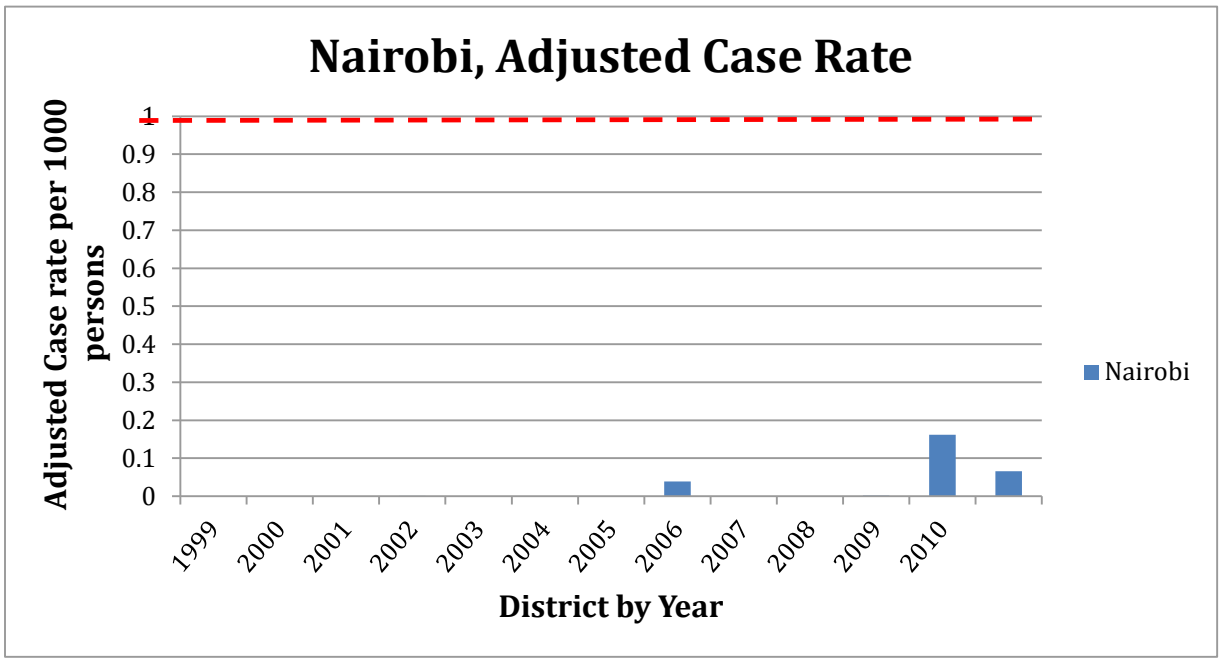

Figure 4.4e: North Eastern Province (NEP) Adjusted Case Rate

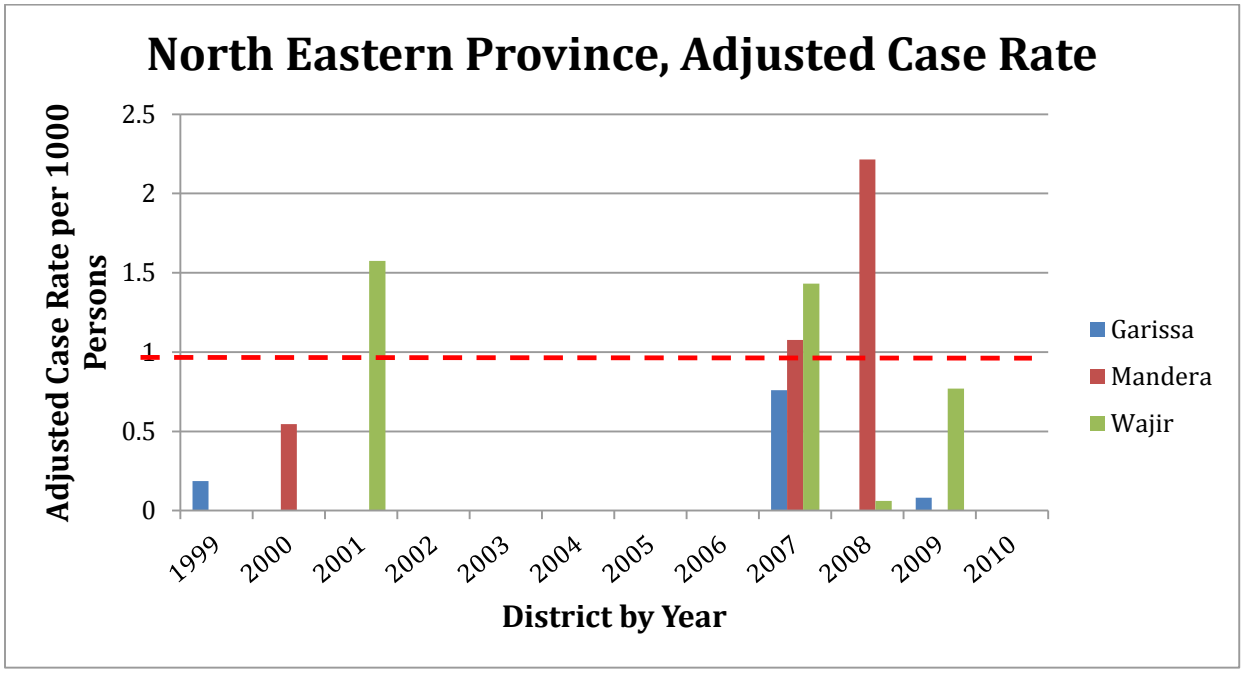

Figure 4.4f: Nyanza Province Adjusted Case Rate

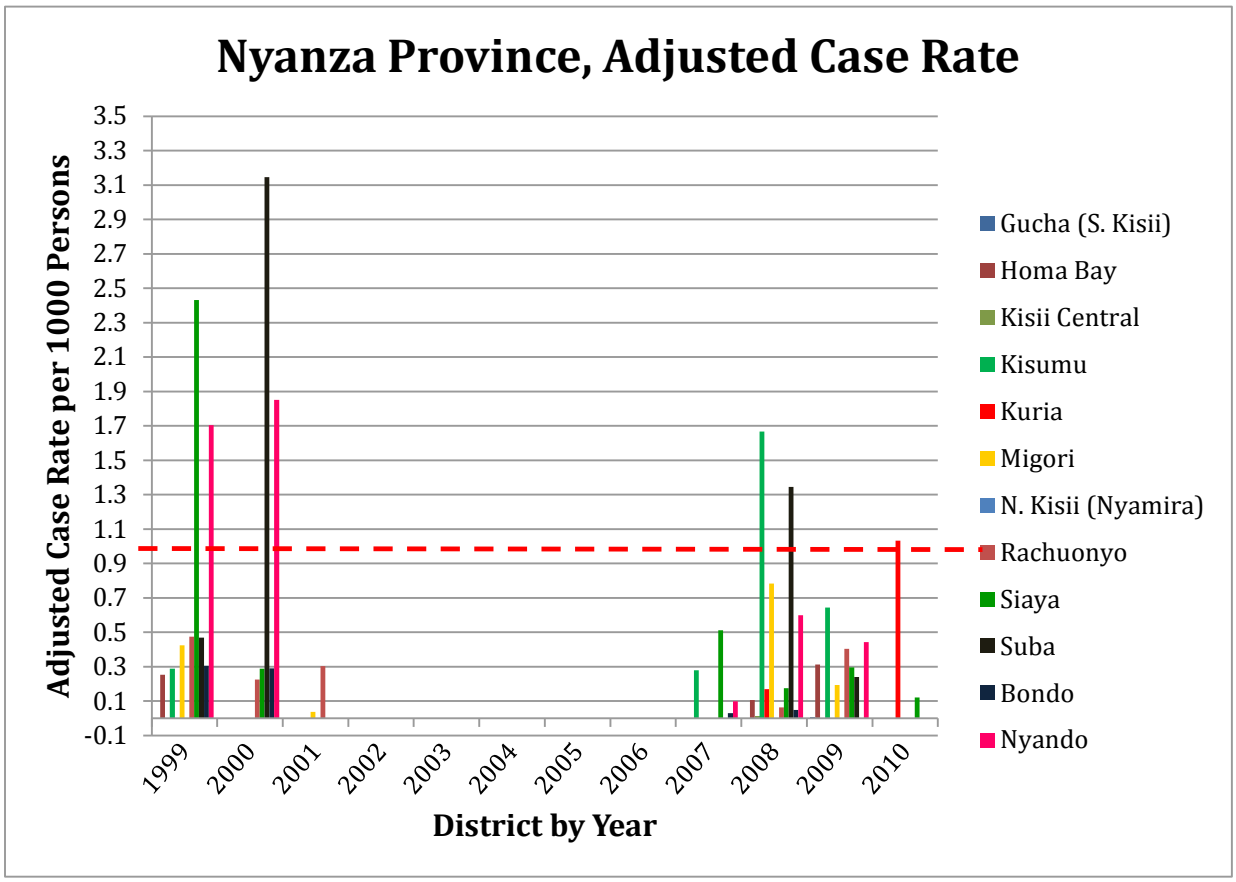

