CHARACTERISATION OF *Vibrio cholerae* ISOLATES FROM THE 2009, 2010 AND 2016 CHOLERA OUTBREAKS IN LUSAKA PROVINCE, ZAMBIA

By

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A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medical Microbiology of the University of Zambia

THE UNIVERSITY OF ZAMBIA

LUSAKA
2017

Declaration

I, Kapambwe Mwape, declare that this dissertation is my own work. It is being submitted for the Master of Science in Medical Microbiology at the University of Zambia, Lusaka, Zambia. It has not been submitted either wholly or partially for any degree at this or other university.

_____ 28th Day of November, 2017

Kapambwe Mwape

SUPERVISOR

I have read this dissertation and approved it for final submission.

Dr J. Mwansa

Signature _____ Date _____

Certificate of Approval

This dissertation of **Kapambwe Mwape** has been approved in partial fulfilment of the requirements for the degree of Master of Science in Medical Microbiology at the University of Zambia.

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Abstract

In the last three decades, Zambia has experienced frequent cholera outbreaks which mainly affect fishing camps around the country and peri urban areas in Copperbelt and Lusaka Provinces where there is unsafe water supply and poor sanitation. In 2009, 2010 and 2016 more than 12000 cases, with about 300 deaths, were reported from Zambia with Lusaka reporting more cases than the rest of the country. However, the antibiogram and genetic diversity of the *Vibrio cholerae* isolates causing these outbreaks in Lusaka Province were unknown. This knowledge is essential to inform cholera prevention and control programmes.

This was a laboratory based cross-sectional study that sought to determine the antibiogram and genetic diversity of *Vibrio cholerae* isolates from the 2009, 2010 and 2016 cholera outbreaks in Lusaka Province. All available isolates from the respective outbreaks were included in this study and they were characterised by serotyping, antimicrobial susceptibility testing and macro-restriction analysis using Pulsed-Field Gel Electrophoresis. The 2009 and 2016 outbreaks yielded only the Ogawa serotype, while the 2010 outbreak only yielded the Inaba serotype. All the isolates were 100% resistant to nalidixic acid and nitrofurantoin. The 2009 and 2010 cholera outbreak isolates showed 100% multidrug resistance while 67 % of 2016 were multidrug resistant. Macro-restriction analysis demonstrated high diversity among the isolates with six clusters of at least 92% similarity. The largest cluster comprised 12 isolates with 97.8-100% similarity, while the smallest cluster comprised 3 isolates with 95.3-98% similarity.

This study demonstrated that both the Inaba and Ogawa serotypes were responsible for the cholera outbreaks with only one of the serotypes responsible for each particular

outbreak; 2009(Ogawa), 2010(Inaba) and 2016(Ogawa). The study also revealed multidrug resistant *Vibrio cholerae* strains circulating in all the three outbreaks. Macro-restriction analysis demonstrated high genetic diversity among the *Vibrio cholerae* isolates, suggesting that the isolates were not from the same source of infection. The findings in this study will be used to guide therapy and track the sources and spread of infection for effective cholera management. There is need to establish a national surveillance system for cholera to monitor antibiotic resistance and to track the virulence and antibiotic resistance genes of *Vibrio cholerae* strains circulating in Zambia to gain insight into their clinical implications.

Dedication

I would like to dedicate this work to my husband, Mr Nathan Kamanga, for his love, encouragement and support throughout the period of my studies.

Acknowledgements

I would like to express my gratitude to my Principal Supervisor, Dr James Mwansa, for the great supervision, material and moral support throughout my project. I also owe deep gratitude to my Co-supervisor, Dr Geoffrey Kwenda, for his motivation, support and guidance in scientific writing. Both were very helpful throughout the research process and writing of this dissertation. Their friendship, advice and encouragement were vital, especially during difficult times in the course of this work.

I am grateful to Ms Annie Kalonda, Lecturer in the Department of Biomedical Sciences in the School of Health Sciences, for guiding me throughout the research process and in drafting this dissertation. She was always willing to help me out whenever I sought advice from her.

I am also indebted to the Head of the Bacteriology Laboratory at the University Teaching Hospital (UTH), Dr Lukwesa Chileshe-Musyani, for providing me with technical support for my laboratory work. I am also grateful to the other staff in the laboratory for their valuable advice and camaraderie.

I am thankful to Professor Trevor Kaile, the former Head of Pathology and Microbiology Department, for giving me an opportunity to pursue my studies in his Department and for supporting my research work; the former Assistant Dean for Postgraduate Studies in the School of Medicine, Dr Selestine H. Nzala, for his encouragement and support.

A special word of gratitude goes to Dr Anthony Smith and Dr Karen Keddy of the National Institute of Communicable Diseases (NICD) in Johannesburg, South Africa, for facilitating the molecular typing of the *Vibrio cholerae* isolates. This work would not have been completed without their support.

I also owe my debt of gratitude to my employer, the Copperbelt University, for financial support and for granting me study leave to purse my MSc studies. Without their support, it would not have been possible to carry on with my studies.

Lastly, I would like to thank my family for their understanding and support during the period of my studies. Their patience, love and encouragement have upheld me. My special gratitude should go to my husband, Mr Nathan Kamanga, for his unconditional love and unwavering support; to my father, Mr Isaac Mwape, for his mentorship and reason to go on.

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List of Abbreviations

APW	Alkaline Peptone Water
AFLP	Amplification Fragment Length Polymorphism
AMR	Antimicrobial Resistance
CSB	Cell Suspension Buffer
CDC	Centers for Disease Control and Prevention
CT	Cholera Toxin
ctxB	Cholera Toxin Subunit B
CLSI	Clinical and Laboratory Standards Institute
cAMP	cyclic Adenosine Monophosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
CTXΩ	*Vibrio cholerae* Bacteriophage
GM	Monosialotetrahexosylganglioside
IFRC	International Federation of Red Cross and Red Crescent
LIA	Lysine Iron Agar
OCV	Oral Cholera Vaccine
ORS	Oral Rehydration Salts
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
rtxC	Repeat in the Toxin Gene
rstR	Repeat Sequence Transcriptional Regulator
SKG	SeaKem Gold
TTGA	Taurocholate Tellurite Gelatin agar

TE	Tris-EDTA
TCBS	Thiosulphate Citrate Bile Salts Sucrose agar
TCP	Toxin Co-regulated Pilus
tcpA	Toxin Co-regulated Pilus Gene
SXT	Trimethoprim/Sulfamethoxazole
TSI	Triple Sugar Iron
TSA-SB	Trypticase Soy Agar-Sheep Blood
VPI	Vibrio Pathogenicity Island
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background

Cholera is a notorious enteric disease affecting 3.4 million people globally every year with a case fatality rate of over 2% (Rabaudet *et al.*, 2013; WHO, 2016). It is an acute disease characterised by severe watery diarrhoea caused by toxigenic *Vibrio cholerae* strains, which colonise the small intestine and produce an enterotoxin known as cholera toxin (CT) (Akoachere *et al*., 2013). Pandemics that are caused by this bacterium have severely affected many countries on multiple continents for many years (Ramazanzadeh *et al*., 2015). Transmitted via the faeco-oral route, cholera is particularly associated with poverty and poor sanitation and is characterised by high morbidity and mortality in crowded urban slums and rural areas (Marin *et al*., 2014).

Several African countries experience cholera outbreaks in a regular seasonal pattern (Marin et al., 2014). Among them are sub-Saharan African countries such as Mozambique (Gujral *et al*., 2013), Tanzania (Kachwamba *et al*., 2017), Zimbabwe (Maponga *et al*., 2015) and South Africa (Kira *et al*., 2013). The current seventh pandemic which was initially of exclusive Asian focus reached Africa in 1970 (De *et al*., 2013). Between 1995 and 2005, Africa experienced a greater upsurge in cholera outbreaks than other continents accounting for over 80% of the global total number of cholera cases (Griffith *et al*., 2006). The trend of Africa reporting more cholera cases continued between 2006 and 2010 (Saidi *et al*., 2014). Between 2015 and 2016, several African countries reported cholera outbreaks and among these are four of Zambia's

neighbouring countries: Zimbabwe, Mozambique, Malawi and Tanzania (Reliefweb, 2015; InterHealth Worldwide, 2016; Nara *et al*., 2017).

Zambia usually experiences cholera outbreaks during the rainy season and most of them have been associated with fishing camps and unplanned settlements in Lusaka and Copperbelt Provinces (MoH, 2011). The first cholera outbreak in Zambia was reported in the 1970s and several other outbreaks have occurred over the years with the worst outbreak being in 1991 that caused 13,000 cases (Dubois *et al*., 2006). In the 2009 cholera outbreak a total of 7,587 cases and 162 deaths were reported (IFRC, 2010) while the 2010 cholera outbreak caused 6,804 cases and 73 deaths in Zambia with majority of the cases being from Lusaka Province (MoH, 2011). In 2016, Zambia experienced an outbreak in Lusaka Province with more than 1000 cases and 22 deaths reported after having been cholera free for five consecutive years (MoH, 2016).

In order to minimize the disease burden caused by cholera, antimicrobials have been used against *V. cholerae* to shorten the duration of illness (Kitaoka *et al*., 2011). However, the increased use of antibiotics against cholera has resulted in the emergence of multiple drug resistance globally (Mwansa *et al*., 2007; Akoachere *et al*., 2013; Njeru *et al*., 2014). Hence, the need for continued monitoring of the characteristics of epidemic strains cannot be over-emphasised.

1.2 Statement of the Problem

Cholera is a serious global public health problem due to its long established ability to infect and kill large numbers of people within a short period of time (Christian *et al*.,

2013; Ismail et al., 2013; Lü *et al*., 2017). The disease is most prevalent in low-income countries where outbreaks occur frequently due to poor sanitation and hygienic practices (Hasan *et al*., 2012; Dixit *et al*., 2014; Miwanda *et al*., 2016). As evidenced by frequent outbreaks in different parts of the country, especially in the rainy season, cholera is a major public health concern in Zambia (Mwansa *et al*., 2007; MoH, 2011). A total of 7,587 cases with 162 deaths were reported during the 2009 cholera outbreak (IFRC, 2010) and the 2010 cholera outbreak caused 6,804 cases and 73 deaths (MoH, 2011). However, the serotypes or strains of the *V. cholerae* involved in these outbreaks are unknown.

Most of these cases are treated empirically to relieve the symptoms of cholera without determining the suitability of the drugs used for treatment during outbreaks (Shrestha *et al*., 2015). The increase in antimicrobial resistance (AMR) among enteric bacterial pathogens, especially multidrug resistant (MDR) strains, is challenging global prospects for fighting these pathogens (Kitaoka *et al*., 2011; Dantas and Sommer, 2014; Barati *et al*., 2015). The emergence and spread of drug-resistant pathogens shrinks the therapeutic arsenal, leading to increased morbidity and mortality and may further complicate cholera management (Sack *et al*., 2001; Rajpara *et al*., 2015). Although, MDR isolates have previously been reported in Zambia (Mwansa *et al*., 2007), there is little or no data describing how drug resistance patterns have changed over time. Furthermore the phenotypic and molecular characteristics of the *V. cholerae* isolates in Zambia are unknown, and this hampers efforts for planning, management and elimination of cholera.

1.3 Rationale of the Study

Frequent seasonal cholera outbreaks in Zambia are a reminder of the huge burden cholera places on the public health system of the country (WHO, 2011). To provide new insights into the epidemiology of the disease during an outbreak or epidemic, it is important to document and characterise the biotype, serotype and antibiotic susceptibility/resistance patterns of the isolates. This is important for effecting efficient counter measures needed to confine outbreaks and to avoid high case fatality rates of the disease.

Determination of antibiotic resistance patterns guides therapy for more effective cholera control. With the growing AMR in toxigenic *V. cholerae* strains (Mandal *et al*., 2012; Marin *et al*., 2014; Baloi *et al*., 2016) with epidemic potential, monitoring of antimicrobial susceptibility patterns and source tracking are important for obtaining data to guide the adaptation of policy for control of cholera at the national and global levels. This is in line with the Ministry of Health (MoH) commitment to mitigating AMR in Zambia (Zambia National Public Health Institute, 2017). Data obtained from this study will provide important insights on how drug resistance patterns have changed over time and the genetic diversity of the epidemic strains circulating in Zambia, which will be important for enhancing cholera control efforts. This is the first study in Zambia to simultaneously investigate the antimicrobial susceptibility patterns and genetic diversity of the *V. cholerae* isolates.

1.4 Research Question

What is the antibiogram and genetic diversity of *V. cholerae* isolates from the 2009, 2010 and 2016 cholera outbreaks in Lusaka Province?

1.5 Objectives

1.5.1 General Objective

To determine the antibiogram and genetic diversity of *V. cholerae* isolates from the 2009, 2010 and 2016 cholera outbreaks in Lusaka Province.

1.5.2 Specific Objectives

i. To determine the serotypes of *V. cholerae* strains isolated at the University Teaching Hospital, Lusaka.

ii. To determine the antibiogram of *V. cholerae* strains isolated at the University Teaching Hospital, Lusaka.

iii. To determine the genetic relatedness of the *V. cholerae* strains isolated at the University Teaching Hospital, Lusaka.

CHAPTER 2

LITERATURE REVIEW

2.1 General Characteristics of *Vibrio cholerae*

Vibrio cholerae, a is a free living bacteria found in aquatic environments such as seawater, fish, sea plankton, coral reefs and chitinous sea animals (Huff *et al*., 2012). It is a Gammaproteobacterium of the order Vibrionales belonging to the family Vibrionaceae (Dikow and Smith, 2013). It is a Gram-negative, motile and non-spore forming curved rod found in coastal waters and estuaries (Silva and Benitez, 2016). It grows best in the presence of salt, although it grows in water of low salinity when it is warm and contains sufficient organic nutrients (Harris *et al.*, 2012). When nutritionally deprived, *V. cholerae* switches to a viable but non-culturable state (Kitaoka *et al*., 2011). Once the organism encounters favourable conditions, it reverts back to infectious transmissible state (Kitaoka *et al*., 2011). When cultured on Thiosulphate Citrate Bile Salts Sucrose agar (TCBS), *V. cholerae* strains grow as yellow, shiny colonies (Ali *et al*., 2014). However, on Taurocholate Tellurite Gelatin Agar (TTGA) they produce grey flattened colonies with opaque zone around each colony on and colourless to pink colonies on MacConkey agar (Harris *et al*., 2012).

Based on the differences in the sugar composition of the O antigen, *V. cholerae* has been classified into more than 200 serogoups but of these only the O1 and O139 have been known to cause epidemics and pandemics (Rajpara *et al*., 2015). The O1 serogroup exists as El Tor and classical biotypes which are further differentiated into three serotypes, Inaba, Ogawa and Hikojima based on antigenic factors, methylation or non-

methylation on the terminal perosamine sugar of the surface lipopolysaccharide (Barnerjee *et al*, 2014; Shrestha *et al*., 2015; Karlson *et al*., 2016). Genetic markers like the cholera toxin subunit B ctxB have been used to distinguish between El Tor and classical biotypes (Son *et al*., 2011). In addition, the repeat sequence transcriptional regulator (rstR) gene and the major toxin coregulated pilus gene (tcpA) possess classical and El Tor-specific alleles, while the repeat in the toxin gene (rtxC) is present in El Tor but absent in classical biotype (Zhang *et al*., 2014). The first six pandemics were caused by the classical strain while seventh cholera pandemic was caused by the El Tor strain (Dixit *et al*., 2014). The figure below shows the classification of *V. cholerae* according to serogroups, serotypes and biotypes.

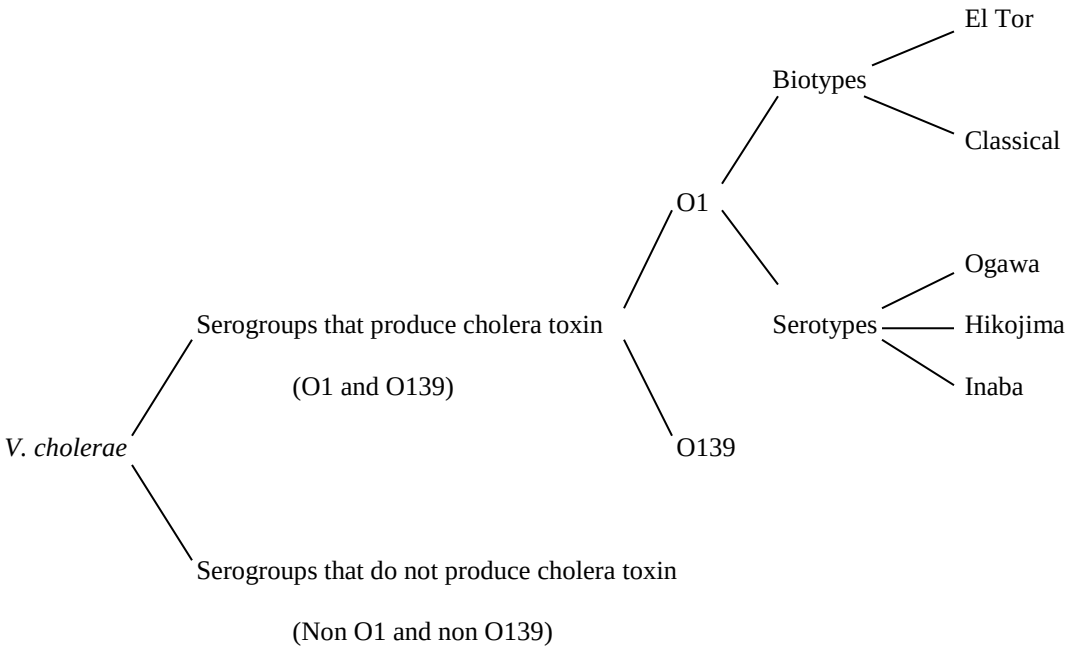

Figure **2.1**. Classification of *V. cholerae* (Barnerjee *et al*., 2014)

2.2 Epidemiology of Cholera

The world has experienced seven cholera pandemics that originated from Asia since 1817 with the seventh pandemic being the most extensive in geographic spread and duration (Kim *et al*., 2014). The first six recorded cholera pandemics (1899-1923) have been attributed to classical strains (Safa *et al*., 2010), while the El Tor strains have been implicated in the seventh pandemic (Naha *et al*., 2013). This pandemic spread throughout Asia and eventually reached Africa in the early 1970s and caused massive outbreaks resulting in more than 400, 000 cases with a high mortality rate mainly due to lack of community immunity in the population and inadequacies in the healthcare infrastructure (Harris *et al*., 2012). In 1992, *V. cholerae* O139 also known as the Bengal strain emerged as a major cause of epidemic cholera in Bangladesh and India by displacing the El Tor biotype strains (Dixit *et al*., 2014). According to recent reports, cholera causes 3 to 5 million cases annually predominantly in Asia and Africa (Uthappa *et al*., 2015).