Figure 4.4g: Western Province Adjusted Case Rate

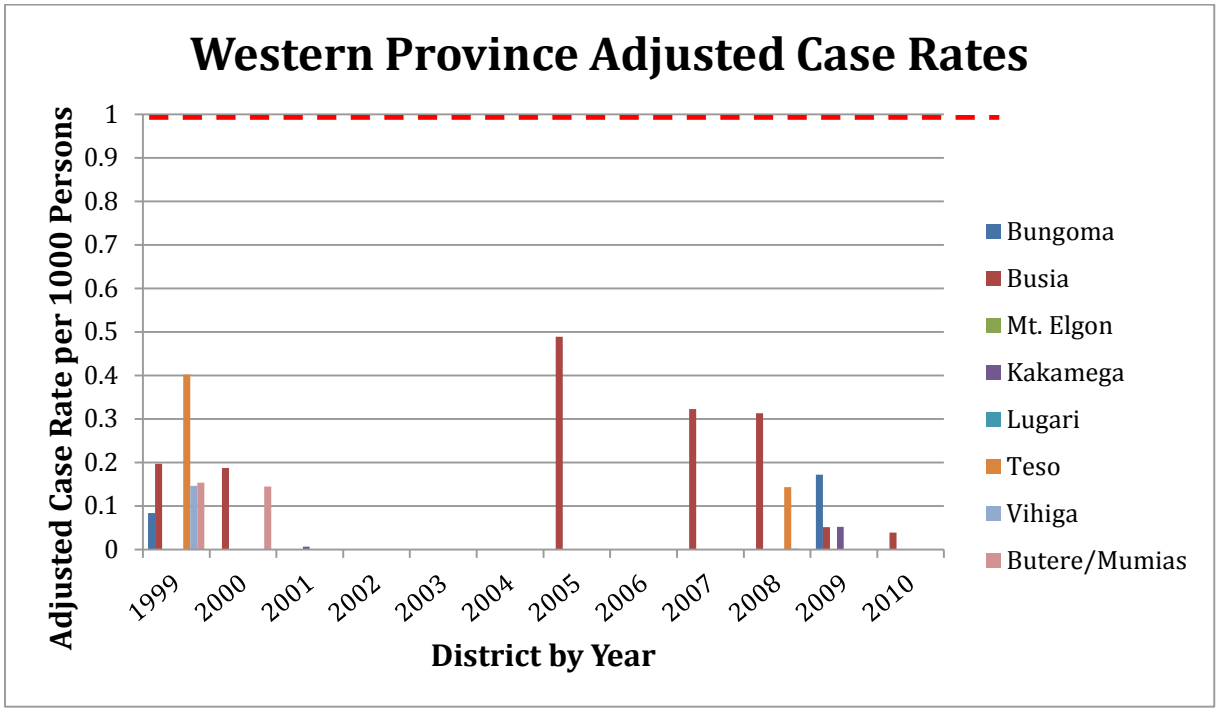

Figure 4.4h: Rift Valley Province (RVP) Adjusted Case Rate

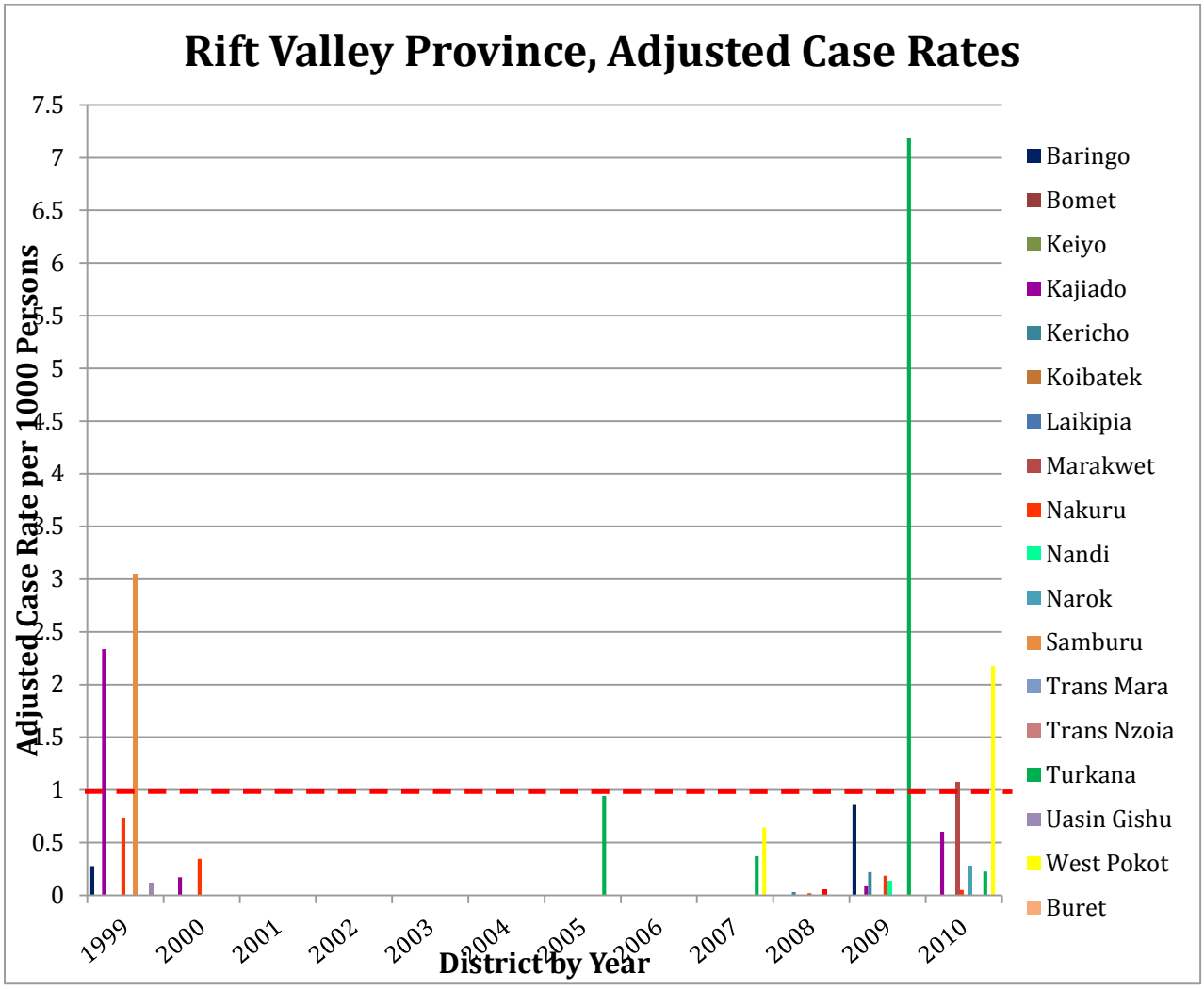

Figure 4.5a: Weighted Cholera Risk by District: Weight according to Cut-off Values

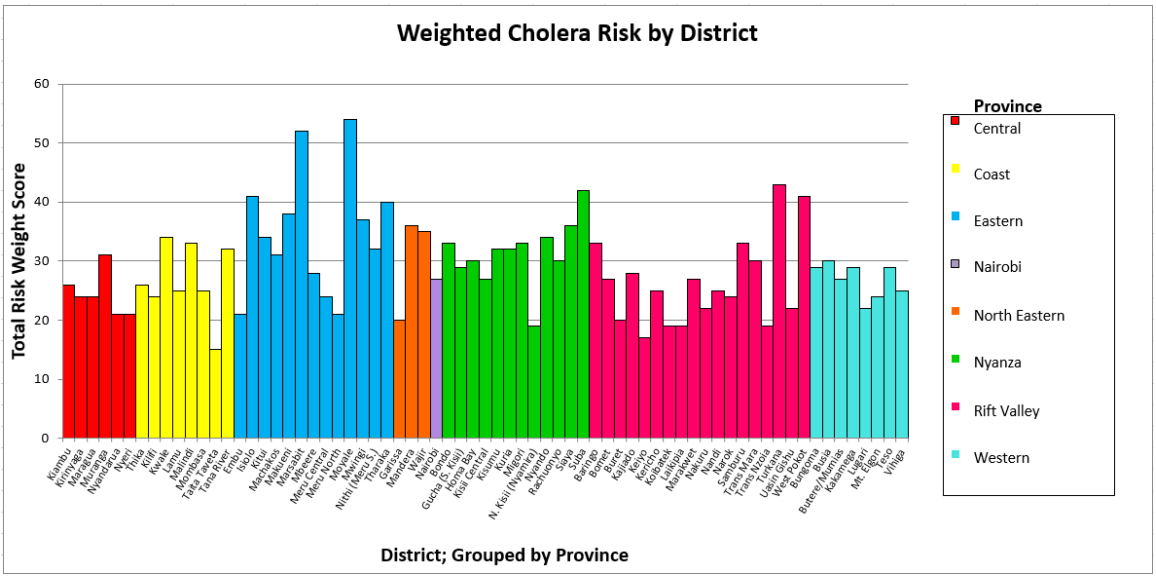

Figure 4.5b: Weighted Cholera Risk by District: Weighted by Scaling

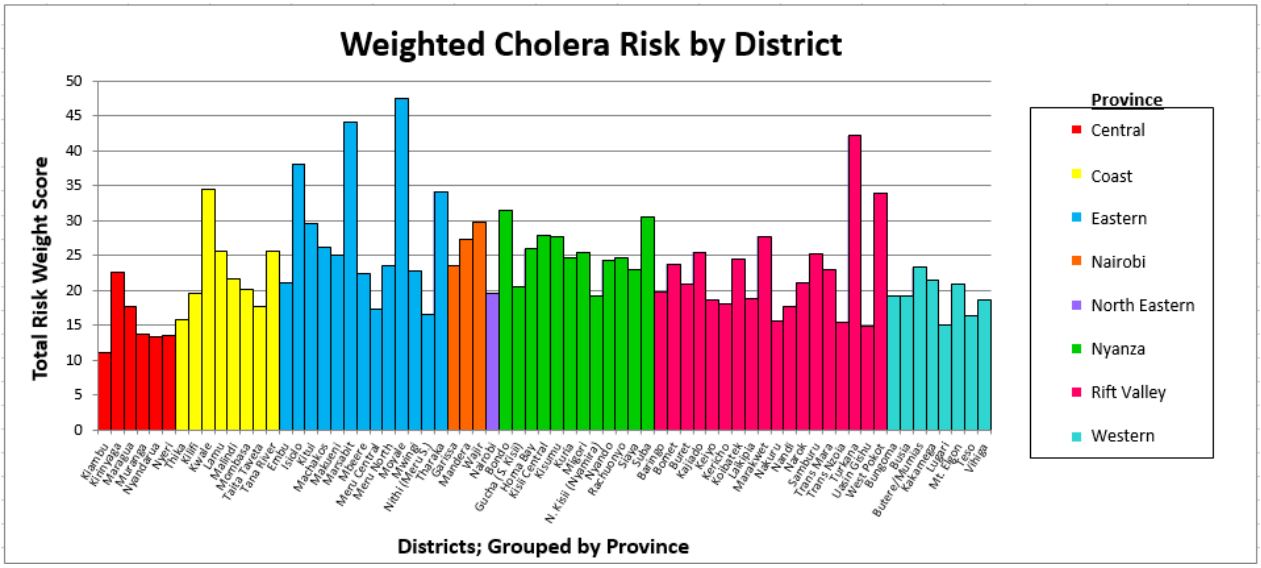

Table 4.1: Number of Districts above threshold 1/1000 from 1997-2010

# of Districts above Threshold (1/1000) from 1997-2010			
Province	**>1 Year**	**1 Year**	**0 Years**
Central			All districts
Coast		Kwale; Lamu; Malindi; Mombasa	Kilifi; Taita Taveta, Tana River
Eastern	Isiolo-2; Tharaka-2	Kitui; Makueni; Marsabit; Moyale; Mwingi	Embu; Machakos; Mbeere; Meru Central; Meru North; Nithi (Meru S.)
North Eastern	Mandera-2; Wajir-2		Garissa
Nairobi			Nairobi
Nyanza	Kisumu-3*ǂ; Nyando-2; Suba-2	Migori*;Kuria; Siaya	Bondo; Gucha (S. Kisii); Homa Bay; Kisii Central; N. Kisii (Nyamira); Rchuonyo
Rift Valley		Kajiado; Marakwet; Samburu; Turkana; West Pokot	Baringo; Bomet; Keiyo; Kericho; Koibatek; Laikipia; Nakuru; Nandi; Narok; Trans Mara; Trans Nzoia; Uasin Gishu; Buret
Western			All districts

*Actual Distribution of cases in Kisumu & Migori is unclear for 1998. Assumptions made that 50% occur in each distribution. ǂ The breakdown of the 1997 outbreak is unclear, however it was noted to have occurred predominately in Kisumu area of Nyanza so this is the only district allotted an outbreak for this year.