Cholera cases are very rare in high income countries (WHO, 2014). According to a World Health Organisation (WHO) report, cholera cases in Europe are very low and are usually imported cases (WHO, 2014). Similarly, most cholera cases reported in the United States of America are acquired during international travel to cholera endemic areas such as parts of Africa, south East Asia or Haiti (CDC, 2014). The few domestically acquired cholera cases in the United States are primarily associated with consumption of raw or undercooked seafood harvested from the United States Gulf coast (Loharikar *et al*., 2015). In 2010, cholera which was not officially reported in Haiti erupted as an epidemic and within a year after the initial cases claimed more than 6,500

lives and caused sickness in 500,000 people (Hassan *et al*., 2012). According to CDC, there has been a decline in the number of reported cases in Haiti with 352,033 cases in 2011 and 15,063 cases in 2014 (CDC, 2015).

Although not common in high income countries, cholera remains a major public health problem in the low income countries in Asia, Africa and Latin America due to poor sanitation and unsafe water (Ali *et al*., 2012; Dixit *et al*., 2014). According to reports from Bangladesh, about 450,000 cases of cholera are reported annually in Bangladesh (Wahed *et al*., 2013). A study conducted in the Philippines revealed that between 2008 and 2013 about 42,071 confirmed and suspected cases were reported affecting all age groups (Lopez *et al*., 2015). A similar study conducted in New Papua Guinea reported that between 2009 and 2011, 15,582 cases and 493 deaths occurred (Horwood *et al*., 2011).

During the past decade, except for the Haitian outbreak, most cholera epidemics cases and deaths have been reported from sub-Saharan Africa (Rabaudet *et al*., 2013). A study conducted in Mozambique showed that between 2008 and 2011, out of the 25, 431 suspected cholera cases, 220 deaths were reported (Gujral *et al*., 2013). A similar study conducted in Kenya between 1997 and 2010, reported 65,522 suspected cholera cases with 2,461 deaths (Mutonga *et al*., 2013). Another study reported that Zimbabwe contributed more than half of the African cholera cases between 2008 and 2009 (Maponga *et al*., 2015). This outbreak of over 100,000 cases in Zimbabwe subsequently spread to neighbouring South Africa and Zambia, causing thousands of additional cases (Kira *et al*., 2013). During the same period, Tanzania experienced an outbreak that

caused 7,700 cases (Naha *et al*., 2013). The last major outbreak in Tanzania occurred between 2015 and 2016 causing 24,000 cases with 378 deaths (WHO, 2016).

Zambia reported the first cholera outbreak in 1977/1978 and then later in 1982/1983 another outbreak occurred (Chirambo *et al*., 2016). The first major outbreak which was the worst ever experienced in Zambia occurred in 1990 and lasted for three years after which cholera became endemic and the interval between outbreaks reduced to every two years (Olu *et al*., 2013; Chirambo *et al*., 2016). Cases are commonly reported in fishing camps in various parts of the country and the rural and peri-urban areas of Lusaka and Copperbelt Provinces (MoH, 2011). This follows the typical pattern of cholera outbreaks occurring in places where access to sanitation and water supply is poor (Ismail *et al*., 2013). Outbreaks usually start in October and end between mid-May and the beginning of June corresponding to the rainy season (WHO, 2011). In 2009 a total of 7,587 cases and 162 deaths were reported from a cholera outbreak (IFRC, 2010) while in 2010, the number of cases reported was 6,804 and 73 deaths with most cases being from Lusaka Province in both outbreaks (MOH, 2011). Most recently after a five year break, a cholera outbreak occurred in 2016 causing more than 1,000 cases and 16 deaths in Lusaka Province (MOH, 2016). Due to the unpredictability of cholera outbreaks, the epidemic response team must always be well equipped in order to combat the spread of the disease.

2.3 Transmission of Cholera

Cholera is a water and food-borne disease transmitted via the faeco-oral route from person-to-person as a result of poor hygiene, poor sanitation and inadequate water

supply (Taylor *et al*., 2015). These conditions propagate transmission as faecal matter from an infected person may be disposed into water bodies from which people draw water for drinking or cooking (Rebaudet *et al*., 2013). The contaminated water may also be used to wash food that is eaten raw or dishes onto which food is placed and eventually eaten. Unhygienic practices such as lack of soap use after defecation and before eating, and hand eating from common plates contribute to cholera transmission (Sasaki *et al*., 2009). The infectious dose of *V. cholerae* is 10^3-10^6 cells when ingested with water and a lower infectious dose of 10^2-10^4 cells when ingested with food (Sharifi-Mood *et al*., 2014).

2.4 Pathogenesis of Cholera

After ingestion of *V. cholerae* cells in contaminated food or water, some organisms are killed by gastric acid but the surviving organisms colonise the small intestine (Sack *et al*., 2004). The organisms use flagella to reach the intestinal mucosa and attach to microvilli of the brush border of gut epithelial cells by pilli (Almagro-Moreno *et al*., 2015). The organisms begin to release the Cholera Toxin (CT), a protein exotoxin that consists of subunit A associated with five B subunits (Sánchez and Holmer, 2011). The B subunit pentamer binds to the ganglioside GM (monosialotetrahexosylganglioside) on eukaryotic cells to promote entry of the A subunit. The A subunit is translocated intracellularly, where it acts to enzymatically activate adenylate cyclase and raise intracellular cyclic Adenosine Monophosphate (cAMP) to abnormal levels thus pumping large amounts of chloride into the intestinal contents (Wernick *et al*., 2010). Water, Sodium and other electrolytes follow due to osmotic and electrical gradients caused by the loss of chloride ion. The lost water and electrolytes are replaced from the blood.

Thus the toxin damaged cells become pumps for water and electrolytes causing diarrhoea, loss of electrolytes and dehydration (Brooks *et al*., 2010).

The pathogenicity of *V. cholerae* rests on its ability to elaborate CT and the colonisation factor Toxin Coregulated Pilus (TCP) (Akoachere *et al*., 2013). These major virulence factors are present in clusters within 2 regions in the *V. cholerae* chromosome (Shrestha *et al*., 2015). Genes for the CT are encoded in the genome of the filamentous bacteriophage (CTXΩ) and the bacterial cell surface receptor for CTXΩ is the TCP which is itself encoded within a genomic island-Vibrio Pathogenicity Island (VPI-1) (Harris *et al*., 2012). Genes that code for CT and TCP are presumed to be exclusively associated with toxigenic *V. cholerae* strains and are acquired by the bacterium from the aquatic environment (Akoachere *et al*., 2013).

2.5 Clinical Presentation of Cholera

The signs and symptoms of cholera are caused by rapid and profound loss of fluid and electrolytes in watery diarrhoea and vomitus. Incubation period is 24-48 hours but may vary from 5 hours to 5 days (Sharifi-Mood and Metanat, 2014). This variation has been attributed to previous exposure which may provide some protection, immune status of an individual or the type of *V. cholerae* strains causing infection (Azman *et al*., 2013). 75% of persons that get infected with cholera are asymptomatic and most of the 25% with symptomatic infections have mild illness (CDC, 2011). Although cholera affects all age groups, severity of disease on admission was reported to directly correlate with older age as immunity lowers with age (Valcin *et al*., 2013).

After the initial intestinal purge, diarrhoea becomes very watery with flakes of mucus and has the appearance of rice water stool (Ngwa *et al*., 2016). This is characteristic of diarrhoea caused by cholera. Dehydration ensues with symptoms and signs such as excessive thirst, hypotension, tachycardia, weakness, fatigue, dry mucous membranes, dry mouth, oliguria, sunken eyes, weak pulse, wrinkled skin, somnolence and coma (Sharifi-Mood and Metanat, 2014). Massive watery diarrhoea up to litre per hour can lead to hypotensive shock and death within hours of the first symptom (Harris *et al*., 2012).

2.6 Diagnosis of Cholera

2.6.1 Clinical Diagnosis

Clinically, cholera is characterised by severe dehydration from acute, severe and watery diarrhoea often without vomiting in any person aged 5 years or older from an endemic area or where an outbreak of cholera has occurred currently (WHO, 2004; Sharifi-Mood and Metanat, 2014). Cholera in children under 5 years of age need laboratory confirmation especially early during an outbreak as they may have diarrhoea due to other aetiologies with similar symptoms to cholera (WHO, 2004; Farthing *et al*., 2013). Such diarroeal disease causing organisms include enterotoxigenic *Escherichia coli* (Sack, 2011) and Salmonella (Said *et al*., 2011).

2.6.2 Laboratory Diagnosis of Cholera

Pathogen identification is crucial to confirm bacterial infection and guide antimicrobial therapy. There are two main laboratory approaches to cholera diagnosis, phenotypic and genotypic methods.