Table 4.2 Risk Factors Weighting Approach

#	District-Based Risk Factor	Cut-Off Value	Total Risk Weight	Comments/Evidence for Risk	Reference
1	Incidence Rate	No cholera = 0; <1 per 1000=1; 1-2/1000=**5**; >2/1000=**9**	9	Endemic countries have demonstrated trends in repeated outbreaks	(6, 22)
2	previous cholera outbreak	0 outbreaks=0; 1-3 outbreaks per 5 years = **4;** >3 outbreaks per 5years = **8**	8	Populations with a history of cholera outbreak, particularly more than one outbreak, is a strong predictor of another outbreak[1]	(23)
3	Recent outbreaks in neighboring areas	Cholera not reported in surrounding areas=0, cholera reported in neighboring areas but those sharing a direct border = 3, cholera reported in bordering areas/towns=7	7	Historical and current outbreaks continue to show spread to neighboring areas	(24)
4	Distance (hours) to the closest health facility	<1 hour=0; 1-4 hours= **4**; >5 hours = **8**	8	Distance to health facilities likely plays a role in health care utilization, in both mild cholera cases and most especially in severe cholera cases, when patients may not have adequate time or transportation to reach care.	(25, 52)
4a	Proximity to health facility (proxy: Health Facility Density)	Weight (1 if >100; **3** if 50-99; **5** if <50)	5	The distance from the health care provider may have an effect on health care utilization. As a proxy for distance, we assess the number of health facilities per district. Per the WHO Health Facility Density Calculation	(25, 26, 52)
5	Water Source	Score is 1 if <2; Score is **4** if ≥ 2 but < 3; Score is **7** if ≥3	7	The percentage of the population that uses water sources characterized as unimproved versus improved	(23, 29, 56)
6	Sanitation	Score is 1 if <2; Score is **4** if ≥ 2 but < 3; Score is **8** if ≥3	8	The percentage of the population that uses sanitation sources characterized as unimproved versus improved sanitation	(28, 29)
7	CFR	if CFR is <1%=1, if CFR is 1-2%=**4**, if CFR is >=2%=**8**	8	Case fatality rates should be less than 1% when cholera is treated promptly; however the high mortality rates registered throughout Africa in the last decade highlights a lack of appropriate access to health care.	(20, 31)
8	ORS use	If >75%=1;, Rate <75% but >25%= **4**; if Rate<25%=7	7	This risk factor is an important measure of health care access as well as knowledge of treatment of diarrheal disease and dehydration.	(32, 33)
9	Presence of vulnerable populations	If 1 group present=1;, If 2 groups present=**3**; if 3 or > present=**6**	6	1) presence of migrant population, internally displaced population, slums, or fishing communities 2) security and safety concerns, 3) frequented by calamities/disasters, 4) poor access to health services due to terrain/transportation issues	(34, 35)
9a	Presence of migrant pop, IDPs, slums, or tribal communities		6		

#	District-Based Risk Factor	Cut-Off Value	Total Risk Weight	Comments/Evidence for Risk	Reference
9b	Security and safety concerns		7		
9c	Frequented by calamities/disaster		6		
9d	Poor access to health services due to terrain/transport issues		7		
10	Population Density	Based on quintile for population Density. Quintile 1(lowest density)=1; quintile 2 & 3=2; quintile 5=4; Quintile 6=6	6	This risk factor has been linked to the lack of sanitation in overcrowded and poverty stricken areas, or potentially because living in close proximity decreases the distance needed for transmission	(36-38)
11	Proximity to large water bodies	Score is 1 if <1; Score is **3** if ≥ 1 but < 2; Score is **6** if ≥ 2	6	Several studies have found that proximity to estuaries as well as lake areas have increased risk of cholera.	(39-41)
11a	Are there lakes or estuaries in the district/area?				
11b	How many lakes or estuaries in the district/area?				
11c	What is the % of the population that use the water body as their primary water source?				
12	SES Disparity/Poverty	If Rate<30%=1;, Rate >30 but <60%=**,3**; if Rate>=60%=**5**	5	Socioeconomic disparities are an important component of cholera risk as cholera has been shown to be more prevalent in low-income countries as compared to middle or high income countries	(42)
13	U5M	Risk Factor WT (<50=1; 50-100=**3**; >100=**5**)	5	Previous studies have shown a relationship between high infant mortality rates and cholera. Both cholera and high infant mortality rates are associated with poor water and sanitation and poverty. The risk factor cut offs are based on	(43)
14	Local health facility trained as a cholera treatment center	Local health facility trained as CTC = 0; Local health facility given basic guidance but no clear plan = 3, no training = 5	5	This risk factor accounts for the presence of a health infrastructure, human resources, accessibility, and supplies	(57)
14a	Ability to set up cholera treatment center and oral rehydration units as separate wards				
14b	Presence of supplies to treat cholera (ORS, ringer lactate and IV lines)				
14c	Presence of healthcare staff trained to support cholera patients				

#	District-Based Risk Factor	Cut-Off Value	Total Risk Weight	Comments/Evidence for Risk	Reference
15	Transit areas	if 1 transit area risk present = 1; if 2 risks present = 3; if you have 3 or more transit area risks present= 5	5	Studies have assessed the relationship among high transit areas and increased risk of cholera due to more dense population but also potential for the increased circulation and transmission of cholera in the surrounding urban area	(44, 45)
	Is the district area an urban area?				
	Is transport readily available, including good roads, train or airplane?				
	Are there busy marketplaces in the district/area?				
16	Water Switching (rainfall?)	Score is 1 if <2; Score is **2** if ≥ 2 but < 3; Score is **4** if ≥3	4	Insufficient infrastructures for safe water is recognized as a major factor that contributes to cholera outbreak, and water switching is one way to measure this risk	(46)
	How often is the water source changed				
	Why does the switch occur				
	Was the switch to a more at-risk water source				
17	Cultural behaviors	if 1 SES or cultural behaviors present = 1; if 2 behaviors present = 2; if you have 3 or more behaviors = 4	4	Cultural behaviors such as hygienic behaviors, funeral practices, large social gatherings and literacy have been linked to increased cholera risk	(47)
	Hygiene practice (e.g. % of people reporting hand washing before eating)				
	Funeral practices that involve risk of cholera infection (ego. Washing of bodies),				
	Seasonal social and religious gatherings.				
18	Rainfall (Flooding/drought)	Risk Score based on p value: score is 0 if >0.1; risk score 1 if 0.05-0.1; Risk Score 2 if <0.05	3	Studies have shown that an over or under-abundance of rain (flooding or drought) can lead to increased cholera risk and conditions	(46, 48)
19	Temperature	Risk Score based on average temp in previous months: score is 0 if >0.1; risk score 1 if 0.05-0.1; Risk Score 2 if <0.05	2	Studies have continued to demonstrate the relationship between elevated temperature and increased cholera risk, given appropriate transmission conditions	(49, 50)
TOTAL RISK POINTS			144		

4.6 References

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Chapter Five: Conclusions and Recommendations

5.1. Summary of Major Findings

The findings of this dissertation highlight several new epidemiological and laboratory diagnostic tools for cholera surveillance and intervention in low resource settings, as well as identifies potential strategies for intervention implementation in endemic areas. There is limited understanding of the true disease burden of cholera in many areas globally due to the difficulty and cost associated with confirming suspected cases, and even more, the complexities of maintaining regular disease surveillance. The lack of knowledge surrounding disease burden has resulted in limited vaccine production in the past, and currently hinders the strategic targeting of the available vaccines and other interventions in both reactive and endemic settings. These simple, affordable, and sustainable tools allow for more accurate disease burden estimates that have widespread application for furthering understanding of cholera transmission dynamics and guiding policy

5.1.1. Paper I

There have been minimal attempts to characterize the burden of cholera the Far North of Cameroon in spite of recurring outbreaks since the first appearance of cholera in Cameroon in the 1970's. Paper 1 demonstrated the successful implementation of a modified sentinel surveillance methodology in a remote area suffering from security issues throughout the entire study period. In addition to demonstrating early identification and confirmation of cases, the presence of surveillance activities in several districts resulted in early notification of cases from surrounding districts, leading to increased dissemination of

cholera prevention measures, disease confirmation and improved reporting and documentation of disease burden information.

Paper 1 successfully demonstrated the routine use of the modified dipstick protocol in a remote setting with a significantly improved specificity of 99.8%. These results highlight the feasibility of this modified methodology in any setting as well as the applicability of low-resource requiring lab diagnostics to improve disease detection and surveillance. Further, the use of readily available laboratory supplies such as gauze and water bottles to replace current expensive filtration methodologies not only decreases overhead costs but increases feasibility of important environmental surveillance that can provide a better understanding of the disease, seasonality and transmission patterns. Finally, the novel application of filter paper as a preservation method for specimens for confirmation testing by PCR not only reduces laboratory costs by eliminating the need for culture as a confirmation and gold standard test, but allows for more advanced molecular epidemiology and characterization of disease transmission pathways.

5.1.2. Paper II

Paper 2 expands upon the findings related to the use of simplified laboratory diagnostics in vulnerable and remote field setting. This study demonstrates that the simplified preservation technique of dried filter paper spots, for crude environmental or stool samples after Alkaline Peptone Water (APW) enrichment or for the preservation of purified isolates for simplified shipping, enables the simplified storage and transport for successful molecular characterization. The simplified transport of non-infectious material results in real-time analysis; allowing for the rapid dissemination of results to inform policy

decisions at the country level regarding implementation of interventions to prevent further spread.

Paper 2 demonstrates that the strains present in the 2014 outbreaks in South-east Asia are distinct from those in Africa. In addition, the sequencing results revealed a distinct relationship between strains present in the 2014 outbreaks in Cameroon and those isolated from Mozambique in 2008. This finding combined with the fact that the strains in the two outbreaks in distinct areas of the Far North of Cameroon in 2014 are similarly related suggests that continued molecular characterization in these areas and in Africa is needed to clarify the relationship among strains on the continent and disease transmission patterns.

5.1.3. Paper III

Paper 3 presents the first cholera rapid risk assessment tool (RAT) to aid agencies and ministries for assessing the risk of a cholera outbreak in an endemic area. The RAT details the step-by-step process for collating case and death data at the national, regional and district levels to include adjustments for missed cases and deaths. The RAT also provides a tool for assessing risk in the absence of disease burden and death information by applying a weighted risk model to identify areas of high risk in the country. The RAT is a simple tool applying readily available data while engaging the Ministry of Health; in comparison to the costs and time associated with setting up a prospective surveillance study, the RAT provides the information in a very short period of time. The process of gathering the data for the RAT encourages discussion regarding cholera disease burden among decision makers and the Ministry of Health. The RAT will hopefully provide evidence necessary for policy makers to implement the use of OCV as an intervention for cholera

outbreaks. The RAT will continue to be improved through further application, currently being targeted for use in Nepal, Uganda and South Sudan in early 2015.

The RAT evaluation using data from the Republic of Kenya identified several provincial level and district level populations at high risk for cholera but with populations too large for vaccination consideration with the current vaccine availabilities. Therefore, the use of the weighted risk factor algorithm demonstrated further stratification of risk among the districts to provide more refined guidance for the decision makers and Ministry of Health to consider as they evaluate vaccination and intervention strategies. Given the detailed district level cholera surveillance information that was available for evaluation in the RAT in Kenya, it is likely that most countries may need to incorporate the use of risk factors for cholera in order to target cholera interventions to areas at greatest risk where cholera vaccination campaigns are financially and politically feasible.

5.2. Study Limitations

The most important limitation of our investigation was the major security risks at our study site in the Far North of Cameroon. Since shortly after our grant was awarded, the Boko Haram terrorist group has established themselves in the Far North region as well as in the communities and islands in Lake Chad. Our study team has worked as diligently as possible in spite of on-going personal risk of harm. Considering the obstacles, the study has been extremely successful. However, these security and safety issues led to loss of data in some study areas, particularly Darak which is located in Lake Chad. In paper 1 we detail the clinical and environmental surveillance among our study sites for the first 14 months of surveillance. The presence of non-toxigenic *V. cholerae* 01 was identified in

the environmental sampling sites in and around the Darak sentinel site in the early months of 2014. It is possible that the presence of these vibrios may have been an early warning sign for the area's impending outbreak in October 2014; however, the team was unable to maintain regular surveillance in this area due to increased security risks and military intervention in the area. While clinical cholera cases were confirmed in Darak health district in October 2014, there were only 2 additional clinical cases detected in the Blangoua health district for the period considered in paper 1. Little analysis could be performed on the results of these clinical cases as only the 2 in Blangoua were detected during our regular study surveillance activities. Therefore, we did not have the study size anticipated to conduct a case-control study in the area. This limited positive sample size also likely negatively affected the reported dipstick sensitivity. Finally, our laboratory diagnostics are only targeted to identify *V. cholerae*, and therefore, were unable to further characterize the cases of non-cholera diarrhea enrolled in the study to potentially evaluate the risk for adult diarrhea in the area.

The research and results supporting paper 2 were also limited as a result of security concerns. Due to the inability to maintain regular surveillance in our study site, or to support cholera response efforts in the Bourrha Health district and surrounding areas, we were unable to collect samples from all suspected cholera cases. In addition, we were unable to enroll all suspected cases into our study to collect epidemiological information to better characterize cholera risk in these situations. However, the molecular characterization of the specimens collected demonstrated a very close relationship among strains analyzed. Additionally, the relationship between the Cameroon strains and those from Mozambique was limited due to the limited number of strains available for

comparison from Mozambique. We are currently working to collaborate with a team in Mozambique to aide in their cholera disease surveillance and characterization.

The major limitation of the cholera RAT is that it has only been evaluated in one endemic country, the development of the tool will continue as the tool is applied in real-time settings. Currently, Nepal and South Sudan are considering targeted vaccination efforts utilizing vaccines supplied by the WHO stockpile. We have on-going collaborations in each country and plan to evaluate the RAT in these settings. In addition, the RAT is limited by the quality of the data available in the country, at national, regional and district level. If the data available is poor, the resulting assessment will be similarly of lower quality. To ensure a quality assessment, the RAT must be conducted with cooperation from local decision-makers and health authorities.

While the dataset from the icddr,b utilized for the analysis in Paper 4 is a rich source of information, a retrospective surveillance cannot provide the same insight as a prospective case-control study to more clearly elucidate the risks of person-to-person transmission among neighbors. In addition, it is not possible to clearly differentiate retrospectively whether transmission was truly person-to-person or from an environmental reservoir. Based on statistical trends, we assume that large clustering of cases coincide with previous studies on person-to-person transmission. Finally, due to the large size of the dataset in consideration, our statistical analysis took considerable time. We will continue to explore this rich dataset to ensure that we have fully explored the study question within this population.

5.3. Recommendations for future research

Continued research will be done to expand upon the findings of this dissertation. The clinical and environmental surveillance being conducted in the Far North of Cameroon will continue through 2016. Cameroon has historically had years of little to no cholera incidence followed by several years of severe cholera disease. During the first 14 months of our study, there was little *V. cholerae* 01 detected in the environmental or clinical aspects of our study. Our original intent was to conduct a case-control study in this region to understand the risk factors as the dry, arid Sahel Desert is not the environment typically associated with endemic cholera. As reported in Paper 2, clinical cholera was confirmed in the Far North region in May and June of 2014, near the Nigerian border and more recently it was confirmed in the Lake Chad area in and around our surveillance sites. Simultaneously to the outbreaks in the Cameroonian portion of Lake Chad, there was also an outbreak in the Lake Chad area of Chad. The cholera outbreaks of 2009 in which 717 cholera cases and 85 deaths (CFR 11.9%) were reported in the North and Far North of Cameroon (1) were much smaller than those in 2010 and 2011, in which a total of 27, 725 cases including 1282 deaths (CFR, 4.6%) were reported (2). With continued a continued surveillance program into 2015, we hope to be able to detect early signs of an outbreak and to aide in intervention strategies now to prevent outbreaks as large as those seen in 2010 and 2011. In addition, in Paper 1, we were unable to determine any seasonality examining nonpathogenic *V. cholerae* 01 patterns. With continued surveillance, we hope to better understand if there truly is not a seasonality in this area. Finally, as described in Paper 2, with continued molecular characterization of strains in this area, we can further elucidate

if the similar sequence type present in both outbreaks in 2014 is present due to its endemicity in the area or whether it was imported through travel/migration of people.