2.6.2.1 Phenotypic Methods

Phenotypic methods for cholera diagnosis include culture, rapid diagnostic tests or biochemical tests. Currently, isolation and identification of *V. cholerae* O1 or O139 by culture from stool specimen remains the method of choice for laboratory diagnosis (Keddy *et al*., 2013). However, this method is lengthy and not favourable for rapid response during outbreaks, hence the need to introduce rapid diagnostic tests for routine detection of cholera. *V. cholerae* grows aerobically at an optimum temperature of 37^{o}C on culture and produces yellow shiny colonies that are 2-4 mm in diameter on TCBS media (Ali *et al*., 2014). For biochemical tests and serology, a pure characteristic colony is subcultured onto non selective media such as Müeller-Hinton agar. Biochemical tests for *V. cholerae* include oxidase test, string test, sulphide indole motility, citrate test, triple sugar iron and lysine iron agar (Mandal *et al*., 2011). Biochemical tests are supplemented by serology that detects the O antigen of *V. cholerae* (Torane *et al*., 2016). Determination of serotypes is not routinely done in peripheral laboratories but reference laboratories due to limited acquisition of antisera. Phenotypic methods are relatively cheaper and do not require highly skilled experts to perform, hence suitable for resource-limited settings such as Zambia**.**

There are several commercial tests available for identification of *V. cholerae* such as Crystal E/NF, Microscan Neg ID2, Vitek ID GNB and API 20E (Keddy *et al*., 2013). In the recent past, the rapid diagnostic test Crystal VC which detects lipopolysaccharide antigens has been used during outbreaks (Martinez-Pino *et al*., 2013). This test was evaluated in 2016 in Sudan and it was reported that with Alkaline Peptone Water (APW) enrichment, it gave results comparable to culture method (Ontweka *et al*., 2016). During

the 2016 cholera outbreak in Zambia, one such test, the SD BIOLINE cholera Ag O1/O139 (Standard Diagnstics Inc, Gyeonggi-do, Republic of Korea) was used for cholera diagnosis along with culture and serology (MoH). This test proved to be faster and most suitable for rapid cholera diagnosis during an outbreak.

2.6.2.2 Genotypic Methods

Molecular methods are an essential requirement to support phenotypic identification of the organism (Keddy *et al*., 2013). DNA Sequencing is regarded as the "gold" standard for the identification of all microorganisms, including *V. cholerae* (Deng *et al*., 2014). It is a superior method as it is able to detect the smallest detail as illustrated in a Chinese study where variations due to point mutation due to deletions in the trimethoprim/sulphamethoxazole (SXT) element were reported (Wang *et al*., 2016).

Polymerase Chain Reaction (PCR) though not widely used in the African setting for diagnosis can be used to detect the two main virulence factors: CT and TCP which also indicate epidemic potential of the organism (Keddy *et al*., 2013). Primers targeted for these virulence factors are ctxA and tcpA (Njeru *et al*., 2014). Isolates are confirmed as *V. cholerae* by detecting the outermembrane protein (ompW) and virulence is determined by detecting cholera enterotoxin gene (ctx), tcpA and haemolysin gene (hlyA) (Keasler *et al*., 1993; Njeru *et al*., 2014; Hounmanou *et al*., 2016). It is of great significance in cholera diagnosis as *V. cholerae* is influenced by factors such as changes in pH and osmolarity during passage in the alimentary canal or antibiotics and may change into a coccoid non-culturable state (Alam *et al*., 2010). There is no current consensus on a single validated PCR method for cholera diagnosis but the combined use

of stool culture and PCR in future could influence the sensitivity of *V. cholerae* detection during cholera outbreaks (Lucien *et al*., 2015).

2.7 Treatment and Control of Cholera

The mainstay of cholera treatment is rehydration through the administration of oral rehydration salts (ORS) or intravenous fluids and electrolyte replacement (Ismail *et al*., 2013). Antibiotics are adjunctive therapy in patients with moderate and severe cholera as they diminish the duration of diarrhoea and reduce the volume of rehydration fluids needed (WHO, 2015). The recommended antibiotics for cholera by WHO include doxycycline, tetracycline, furazolidone, trimethoptim-sulfamethoxazole, ciprofloxacin, ampicillin and erythromycin (WHO, 2016). The choice of antibiotics varies according to the current susceptibility profile of the organism in a region, hence the need to monitor antibiotic resistance periodically. The recommended antibiotics for cholera treatment by Ministry of Health Zambia include azithromycin, ciprofloxacin, doxycycline and erythromycin (MoH, 2011).

Effective prevention of cholera involves a comprehensive approach that includes complementary use of Oral Cholera Vaccine (OCV) to water, sanitation and hygiene (WASH) interventions such as latrine construction and use, water chlorination, hand washing campaigns with soap and compact water filtering (Bwire *et al*., 2017). Other factors that contribute to cholera control include strengthening epidemiological surveillance, timely alert of outbreaks, improved case management and operational research (Muyembe *et al*., 2013). Operational research is defined as knowledge on

interventions, strategies or tools that can enhance the quality, effectiveness or coverage of programs (Zachariah *et al*., 2009). This will inform policy makers as to whether the interventions that have been put in place are effective and thus make improvements if not. In places where provision of safe portable water and good sanitation have proven insufficient in preventing cholera, including vaccination campaigns will enhance cholera control efforts (Seidlein *et al*., 2013).

The oral cholera vaccine has proven to be safe and effective, and is now considered part of the comprehensive and multi-disciplinary approach to the prevention of cholera and response to cholera outbreaks (WHO, 2014). Commonly used vaccines include Shancol and Dukoral, both killed whole cell vaccines. Shancol contains *V. cholerae* O139 and Dukoral is a recombinant cholera toxin B subunit vaccine (Luquero *et al*., 2013). Shancol is the most commonly used vaccine in low-income countries, and it works by reducing internal colonisation of *V. cholerae*, thus making people less likely to spread the infection (International Vaccine Institute, 2012). Several studies looking at the safety and feasibility of implementation of OCV have been carried out in the most recent past. A study in Iraq during the 2015 cholera outbreak demonstrated that OCV campaigns can be successfully implemented as part of cholera outbreak response in conflict settings (Lam *et al*., 2017). Another study in Ethiopia demonstrated that the OCV was safe and elicited a robust immune response against *V. cholerae* O1 (Desai *et al*., 2015). The OCV was used during the 2012 cholera outbreak in Guinea and demonstrated that it poses very low risk in pregnant women (Grout *et al*., 2015). A similar study conducted in Malawi in which a vaccination campaign was implemented during the 2015 cholera outbreak demonstrated that a large scale mass OCV campaign was feasible and

acceptable among the Malawians (Msyamboza *et al*., 2016). This vaccine is currently being evaluated in Zambia and results are expected soon. .

2.8 Antimicrobial Resistance

The widespread usage of antibiotics has increased the threat of emergence of antimicrobial resistant strains that are a serious cause for concern. Resistance is achieved by exporting drugs through efflux pumps, chromosomal mutations or through genetic exchange using conjugative plasmids, transposons, integrons or self-transmissible chromosomally integrating SXT (Trimethoprim/Sulfamethoxazole) element (Kitaoka *et al*., 2011). The SXT element and integrons have been implicated in the emergence and transmission of multidrug resistant *V. cholerae* (Wang *et al*., 2016; Jain *et al*., 2016). A study in India revealed the occurrence of both the SXT element and class 1 integrons in all the isolates studied from the 2010 cholera outbreak (Jain *et al*., 2016). Another study in Nigeria demonstrated that strains from the 2009/2010 cholera outbreak harbour the SXT element (Marin *et al*., 2013). The occurrence of the SXT element has also been reported in Kenya (Mutreja *et al*., 2011) and Mozambique (Taviani *et al*., 2009). A study previously carried out in Zambia revealed that the 1996/1997 isolates harboured the class 1 integron but lacked the SXT element while the 2003/2004 isolates harboured the SXT element but lacked the class 1 integron (Mwansa *et al*., 2007). The SXT element harbours *sulI*, *floR*, *strA*, *dfrA1* and *dfrA18* genes (Sambe-Ba *et al*., 2017) while the integron harbours *dfr*A1, *sat*1 and *aad*A1 (Canto de Sá *et al*., 2010). It is not known whether antibiotic resistance genes carried by these elements are currently circulating in Zambia.

Multidrug resistant (MDR) *V. cholerae* in epidemic cholera outbreaks have been reported worldwide (Okeke *et al*., 2007; Mandall *et al*., 2011: Rashed *et al*., 2012; Yu *et al*., 2012; Marin *et al*., 2014). India reported resistance to ampicillin, tetracycline, nalidixic acid, furazolidone, streptomycin, erythromycin, cotrimoxazole, neomycin and chloramphenicol with resistance to tetracycline being 100% in 2014 (Kar *et al*., 2015). Shrestha recently reported multi drug resistance in Nepal with all the isolates resistant to ampicillin, nalidixic acid, cotrimoxazole and erythromycin but 100% susceptible to tetracycline (Shrestha *et al*., 2015). A similar study in Bangladesh reported 93% MDR isolation rate with most isolates being resistant to streptomycin, nalidixic acid, tetracycline and cotrimoxazole (Rashed *et al*., 2017).

In Nigeria, a study reported that the 2009/2010 cholera outbreak was caused by MDR strains of *V. cholerae* (Marin *et al*., 2013). A similar study in Kenya demonstrated that out of the six recommended drugs for cholera by WHO, two were reported resistant in Kenya between 2007 and 2010 (Njeru *et al*., 2013). In another study looking at the 2009 cholera outbreak in South Africa, organisms resistant to ampicillin, tetracycline, chloramphenicol and erythromycin were detected, with low level extended beta lactamase activity and third generation cephalosporin resistance (Ismail *et al*., 2013). A similar study in Mozambique revealed 100% resistance to trimethoprim-sulfamethoxazole,100% to ampicillin, 99% to nalidixic Acid, 97% to chloramphenicol, 95% to nitrofurantoin, 82% to tetracycline, 56% to azithromycin and no resistance ciprofloxacin (Baloi, 2016).