The cholera RAT will continue to be evaluated in endemic countries, globally, particularly those who have interest in vaccination campaigns in the coming year. In addition, we plan to expand the cholera RAT to consider alternative scenarios in reactive and pre-emptive situations. The current WHO stockpile minimum requirements for access to doses in the stockpile includes; the report of a culture-confirmed cholera case in a given area and the proposal to implement a reactive vaccination plan (3). In addition, this year the Stockpile supplied vaccines for a pre-emptive vaccination approach in South Sudan (4). Countries that are currently accessing the stockpile need a risk assessment tool to aide in decision making regarding vaccination campaigns. Recently, the WHO has begun seeking advice on developing a RAT for these various scenarios and we intend to work with them and other partners to adapt our tool for application in these settings.

5.4 Policy Implications

This dissertation has several policy level implications for cholera surveillance and intervention strategies. The findings from paper 1 and 2 demonstrate the successful application of simplified epidemiological surveillance methodologies and laboratory diagnostics to improve the capability and sustainability of cholera surveillance in even the most remote and vulnerable settings. These findings may be expanded to enable surveillance in many other areas of Africa, and globally, where cholera disease burden remains uncharacterized. Similarly, countries recognizing their cholera burden but without the budget or personnel to build full microbiological laboratory capacity may employ these

simplified methods to ensure either environmental and/or clinical cholera surveillance to improve their public health response networks.

Findings from paper 3 have policy implications for the strategic approach to cholera prevention and intervention programs, including vaccine campaigns. Findings from paper 3 suggest that while incidence rate and fatality rates are vital to targeting at risk areas, in the current state of limited vaccine availability, it is probable that most countries utilizing the RAT for risk assessment will have to employ the weighted risk factor approach to further guide their consideration of where to target high level interventions such as vaccination. The WHO recognizes the need for this risk assessment tool, and have proposed the development of this tool to assess the risk and response to cholera in hotspots, as well as to define cholera control interventions. The findings from Paper 3 will be shared with the WHO and it is our hope that we can employ and adapt our tool to fit their needs.

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EDUCATION AND TRAINING

2002	BA in Biological Sciences	University of Delaware, Newark, DE
2005	MS in Biotechnology	Johns Hopkins University, Baltimore, Maryland
2015	PhD in International Health	Johns Hopkins University, Baltimore, Maryland

PROFESSIONAL EXPERIENCE

Aug2010 – Present **PhD Candidate, Student Investigator** - Johns Hopkins School of Public Health, Baltimore, MD & Kousseri, Cameroon

- Doctoral student investigator in support of the operational research project "Delivering Oral Cholera Vaccine Effectively (DOVE)" funded by the Bill and Melinda Gates Foundation.
- Thesis research is focused on the epidemiology of cholera in Cameroon using simplified epidemiological and laboratory methods to guide the use of OCV in Africa, and the development and validation of a cholera risk assessment tool.

Feb2011-Sept2012 **Graduate Research Assistant –** Institute for International Programs (IIP), Johns Hopkins Bloomberg School of Public Health

Oct2010-Aug2012 **Graduate Research Assistant -** International Vaccine Access Center (IVAC), Johns Hopkins Bloomberg School of Public Health

- Research Assistant in the development of the "Literature Review and Qualitative Interview Findings for Hepatitis A Vaccine Adoption in Six Countries".
    - Responsible for literature review, conducting interviews, and qualitative analysis of data for Russia and India
- Literature Review & Meta Analysis of the incidence and mortality of Meningococcal and pneumococcal disease to estimate the public health burden in children and adults globally and by region.

Jun2011–Aug2011 **Student Intern** – USDA FSIS Office of Public Health

- Statistical analysis of Baseline study data utilizing R and SAS analysis programs

Feb2008-Feb2014 **DTRA-CBEP Assistant Manager** – Research Scientist, Bacteriology Division, USAMRIID - Ft. Detrick, MD

- Develop training and research programs to establish and evaluate public health surveillance networks in the Cooperative Biological Engagement Program- CBEP), Defense Threat Reduction Agency (DTRA) supported countries.
- Design, develop and implement clinical research protocols to investigate aspects of pathogenesis, immunology and prophylaxis of bacterial and viral infections of military relevance.
- Diagnostic research for development and validation of assays to be used in research protocols and subsequent use in public health networks in BTRP countries
- Development of technique based training program for BTRP DTRA-TADR Azerbaijan
- Primary trainer for level 1 training for Azerbaijan BTRP DTRA/TADR program, including development of training materials and train-the-trainer capacity
- Logistical coordination and support, including procurement, shipping and laboratory management for all current clinical research protocols
- Laboratory training in all laboratories supporting CRU research protocols; including training plan design, training material development, training planning and coordination and implementation.
- Development of Proficiency testing program in support of the surveillance network in BTRP countries.
- Development of University Recruitment Strategy in Azerbaijan in coordination with Baku State University and local Ministry of Health
- Science lead/support on all CRU clinical research protocols funded by BTRP, GEIS, CBD and other funding sources

Sept2006-Feb2008 **Research Biologist**, Bacteriology Division, USAMRIID – BTRP/DTRA-TADR, Bacterial Therapeutics Lab - Ft. Detrick, MD

- Work in cooperation with the **Biological Weapons Prevention Program BWPP), Defense Threat Reduction Agency (DTRA) on the Threat Agent Detection and Response System (TADR) for Central Asia. The goals of this project are to:**
- Establish cooperative research projects on bacterial virulence and bacterial therapeutic countermeasures to Select Agents. Perform *In vitro* and *in vivo* screening of antibiotic resistance.
- Provide consultation and lectures on Biosurety, Biosecurity and Biosafety issues and designs of biocontainment facilities. Utilize existing Standard Operating Procedures (SOP) established at USAMRIID, expertise and our experience to

facilitate development of Laboratory Response Network for Select Agents.

- Establish Medical Collaboration for the use of Investigational New Drug Therapies for the treatment and prevention of naturally occurring diseases caused by Select Agents. The FDA approved IND protocols will be for treatment and prevention of diseases caused by Select Agents. Protocol sample testing will be performed by the personnel we train overseas in collaboration with our laboratory in the U.S.
- Investigate aspects of pathogenesis, immunology and prophylaxis of bacterial infections of military relevance, and to perform preclinical research and development studies on potential candidates for vaccines and therapeutics directed against infectious agents of military relevance. This research involves:
- Animal work including immunizing, bleeding, and euthanasia of mice, rabbits, guinea pigs and monkeys.
- Biological safety level 3 containment clearance and subsequent participation in aerosol challenges of animals by infectious agents
- Production, harvest, purification and storage of bacterial cells and spores and Determination of the viability of suspensions of infectious agents
- Performance of ELISA and toxin neutralization assays on sera from animals
- Preparation of DNA/RNA, Real time PCR analysis of samples
- Collection and analysis of telemetry data derived from challenges
- Security Clearance

Nov2003-Sept2006 **Senior Research Technician** - National Cancer Institute-Gene Expression Lab, SAIC-Frederick–Ft Detrick, MD

- Project Manager for RNAi Technology Development. Establishment of RNAi procedures for the lab including: primer and vector design, shRNA plasmid DNA production, sequencing analysis, transient transfections, virus production, development of stable cell lines, reporter assays, cell-cycle assays and FACs sample preparation, data quantification and establishment of SOPs. Have established several collaborations providing shRNA technology to other scientists at the NCI/SAIC-Frederick Campus.
- Project Manager for establishing Lentivirus production services in the GEL. Worked to establish Lentiviral procedures and SOPs in order to provide Lentivirus production, purification and amplification as a service to the NCI/SAIC-Scientific Community.
- Adenovirus production, purification and amplification as well as crude viral lysate production.

- Participation in collaborative projects involving gene cloning, plasmid DNA preparation and Real Time PCR & data analysis.
- Supervisor to student intern from 6/05-8/06. Includes establishing the Student's project, training & supervising student in all applicable areas.
- MGC-gene rescue project: primer design, RT-PCR, PCR product purification, transformation of purified PCR products for screening, preparation of glycerol stocks and plates for gene sequencing.
- Responsible for the maintenance and renovations of the GEL website. Work closely with Webmaster to make the website User-Friendly.
- Lab duties also include: Radiation Safety Supervisor, ordering laboratory supplies, GEL personnel training in lentivirus and shRNA production techniques, inventory and lab maintenance.
- Participated in Computer Training Programs offered through the NCI/Frederick: taking Microsoft Excel I, II and Data Manipulations in the fall of 2005.

Dec2002-Nov2003 **Research Technician** - National Cancer Institute/AIDS Monitoring Laboratory, SAIC-Frederick–Ft. Detrick, MD

- Perform immunological procedures to evaluate immune functions in persons with HIV disease who are being treated with a variety of anti-viral and immunomodulatory agents.
- Duties include: processing blood and body fluids from HIV-infected patients, cryopreservation of peripheral blood mononuclear cells, lymphocyte proliferation assays, ELISA techniques, data analysis and performing quality control procedure. BSL-3 safety procedures observed.

Jul2002-Oct 2002 **Research Assistant** - United States Army Research Institute of Infectious Diseases – Frederick, MD

- Performed general laboratory techniques pertaining to the development of an assay to screen for inhibitors of anthrax lethal toxin.
- Techniques performed include the following: PCR, agarose gel electrophoresis, DNA purification, molecular cloning, sequence analysis, protein expression in bacterial systems, protein purification and quantification, FPLC, HPLC, western blotting, SDS-PAGE, fluorescent labeling.

Apr2001-May2002 **Intern/Laboratory Technician -** Charles Peter White Fellowship with support from Dr. Daniel T. Simmons and the National Institute of Health, University of Delaware – Newark, DE

- Performed general laboratory techniques pertaining to research regarding cell replication.
- Techniques performed include, but not limited to, the following: PCR, agarose gel electrophoresis, DNA purification, molecular cloning, protein expression (baculovirus systems), Western blotting, SDS-PAGE, protein purification, Recombinant baculovirus production, immunofluorescence assays, biotinylated DNA binding assays, dot blotting, protein quantification, DNA transfection, viral plaque assays.

PROFESSIONAL ACTIVITIES

Feb2012-present **Non-dissertation research**: Cost Effectiveness Analysis of Pediatric Tuberculosis Diagnostics

PUBLICATIONS

1. **Debes, AK**; Kohli, A; Walker, N; Edmond, K; Mullany, LC. Time to initiation of breastfeeding and neonatal mortality and morbidity: a systematic review. *BMC Public Health,* 2013, 13(Suppl 3):S19.
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3. Chitadze N, Kuchuloria T, Clark DV, Ekaterine T, Chokheli M, Tsertsvadze N, Trapaidze N, **Lane A**, Bakanidze L, Tsanava S, Hepburn M, Imnadze P. Water-borne outbreak of oropharyngeal and glandular tularemia in Georgia: investigation and follow-up. *Infection*, 2009;37(6):514-521
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7. Clark DV, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov Koehler, J., **Debes AK**, Qasimov M, Schmaljohn, C., Hepburn MJ. Seroprevalance of Crimean-Congo Hemorrhagic Fever in Rural Azerbaijan. *Submitted to Transactions of the Royal Society of Tropical Medicine and Hygiene.*
8. **Amanda K. Debes**, Jerome Ateudjieu, Walter Ebile, Malathi Ram, Etienne Guenou, David Sack. Clinical and Environmental Surveillance for *Vibrio Cholerae*

in Resource Constrained Areas: Application during a one year surveillance in the Far North Region of Cameroon. *In preparation for submission*.

9. **Amanda K Debes**, Daniel R Feikin, Andrew Azman, Francisco Luquero, David A Sack. Overview of a Rapid risk Assessment Tool (RAT) for describing geographic rapid risk of cholera burden in developing countries to assist in implementation of Oral Cholera Vaccine. *In preparation for submission*.
10. **Amanda K. Debes**, Jerome Ateudjieu, Etienne Guenou, Shan Li, Colin Stine, David Sack. Molecular Epidemiology of Vibrio Cholerae, Cameroon, 2014 using novel specimen preservation methodologies. *In preparation for submission*.
11. **Amanda K. Debes**, Mohammad Yunus, David Sack, Mohammad Ali. Characterization of the transmissions of cholera among neighborhood contacts in Matlab, Bangladesh *In preparation for submission*.

ABSTRACTS OF PRESENTATIONS

A.K. Debes, J. Ateudjieu, E. Guenou, W. Ebile, M.Ram and D.A. Sack. Environmental Surveillance for Vibrio cholerae in Resource Constrained Areas: Application during a one year surveillance in the Far North Region of Cameroon. US-Japan Cholera Conference. Gainesville, FL January 14-16, 2015.

WM Webster, T Kuchuloria, **AK Debes**, SF Little, S Tsanava, N Chitadze, S Saghinadze, N Tsertsvadze, P Imnadze, TR Laws, EH Dyson, AJH Simpson, RG Rivard, MJ Hepburn, N Trapaidze. Assessment and comparison of the human immunological response to anthrax infection and vaccines. American Society of Tropical Medicine and Hygiene Conference, Washington, DC, November 13-17, 2013.

N. Trapaidze, **A.K. Debes**, W. Webster, S. Little, N. Chitadze, T. Kuchloria, S. Saghinadze, N. Tsertsvadze, R. Rivard, R. Mody, S. Tsanava, P. Imnadze, E.H. Dyson, A.J.H. Simpson, M. Hepburn. Serological Immune Response to Cutaneous Anthrax Infection and Booster of Live Attenuated Anthrax Vaccine. ASM Biodefense Conference, Washington, DC, February 6-8, 2011.

E. Jgenti, K. Sidamonidze, T. Akhvlediani, I. Kokaia, S. Tsanava, S. Francesconi, M.Nikolich, **A. Debes**, M.Hepburn, R. Mody, P.Elzer, N. Trapaidze. Evaluation of real-time PCR assays for the detection of Brucella spp. in human clinical samples in Georgia. ASM Biodefense Conference, Washington, DC, February 6-8, 2011.

Clark DV, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Richards A, Habashy EE, Maksoud MA, Rahman BA, Pimentel G, Koehler J, **Lane A**, Qasimov M, Hepburn MJ. Seroprevalence of Select Arthropod-borne and Zoonotic Infections in Rural Azerbaijan. International Conference on Emerging Infectious Diseases. Atlanta GA, July 11-14, 2010.

Ismayilov A, Clark DV, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, **Lane A**, Hepburn MJ. Human seroprevalence of brucellosis in three regions of Northern Azerbaijan. 12th World Congress on Public Health. Istanbul, April 27-30, 2009.

Clark DV, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Presentations Piraliyev S, Bagirov S, Aslanova A, **Lane A**, Qasimov M, Hepburn MJ. Emerging Viral Zoonoses in Azerbaijan: A Cross-Sectional Study. American Society of Tropical Medicine and Hygiene 58th Annual Meeting. Washington DC, November 18-22, 2009.