A Zambian study carried out on isolates from outbreaks that occurred between 1990 and 2004 revealed increasing antimicrobial resistance to tetracycline (95%), chloramphenicol (78%), doxycycline (70%) and trimethoprim–sulphamethoxazole (97%) (Mwansa *et al*., 2007). The increase in drug resistance to these antibiotics led to fluoroquinolones becoming the drug of choice for cholera treatment (Okeke *et al*., 2007; MoH, 2011). Flouroquinolones have been reported to induce horizontal transfer of SXT elements (MoH, 2011; Jain *et al*., 2015), therefore this calls for close monitoring of the usage of antimicrobial drugs (Wang *et al*., 2016). Despite identification of this problem of AMR in Zambia, the antibiotic susceptibility patterns of *V. cholerae* isolates in the most recent cholera outbreaks remain unknown.

2.9 Molecular Typing of *Vibrio cholerae* Isolates

The rapid genesis of antibiotic resistance and changes at genomic level make genotypic methods very relevant for the detection of the most recent changes at the genetic level, (Keddy *et al*., 2013). For epidemiological purposes, genotypic methods help in discriminating between clones, identifying clusters, understanding phylogeny, tracking spread and transmission, and tracing the origin or the source of infections (De *et al*., 2013). Traditional typing methods based on phenotype such as serotype, biotype, phage-type or antibiogram have been used for many years. However, newer methods have been developed that have high discriminatory power (Sabat *et al*., 2013). These methods include ribotyping, Amplification Fragment Length Polymorphism (AFLP), Multi locus variable analysis (MLVA) and Pulsed-Field Gel Electrophoresis (PFGE), and the selection of these methods depends on the purpose of analysis (Okeke *et al*., 2003; Keddy *et al*., 2013). Of these methods, PFGE is considered a "gold standard" for

molecular typing methods because of its high discriminatory power compared to the other methods (Rahaman *et al*., 2015). However, in the most recent past there has been a shift to Whole Genome Sequencing (WGS) as the above methods have limited thoroughness and resolution (Rahaman *et al*., 2015). The WGS approach is not feasible in most laboratories because of the high cost involved.

Ribotyping is an epidemiological tool used to study the clonal structure of bacterial populations using labelled Escherichia coli rRNA to probe ubiquitous and polymorphic rDNA loci (Taneja *et al*., 2012). A study in Mexico demonstrated two distinct epidemic strains with different ribotypes between 1991 and 1993 after which one clonal population gradually disappeared and only one ribotype was present from 1994 to 2000 (Lizárraga-Partida and Quilici, 2009). A study in India revealed three ribotype patterns with three of the 2001 isolates having a new ribotype VIII (Ghosh et al., 2016). A similar study in Kenya revealed a predominant ribotype from different parts of the country demonstrating that the epidemic strains responsible for the outbreak betwwen 1998 and 1999 had a clonal origin (Scrascia *et al*., 2006).

Amplified Fragment Length Polymorphism fingerprinting is a technique to detect genomic restriction fragments by PCR amplification (Jiang *et al*., 2000). An AFLP study on *V. cholerae* strains from many different countries demonstrated that the two epidemic foci in the 1991 cholera upsurge in Africa were due to two separate introductions (Lan and Reeves, 2002). A study on isolates from various parts of the world demonstrated that the African O1 strain was closely related to the Asian O139 *V. cholerae* suggesting that the O1 African strain may be in a transition state (Jiang *et al*.,

2000). Another study in a cholera endemic region of India indicated that environmental and clinical *V. cholerae* isolates belonged to a single cluster thus demonstrating the role of acquatic ecosystem in the spread of cholera (Mishra *et al*., 2011).

Multilocus Variable Analysis is a useful tool for tracking *V. cholerae* and resolving distinct populations of clinical isolates from different geographic locations (Hasan *et al*., 2012). An MLVA study showed the presence of five different clonal complexes in a set of isolates from Kenya (Mohamed *et al*., 2012) contrary to the hypothesis of a clonal introduction of outbreak causing strains (Stine and Moris, 2014). However, a Haitian study revealed limited Variable Number Tandem Repeat (VNTR) diversity among outbreak strains indicating a possible single source of infection (Hasan *et al*., 2012). A study conducted in Thailand revealed one MLVA type that survived for three years and this long term survival can only be attained in watery environments or humans who chronically carry the organisms with no signs or symptoms of cholera (Okada *et al*., 2012)

A study carried out in Ghana on isolates from three cholera outbreak years revealed major annual outbreak clusters with co-circulating genetically distant genotypes suggesting an endemic reservoir of *V. cholerae* in Ghana (Eibach *et al*., 2016). A similar study in Nigeria revealed four different clusters among water and clinical isolates from 2007 to 2013 with 95% similarity suggesting contamination of water sources by this pathogen and thus may act as a reservoir in transmission of the disease (Adewale *et al*., 2016). Another study carried out on isolates from several sub-Sahara African countries showed that certain pulsotypes were shared among different countries while other

pulsotypes were unique to particular countries (Smith *et al*., 2015). A cluster of isolates from Togo, Democratic Republic of the Congo (DRC), Côte d'Ivoire and Guinea shared a similar pulsotypes and clustered together with an overall similarity of 97.7% (Smith *et al*., 2015). A second cluster of isolates from DRC, Côte d'Ivoire and Guinea shared a similar PFGE pattern and clustered together with an overall PFGE pattern similarity of 97.7% (Smith *et al*., 2015). A cluster comprising six isolates from Côte d'Ivoire and one isolate from Mozambique displayed the PFGE pattern for the Haiti cholera outbreak strain which was also seen in Cameroon and South Africa (Smith *et al*., 2015).

A study carried out on Zambian isolates collected between 1996 and 2004 showed that typical El Tor strains were prevalent in Zambia but were replaced completely by an altered variant from the 2003-2004 outbreak (Bhuiyan *et al*., 2014). The 1996-1997 isolates clustered with El Tor reference strain N16961, a Bangladeshi seventh-pandemic El Tor strain isolated in 1971, while the 2003-2004 isolates clustered together with El Tor variant strains from Bangladesh (Bhuiyan *et al*., 2014). This result indicated that the new strains that emerged in the 2003-2004 outbreak may have originated from the progenitor strain *V. cholerae* O1 El Tor strain (Bhuiyan *et al*., 2014). However, the genetic relatedness of isolates from recent cholera outbreaks in Zambia remains unknown.

CHAPTER 3

MATERIAL AND METHODS

3.1 Study Design

This was a cross sectional study conducted on archived isolates identified as *V. cholerae* previously during the 2009 and 2010 cholera outbreaks. The 2016 *V. cholerae* strains were collected as the study was ongoing and all the available isolates were included. The isolates were obtained from samples that came to the laboratory for diagnosis.

3.2 Sample Size

Due to lack of previous prevalence of laboratory confirmed cholera cases the following formula was used to calculate the sample size.

$$N=\frac{Z^2*P*(1-P)}{d^2}$$

Where N= minimum sample size

Z= 1.96 (standard error)

P= 0.5

d= 0.05 (absolute precision)

$$N=\frac{1.96^2*0.5(1-0.5)}{0.05}=384$$

Sample size= 384

However only 83 isolates that were available were included in this study.

3.3 Study Site

This study was carried out on isolates from Lusaka Province at the University Teaching Hospital in Lusaka. Based on a national census in 2010, Lusaka Province has a population of 2, 191, 255 people (CSO, 2010). More than 70% of this population is based in Lusaka District. The city has been growing rapidly through the influx of immigrants from the rural areas to the peri urban areas of Lusaka where access to safe water and to sanitation facilities is limited. The areas captured in this study include Kafue, Kanyama, Chawama, Bauleni, Kalingalinga, Matero, Chipata, John Laing, George and Mtendere.

3.4 Sampling Technique

Convenience sampling was employed in this study. Eighty-three available clinical isolates collected from Lusaka Province during the 2009 and 2010 outbreaks, and incoming 2016 isolates were used in this study.

3.4.1 Inclusion Criteria

V. cholerae strains isolated during the 2009, 2010 and 2016 cholera outbreaks in Lusaka Province from all ages were included in this study.

3.4.2 Exclusion Criteria

All suspected *V. cholerae* isolates that did not agglutinate polyvalent O1/O139 antisera were excluded from the study.

3.5 Determination of *V. cholerae* Serotypes

3.5.1 Specimen Collection, Isolation and Revival of Stored Isolates

V. cholerae isolates used in this study were archived isolates obtained from rectal swabs and stool specimen during the 2009, 2010 and 2016 cholera outbreaks in Lusaka Province, Zambia.

V. cholerae isolates in Skim milk Tryptone Glucose Glycerol (STGG) (20% glycerol) vials previously stored at -80°C were allowed to stand at room temperature to facilitate thawing. A loopful of the bacteria was inoculated into alkaline peptone water (APW) for enrichment. The tubes were incubated with caps loosened at 35-37°C for 6 hours. This culture was then inoculated aseptically on Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar (HiMedia laboratories, Mumbai, India), a selective medium for *V. cholerae*, at 37°C for 18-24 hours. Single, shiny, yellow colonies characteristic of *V. cholerae* were subcultured onto nutrient agar and incubated at 35-37°C for 18-24 hours.

3.5.3 Biochemical Identification

Conventional biochemical tests, Oxidase, Triple Sugar Iron (TSI) agar, Lysine Iron Agar (LIA), citrate and sulphide indole motility tests were used to identify *V. cholerae*. These tests were carried out as described in the Center for infectious Disease Control manual for cholera diagnosis (CDC, 2015). For the sugar fermentation tests, pure isolated colony was picked from Müeller-Hinton agar a non-selective media and inoculated into test media then results read after a 24 hour incubation period. The oxidase test was carried out by dipping a sterile swab into Kovacs oxidase reagent solution (Sigma Aldrich,

Steinheim, Germany) then touching an isolated colony and observing for colour change. If colour changed to purple then the isolate is oxidase positive.

3.5.4 Serological Typing

Serological typing was done by slide agglutination with polyvalent antisera for *V. cholerae* O1, monovalent antisera for Inaba and Ogawa serotype and antisera for *V. cholerae* O139 (Mast Diagnostics, Merseyside, United Kingdom). A glass slide was divided into two test sections using wax pencil and a small drop of normal saline placed in each section. A well isolated colony was picked using a sterile applicator stick and mixed in a drop of normal saline on a glass slide making a milky suspension. A drop of antisera was then added to the drop in one of the sections, a second section served as a control. The suspension and antisera were mixed thoroughly and the slide rocked to observe for agglutination. Agglutination in 30 seconds to 1 minute confirmed the serogroup and serotype of the organism.

3.6 Determination of Antibiogram of *V. cholerae*

3.6.1 Antimicrobial Susceptibility Testing

Antibiotic susceptibility patterns were determined using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2012). Drugs tested for susceptibility included ampicillin (10µg), azithromycin (15µg), ciprofloxacin (5µg), erythromycin (15µg), nitrofurantoin (300µg), norfloxacin (10µg), tetracycline (30µg), trimethoprim-sulphamethoxazole (25µg) and Nalidixic acid (30µg) (Oxoid Limited, Hampshire, United Kingdom). The selection of disks to be tested was based on recommended drugs for cholera (MoH, 2011; WHO, 2016) A well isolated

colony was selected from an overnight culture on nutrient agar with a sterile swab and suspended in 5ml of sterile normal saline. The turbidity of the suspension was adjusted with sterile saline to obtain a suspension similar to that of 0.5 McFarland standard. A new sterile swab was then dipped into the suspension and after removal of excess inoculum by pressing against the wall of the tube, bacteria was evenly spread on Müeller-Hinton agar (HiMedia labs, India). An automatic dispenser was employed to ensure discs were not less than 24mm apart from centre to centre. Each disc was then pressed gently with sterile forceps to ensure complete contact with the agar. The inoculated plates were incubated for 16-18 hours at 37°C. The zones of inhibition were measured using a transparent ruler and endpoints determined based on the areas showing no bacterial growth visible to the naked eye. Results were interpreted according to the CLSI guidelines (CLSI, 2015). *Escherichia coli* control strain ATCC 25922 was included with each test.

3.7 Determination of Genetic Diversity by Macrorestriction Analysis

3.7.1 Preparation of Bacterial DNA

To determine the clonal relationship amongst the *V. cholerae* isolates, macrorestriction analysis using Pulsed-Field Gel Electrophoresis (PFGE) was employed as described under PulseNet (https://www.cdc.gov/pulsenet/PDF/vibrio_pfge_protocol-508c.pdf) Thirty eight (38) of the *V. cholerae* isolates were analysed and compared with strains from other countries. Briefly, isolated colonies from test culture was streaked onto Trypticase Soy Agar containing 5% defibrinated sheep blood (TSA-SB) for confluent growth. The cultures were then incubated for 18 hours at 37°C. Loopfuls of each isolate were transferred to a labelled transparent tube containing 2ml of Cell Suspension Buffer

(CSB) (100 ml of 1 M Tris, pH 8.0 and 200 ml of 0.5 M EDTA, pH 8.0 diluted to 1000 ml with sterile ultrapure water). The concentration of the cell suspension was adjusted to OD 1.0 at 610nm on a spectrophotometer (MRC Ltd, London, United Kingdom). After warming 400µl cell suspension to 55°C for 15min in a waterbath, an equal volume of 1% low melting agarose (Bio-Rad Laboratories, Hercules, CA, USA) in cell suspension buffer containing 20µl Proteinase K (Sigma-Aldrich, Steinheim, Germany) was added and the mixture was then poured into pre-cooled moulds (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to set for approximately 20 to 30min. The cells in the agarose plugs were then incubated in TE (Tris-EDTA, ie 1M Tris, pH 8.0, 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0) buffer containing 4mg/ml of lysozyme (Sigma-Aldrich, Steinheim, Germany) for 10- 15 minutes and then in freshly prepared cell lysis buffer(TE buffer pH 8.0, 2mg/ml proteinase K and 1% N-lauryol sarcosine (Sigma-Aldrich, Steinheim, Germany) at 55°C in shaking waterbath for 2 hours. At the end of the incubation period, the plugs were topped up with 2ml TE buffer and sealed with parafilm (Sigma-Aldrich, Steinheim, Germany) and stored at 4°C until required.

3.7.2 Restriction Endonuclease Digestion

Slices 5mm thick were cut from the plugs in a Petri dish, washed 7 times with 1hr changes in TE buffer (pH 8.0), pre-equilibrated in 300ml of 1X restriction buffer containing 0.1mg/ml BSA (Fermentas Life Sciences, Glen Burnie, MD, USA) for 1hr at room temperature and then incubated in 200ml of 1X restriction buffer containing 0.1mg/ml BSA and 30U *NotI* (Fermentas Life Sciences, Glen Burnie, MD, USA) overnight.

3.7.3 Electrophoresis

The restricted sample was loaded onto a 1% (wt/vol) low-melting point agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5x TBE (10x stock: 89mM Tris, 89mM boric acid, 2mM EDTA [pH 8.0]), sealed with 1% agarose in 0.5x TBE and allowed to set. PFG MidRange II DNA markers (New England BioLabs, Ipswich, USA) were loaded onto gels as molecular size standards and the gel was pre-equilibrated for 1hr at 14°C in a CHEF-DRIII electrophoretic tank (Bio-Rad Laboratories, Hercules, CA, USA) filled with 2 litres of 0.5x TBE. Following electrophoresis, the gels were stained with 0.5mg/ml ethidium bromide for 30min, and after destaining for 30min, the gels were imaged with a Gel Doc XR Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

3.7.4 Macrorestriction Pattern Analysis

A TIFF image of each gel was exported to the Gel Compar 6.0 System (Applied Maths, Kortrijk, Belgium) for analysis. A PFG MidRange II DNA marker (New England BioLabs, Ipswich, Glen Burnie, USA) was included on each gel to facilitate molecular weight assignments and inter-gel comparisons. For inter-gel comparisons, the two outermost and centre lanes of each gel were used.

3.8 Data Analysis

Descriptive data collected in this study included antibiotic resistance and serotypes. The data was stored in excel spreadsheet and plotted in frequency tables and graphs to make comparisons and monitor trends among the three outbreaks.

PFGE patterns were analyzed using GelCompar II Software (Applied Maths, Sint-Martens-Latem, Belgium). For inter-gel comparisons, the two outermost and centre lanes of each gel were used. The phylogenetic relationships among isolates was based on dendrograms constructed with unweighted pair group method with arithmetic averages (UPGMA) of 1% position tolerance. Two isolates with a similarity of ≥ 90% were defined as clones. Strains with less than 90% similarity were each assigned a distinct pulsotype number. Strains above 95% similarity were considered identical. These thresholds were established by cluster analysis of at least two molecular weight standards from each gel followed by calculation of the percentage similarity among each set of standards.

3.9 Ethical Approval

This study was laboratory-based and involved no direct contact with patients. All participant specimens were de-identified and given study specific identification codes. Permission to conduct the study was sought from the University Teaching Hospital Management in Lusaka, whilst ethics clearance was sought from the University of Zambia Biomedical Research and Ethics Committee (UNZABREC). The Ethics Clearance Certificate Number was 014-11-15.

CHAPTER 4

RESULTS

Findings from this study demonstrated that both serotypes Inaba and Ogawa were circulating during the three outbreaks. Multidrug resistance was detected during all the outbreaks and the isolates also showed high genetic diversity.

4.1 Determination of *V. cholerae* Serotypes

A total of 83 isolates were successfully revived and all were identified as *V. cholerae* O1. The figure below shows the distribution of serotypes isolated in this study. The Ogawa serotype comprised 70% of all the isolates in this study, while Inaba comprised 30% (Fig 4.1).

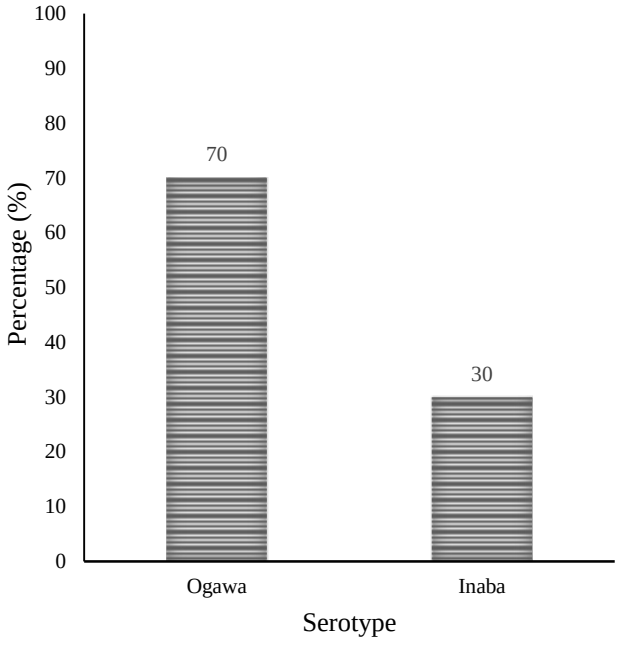

Fig 4.1 Distribution of serotypes of *V. cholerae* isolated

The table below shows the distribution of serotypes according to each outbreak. All the 2009 and the 2016 cholera outbreak isolates were identified as Ogawa serotype, while all those from the 2010 outbreak were identified as Inaba serotype (Table 4.1).