Clark DV, Ismayilov A, Seyidova E, Hajiyeva A, Bakhishova S, Hajiyev H, Nuriyev T, Piraliyev S, Bagirov S, Aslanova A, **Lane A**, Qasimov M, Hepburn MJ. Identification of an endemic infection: Q fever in rural Azerbaijan. Infectious Diseases Society of America 47th Annual Meeting. Philadelphia, October 29-November 1, 2009.
N. Chitadze, **T. Kuchuloria**, **A. Lane**, S. Saginadze, N. Tsertsvadze, M. Chubinidze, R. Rivard, Sh.Tsanava, EH. Dyson, TR. Laws, AJH. Simpson, MJ. Hepburn, N. Trapaidze Cutaneous anthrax infection elicits a robust long-term cellular immunologic response. Infectious Diseases Society of America 47th Annual Meeting. Philadelphia, October 29-November 1, 2009.
Presentations from 2009-2001 available upon request.

RESEARCH GRANT PARTICIPATION

Title of Grant, Dates and Sponsoring Agency: "Delivering Oral Cholera Vaccine Effectively (DOVE)" – Bill and Melinda Gates Foundation. 2012-2016
Principal Investigator and Funding Level: David A Sack - $5 million over 4 years
Main Grant Objective: To promote the effective use of oral cholera vaccine globally.
Principal Responsibilities of Individual: Student Investigator to include development and evaluation of rapid and practical tools for OCV delivery, and to aide in management of epidemiological and laboratory surveillance in African countries engaged in surveillance.

Title of Grant, Dates and Sponsoring Agency: "Immunologic assessment of vaccination and endemic infections from threat agent organisms in the countries of the former Soviet Union" – Chemical and Biological Defense: 2009-2011
Principal Investigator and Funding Level: Matthew Hepburn - $150,000
Main Grant Objective: To understand protective efficacy of vaccines in various endemic areas in the former Soviet Union.
Principal Responsibilities of Individual: Associate Investigator and head of laboratory science aspects of the project.

Title of Grant, Dates and Sponsoring Agency**: "**Anthrax Immunology of Primary Vaccinees in the Republic of Georgia" - Chemical and Biological Defense: 2005-2010
Principal Investigator**:** Matthew Hepburn
Main Grant Objective: To evaluate the effectiveness of anthrax vaccination in Georgia.
Principal Responsibilities of Individual: U.S. Science lead - ELISA & Western Blotting assay development and testing; antigen cloning and production; Cell Mediated Immune Response diagnostic testing; data compilation and analysis; training for in-country personnel.

Title of Grant, Dates and Sponsoring Agency**: "**Plague and Tularemia Immunology of Primary Vaccinees in the Republic of Georgia" - Chemical and Biological Defense: 2006-2010
Principal Investigator**:** Matthew Hepburn
Main Grant Objective: To evaluate the effectiveness of tularemia and plague vaccination in Georgia.

Principal Responsibilities of Individual: U.S. Science lead - ELISA & Western Blotting assay development and testing; antigen cloning and production; Cell Mediated Immune Response diagnostic testing; data compilation and analysis; training for in-country personnel.

Title of Grant, Dates and Sponsoring Agency: "Retrospective Investigation of Anthrax infection Immune Profiles in the Republic of Georgia" - Chemical and Biological Defense: 2006-2013
***Principal Investigator*:** Robert Rivard
Main Grant Objective: To improve the understanding of clinical anthrax infection immunology through a retrospective laboratory analysis of protection.
Principal Responsibilities of Individual: U.S. Scientific support - ELISA & Western Blotting assay development and testing; antigen cloning and production; Cell Mediated Immune Response diagnostic testing; data compilation and analysis; training for in-country personnel.

***Title of Grant, Dates and Sponsoring Agency*:** " Prospective Investigation of Anthrax infection Immune Profiles in the Republic of Georgia" - Chemical and Biological Defense: 2006-2013
***Principal Investigator*:** Robert Rivard
Main Grant Objective: To prospectively evaluate persons infected with anthrax infection to better understand the immunological response during active infection.
Principal Responsibilities of Individual: U.S. Scientific support - ELISA & Western Blotting assay development and testing; antigen cloning and production; Cell Mediated Immune Response diagnostic testing; data compilation and analysis; training for in-country personnel.

***Title of Grant, Dates and Sponsoring Agency*:** " A Clinical Observational Study evaluating diagnostic modalities and case definitions for human brucellosis in Georgia" - Chemical and Biological Defense/Defense Threat Reduction Agency: 2007-2010
***Principal Investigator*:** Matthew Hepburn
Main Grant Objective: To prospectively evaluate persons infected with brucellosis infection to compare Brucella diagnostics and to improve clinical case definitions in Georgia.
Principal Responsibilities of Individual: U.S. Scientific support - ELISA and molecular laboratory training and support for in-country personnel; data compilation and analysis.

***Title of Grant, Dates and Sponsoring Agency*:** "A Clinical Observational Study evaluating diagnostic modalities and case definitions for human brucellosis in Azerbaijan" - Chemical and Biological Defense/Defense Threat Reduction Agency: 2007-2011
***Principal Investigator*:** Matthew Hepburn
Main Grant Objective: To prospectively evaluate persons infected with brucellosis infection to compare Brucella diagnostics and to improve clinical case definitions in Georgia.
Principal Responsibilities of Individual: U.S. Scientific support - ELISA and molecular laboratory training and support for in-country personnel; data compilation and analysis.

***Title of Grant, Dates and Sponsoring Agency*:** "Acute Febrile Illness in the Republic of Georgia" - Global Emerging Infections Surveillance and Response System (GEIS): 2007-2010
***Principal Investigator*:** Matthew Hepburn
Main Grant Objective: To prospectively evaluate persons with Acute Febrile Illness of Unknown origin in Georgia.
Principal Responsibilities of Individual: U.S. Scientific support - ELISA and support for in-country personnel; data compilation and analysis.

***Title of Grant, Dates and Sponsoring Agency*:** "Brucellosis Immunology" - Chemical and Biological Defense/United Kingdom Ministry of Defense: 2007-2010
***Principal Investigator*:** Matthew Hepburn and Huge Dyson
Main Grant Objective: To prospectively evaluate persons with brucellosis to improve immunological understanding of disease progression in Georgia.
Principal Responsibilities of Individual: U.S. Scientific support - ELISPOT, ELISA and Western Blot development, training and support for in-country personnel.

***Title of Grant, Dates and Sponsoring Agency*:** "A seroprevalence study of prior exposure to select arthropod-borne and zoonotic infections among rural populations in Northern Azerbaijan" - Defense Threat Reduction Agency: 2007-2010
***Principal Investigator*:** Matthew Hepburn
Main Grant Objective: To conduct a retrospective seroprevalence study to improve understanding of arthropod-borne and zoonotic infections among the rural populations in Northern Azerbaijan.
Principal Responsibilities of Individual: Associate Investigator - Scientific management, ELISA diagnostic development, training and support for in-country personnel; Data compilation and analysis.

ACADEMIC SERVICE

Mar-May2014 Teaching Assistant, Clinical and Epidemiological Aspects of Tropical Disease, Johns Hopkins Bloomberg School of Public Health
Mar-May2012 Teaching Assistant, Clinical and Epidemiological Aspects of Tropical Disease, Johns Hopkins Bloomberg School of Public Health

ADDITIONAL INFORMATION

Proficient in French; conversational Russian
Proficient in use and application of : Stata, Tree-Age for economic modeling, Atlas.ti for qualitative analysis, ArcGIS
Additional Experience in the use and application of R for Statistical computing and SAS