Table 4.1. Distribution of serotypes *V. cholerae* isolated in each cholera outbreak

Outbreak	Serotype (%, n)	
	Ogawa	Inaba
2009	100(6/6)	0
2010	0	100(25/25)
2016	100(52/52)	0

4.2 Determination of Antibiotic Resistance Patterns

Results showed that the 2009 and 2010 isolates were 100% resistant to cotrimoxazole, nalidixic acid, and nitrofurantoin. These isolates were sensitive to ampicillin, ciprofloxacin, norfloxacin and azithromycin. Reduced sensitivity to tetracycline was observed with 67% for 2009 and 64% for 2010 of the isolates being in the intermediate range of sensitivity. Similar results were obtained for erythromycin that had 100% of the 2009 isolates and 92% of the 2010 isolates being in the intermediate range. Results also showed that the 2016 isolates were 98% resistant to nalidixic acid and nitrofurantoin with low level resistance to cotrimoxazole (10%), norfloxacin (4%), erythromycin (4%), tetracycline (2%) and azithromycin (2%). Reduced susceptibility to both ciprofloxacin and norfloxacin (fluoroquinolones) was also observed (Table 4.2).

Table 4.2. Susceptibility patterns of *V. cholerae* O1 strains

Susceptibility	Frequency n (%)		
	2009 (6)	2010 (25)	2016 (52)
Tetracycline (30µg)			
Sensitive	2(33)	9(36)	49(94)
Intermediate	4(67)	16(64)	2(4)
Resistant	0	0	1(2) CI(3.81)
Cotrimoxazole (5µg)			
Sensitive	0	0	47(90)
Intermediate	0	0	0
Resistant	6(100)	25(100)	5(10) CI(8.15)
Nalidixic acid (30µg)			
Sensitive	0	0	1(2)
Intermediate	0	0	0
Resistant	6(100)	25(100)	51(98) CI(3.81)
Ampicilin (10µg)			
Sensitive	6(100)	25(100)	21(40)
Intermediate	0	0	0
Resistant	0	0	31(60) CI(13.32)
Ciprofloxacin (5µg)			
Sensitive	5(83)	22(88)	3(6)
Intermediate	1(17)	3(12)	43(83)
Resistant	0	0	2(4) CI(5.33)
Norfloxacin (10µg)			
Sensitive	6 (100)	25(100)	7(13)
Intermediate	0	0	43(83)
Resistant	0	0	2(4) CI(5.33)
Erythromycin (15µg)			
Sensitive	0	2(8)	0
Intermediate	6(100)	23(92)	50(96)
Resistant	0	0	2(4) CI(5.33)
Nitrofurantoin (30µg)			
Sensitive	0	0	1(2)
Intermediate	0	0	0
Resistant	6(100)	25(100)	51(98) CI(3.81)
Azithromycin (15µg)			
Sensitive	6(100)	25(100)	51(98)
Intermediate	0	0	0
Resistant	0	0	1(2) CI(3.81)

The majority of the *V. cholerae* isolates exhibited multidrug resistance (MDR) (79%, 66/83) with 7 different patterns. The commonest pattern was SXT-NAL-NIT (50%, 33/66, comprising all the 2009 and 2010 outbreak isolates), followed by NAL-AMP-NIT

(42%, 28/66), and 1.5% (1/66) for all the remaining patterns (Table 4.3). MDR was defined as resistance to three or more drugs from different drug classes (Magiorakos *et al*., 2012).

Table 4.3. Antimicrobial resistance profile of *V. cholerae* isolates from the 2009, 2010 and 2016 cholera outbreaks

Antimicrobial Resistance Patterns	**No. of Isolates (%)**
2016	
SXT-NAL-CIP-NOR-ERY-NIT	1 (1.9%)
TET-SXT-AMP-ERY-AZM	1 (1.9%)
NAL-AMP-NOR-NIT	1 (1.9%)
NAL-AMP-CIP-NIT	1 (1.9%)
NAL-AMP-NIT	28 (53.8%)
NAL-CIP-NIT	1 (1.9%)
SXT-NAL-NIT	3 (5.8%)
NAL-NIT	16 (30.8%)
Total MDR	**35 (67%)**
2009	
SXT-NAL-NIT	6 (100%)
Total MDR	**6 (100%)**
2010	
SXT-NAL-NIT	25 (100%)
Total MDR	**25 (100%)**
Grant Total MDR	**66 (79%)**

SXT-Trimethoprim sulfamethoxazole, NAL-Nalidixic acid, CIP-Ciprofloxacin, NOR-Norfloxacin, ERY-Erythromycin, NIT-Nitrofurantoin, TET-Tetracycline, AMP-Ampicilin, AZM-Azithromycin

4.3 Determination of Genetic Relatedness

To investigate whether the isolates were genetically related, macrorestriction analysis by PFGE was performed on the strains with *NotI*.

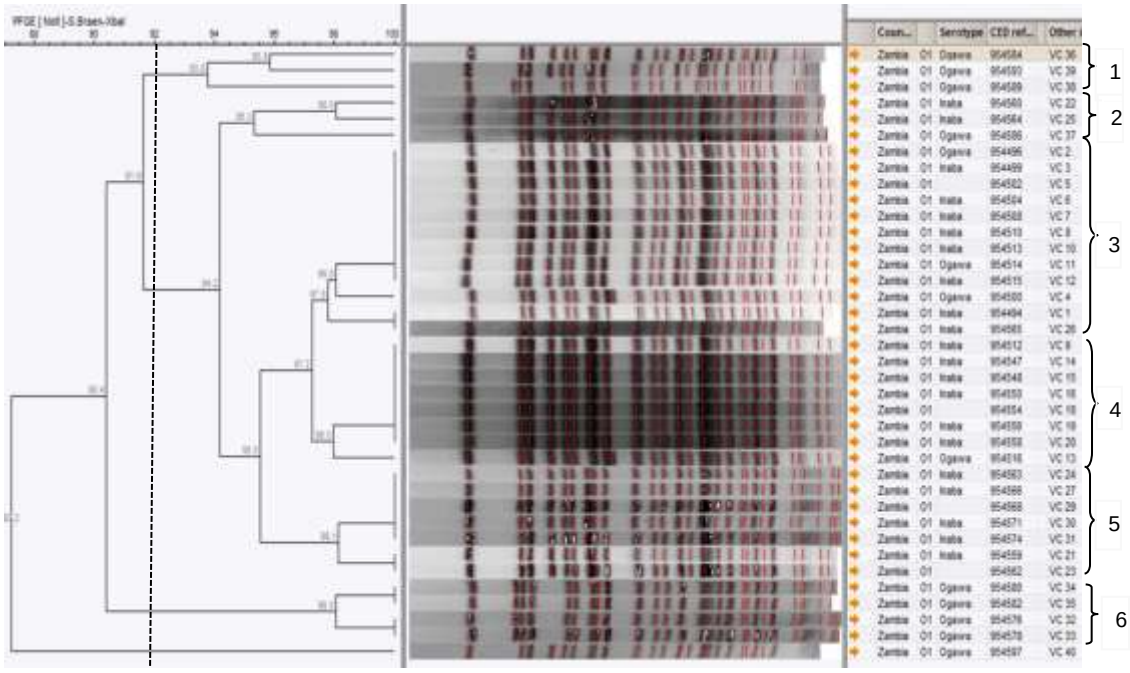

Fig 4.2 Genetic relationships amongst the PFGE *NotI* macrorestriction profiles of *V. cholerae* 01 isolates from Lusaka Province. The dotted vertical line on the left of the dendrogram shows a 92% Similarity Index (SI).

In general, the PFGE analysis demonstrated diversity amongst the isolates. When electrophoretic profiles were compared using the GelCompar Software, 6 clusters, 1-6 of at least three isolates whose PFGE patterns were similar by at least 92%, could be observed (Figure 4.2). This similarity index (SI) was determined by comparing three possible cut-offs, 90%, 92% and 95%. The SI cut-off that provided the best discrimination, after visual inspection was 92% as it correctly assigned the isolates to their respective clusters. Cluster 3 contained the largest number (twelve) of strains, which were 97.8 to 100% similar, followed by Cluster 4 with eight strains (98 to 100% similarity), Cluster 5 with seven strains (98.1 to 100% similarity), Cluster 6 with four strains (98 to 100% similarity), Cluster 1 with three strains (93.8 to 95.8% similarity) and Cluster 2 also with three strains (95.3 to 98% similarity). Only one isolate, VC40, remained outside the cluster.

CHAPTER 5

DISCUSSION

5.1 Discussion

Cholera outbreaks continue to be a major public health problem in Lusaka Province even after three decades since the first outbreak in Zambia. Outbreaks occur in peri-urban slums that are as a result of rural-urban migration due to poor social services in rural areas. Because these are unplanned settlements, they are characterised by overcrowding and hence poor access to safe water and good sanitation. The main sources of water in peri-urban areas of Lusaka are wells which are closely located to pit latrines, hence they get contaminated during the rainy season thus causing cholera outbreaks. Contaminated wells have been reported in areas like Chipata compound, Mazyopa and SOS village (MoH).

V. cholerae O1 strains belonging to either serotype Ogawa or Inaba were identified in this study. Our results are in agreement with other studies that reported serotypes Ogawa and Inaba as the most common serotypes in other African countries such as Tanzania (Kachwamba *et al*., 2013), South Africa (Ismail *et al*., 2013), Kenya (Njeru *et al*., 2014) and Ghana (Eibach *et al*., 2016). However, a study in DRC reported some strains belonging to the Hikojima serotype during the 2009 cholera outbreak (Miwanda *et al*., 2015). Our study also showed a single serotype circulating during each outbreak. This has been reported in several studies; 2011 outbreak in Iran (Barati *et al*., 2015), 2012-2015 outbreaks in Mozambique (Baloi *et al*., 2017) and 2009 outbreak in Tanzania (Naha *et al*., 2013).

The increase in AMR among enteropathogens, especially from MDR strains, is challenging global prospects for fighting diarrhoeal pathogens (Rashed *et al*., 2012). The emergence and spread of MDR pathogens outstrips the development of drugs, shrinking the therapeutic arsenal (Dantas and Sommmer, 2014). In this study all the isolates were resistant to nitrofurantoin and nalidixic acid and partially resistant to ampicillin. A previous study in Zambia during the 1990-1991 major cholera outbreak showed that there was an emergence of *V. cholerae* O1 strains that were resistant to cotrimoxazole (97%), tetracycline (95%), chloramphenicol (98%) and doxycycline (70%) (Mwansa *et al*, 2007). The high resistance observed with cotrimoxazole supports our findings among the 2009 and 2010 isolates, but interestingly very low resistance to this drug was observed among the 2016 isolates. This can be attributed to the fact that the antibiotic was not used for cholera treatment for many years in Zambia due to previous reports of resistance (Mwansa *et al*., 2007).

In consonance with our findings from all three outbreaks, a Nepalese study reported 100% resistance to nalidixic acid (Dixit *et al*., 2014). High rates of sensitivity to azithromycin were reported in a study carried out in Iran which was in accordance with our findings here where only 1.2% resistance was reported (Barati *et al*., 2015). Unlike the findings in this study high resistance levels to tetracycline (82%) were reported in Mozambique (Baloi *et al*., 2016). In the present study, it was also found that 79% of the strains were MDR with the common pattern being cotrimoxazole-nalidixic acid-nitrofurantoin and nalidixic acid-ampicilin-nitrofurantoin. These findings were similar to a study conducted in Cameroon where the MDR detection rate was 92% (Akoachere *et al*., 2013). There was a drop in MDR detection rate between 2010 and 2016 in our study

(100% to 70%). However, strains with new patterns of resistance emerged with reduced sensitivity to fluoroquinolones among the 2016 isolates. *V. cholerae* strains with reduced fluoroquinolone sensitivity in Africa were first reported in a Zimbabwean study (Islam *et al*., 2009) and later similar findings were reported in Nigeria (Marin *et al*., 2013), Kenya (Njeru *et al*., 2014) and Ghana (Eibach *et al*., 2016). Differences in the antibiotic profile pattern observed during the three outbreaks and emergence of MDR strains may be attributed to spontaneous mutation as a result of abuse of antibiotics and horizontal gene transfer (Shrestha *et al*, 2015).

Molecular epidemiology of human bacterial pathogens provides valuable information for understanding the reservoir, pathogenicity and control of bacterial pathogens (De *et al*., 2013). Macro restriction analysis revealed six clusters among the 2009, 2010 and 2016 outbreak strains, with the 2009 and 2010 outbreak strains clustering together. However, the 2016 outbreak strains formed unique clusters suggesting the emergence of new strains that caused this outbreak. Similar findings were reported in a previous study on outbreak strains isolated between 1996 and 2004 in Zambia, which revealed the emergence of new *V. cholerae* strains in 2004 (Bhuiyan *et al*., 2012). The strains in this study generally exhibited high genetic diversity with one isolate from the 2016 outbreak which was not related to any of the other isolates. This high genetic diversity seem to suggest that the isolates did not have the same origin (Hasan *et al*., 2012).

V. cholerae isolates from the 2009 outbreak were closely related to those from the 2010 outbreak, suggesting that the strains that appeared in 2009 may have continued to cause infection during the 2010 cholera outbreak. Similar findings were reported in India

where strains that caused an outbreak in 2003 were reported to have continued to cause infection in 2005 (Bhotra *et al*., 2016). A Kenyan study reported clonally related strains from different outbreaks between 2007 and 2010 comprised both serotype Ogawa and Inaba (Njeru *et al*., 2014). The existence of clonally related serotype Inaba and Ogawa in our study suggests a serotype switch that occurred between 2009 and 2010 from Ogawa to Inaba. This phenomenon has been reported by other studies carried out in South Africa (Keddy *et al*., 2007) Haiti (Alam *et al*., 2016), India (Torane *et al*., 2016) and Kenya (Njeru *et al*., 2014). The switch from Ogawa to Inaba between is attributed to *V. cholerae* evading Ogawa-induced host immunity thereby causing cholera in individuals who may have previously suffered from the disease (Alam *et al*., 2016). Serotype switch occurs due to a mutational disruption in the methyl transferase encoded wbeT gene due to selective pressure at the level of host's immune response (Lizárraga-Partida and Quilici, 2009; Karlsson *et al*., 2016). The 2016 strains, however, could be a new Ogawa clade causing cholera in Lusaka Province but the confirmation of this was not possible due limitations in resources.

CHAPTER 6

CONCLUSION

6.1 Conclusion

Data in this study demonstrated that the 2009, 2010 and 2016 cholera outbreaks were caused by *V. cholerae* serogroup O1, with Ogawa being the predominant serotype. Serotype switching may have occurred in the three outbreaks. However, it is unclear as to what mechanisms could have triggered this process.

This study revealed MDR *V. cholerae* strains in all three outbreaks with cotrimoxazole, nalidixic acid and nitrofurantoin being the most ineffective drugs for 2009 and 2010 strains, while ampicillin, nalidixic acid and nitrofurantoin were the most ineffective drugs in 2016. An interesting observation was the reversion to cotrimoxazole sensitivity and reduced fluoroquinolone sensitivity in 2016. Despite the detection of MDR strains of *V. cholerae*, cheaper antibiotics such as cotrimoxazole, tetracycline and azithromycin proved to be potent drugs for cholera treatment.

Another significant finding was that *V. cholerae* strains showed high genetic diversity, and therefore, were not from a single source of infection. Source tracking is very important for effective cholera control. Also, the strains that caused the 2009 cholera outbreak also emerged during the 2010 outbreak but new strains of *V. cholerae* emerged during the 2016 outbreak evidenced by the new antibiotic resistance pattern and the PFGE patterns.

This study presents data that underscores the need for close monitoring of *V. cholerae* that causes cholera outbreaks in Zambia. This is important to ensure that the correct antibiotic is chosen according to resistance variations, considering the increasing global burden of cholera, and the emergence and spread of new variants (Dixit *et al*., 2014) that will significantly influence the clinical management of cholera and its prevention.

6.2 Limitations

There were some limitations to this study. The results in this study cannot be generalized to the whole country as only isolates from outbreaks in Lusaka Province were studied.

The sample size in this study was limited by the number of available isolates. This limitation was because only few samples were sent to the laboratory for cholera confirmation.

Owing to the limited resources, biotypes, virulence genes and antibiotic resistance genes could not be determined in this study. This required the use of molecular methods, which are very costly.

6.3 Recommendations and Future Directions

This study was limited to *V. cholerae* isolates collected in Lusaka Province. It would be of interest to include isolates from other parts of Zambia so as to give an accurate picture of the characteristics of the strains circulating in the country. It is important to monitor circulating strains so as to determine their clinical and epidemiological implications.

Future studies should be prospective and include other parts of Zambia that experience cholera outbreaks.

It was evident that in this study most of the *V. cholerae* strains isolated were MDR strains. Hence, an effective national surveillance system for cholera outbreaks should be set up for their monitoring and control as well as control of antimicrobial resistance by the MoH. Furthermore, MoH should endeavour to improve laboratory capacity to improve diagnostic capacity of the country.

It was also not possible to determine virulence and resistance genes due to limitations in the techniques used and associated costs. Whole genome sequencing would provide insights into the common circulating virulence and resistance genes plus many other genes and also track evolutionary changes that have occurred in *V. cholerae* unlike PFGE that is limited to genetic relatedness.

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APPENDICES

Appendix A: CLSI interpretation Guidelines

Antibiotic	Susceptible	Intermediate	Resistant
Tetracycline	≥19	15-18	≤14
Cotrimoxazole	≥16	11-15	≤10
Nalidixic acid	≥19	14-18	≤13
Ampicilin	≥17	14-16	≤ 13
Ciprofloxacin	≥30	21-30	≤20
Norfloxacin	≥17	13-16	≤12
Erythromycin	≥23	14-22	≤13
Nitrofurantoin	≥17	15-16	≤14
Azithromycin	≥17	13-16	≤12

Appendix B: Recipes for Culture Media Used

Alkaline Peptone Water

Suspend 20g of alkaine peptone water powder (HiMedia, India) into 1000ml distilled water. Dispense as desired and autoclave at 15 lbs pressure (121^{O}C) for 15 minutes to sterilize.

Thiosulphate Citrate Bile salts Sucrose (TCBS) agar

Suspend 89.08 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Do not autoclave. Cool to 50^{O}C and pour into sterile petri dishes

Müeller Hinton agar

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{O}C) for 15 minutes. Cool to 50^{O}C, mix well and pour into sterile dishes.