`

CHARACTERIZATION AND SEASONALITY OF ENTERIC BACTERIA AND ANTIBIOTIC RESISTANCE AMONG CHILDREN ≤ 5 YEARS ATTENDING MBAGATHI HOSPITAL, NAIROBI CITY, KENYA

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A Thesis Submitted to the School of Public Health, Biomedical Sciences and Technology in Partial Fulfillment of the Requirements for the Award Degree of Doctor of Philosophy in Biomedical Sciences (Medical Microbiology) of Masinde Muliro University of Science and Technology

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DECLARATION

This thesis is my original work prepaired with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

Signature _____ Date _____

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SBM/H/09/2015

CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and technology a thesis desertation entitled "C**haracterization and Seasonality of Enteric Bacteria and Antibiotic Resistance among Children ≤ 5 Years Attending Mbagathi Hospital, Nairobi City, Kenya**."

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DEDICATION

To the diarrheic children who visited Mbagthi Hospital, Nairobi city, Kenya and consented to participate in the study.

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I thank God for the knowledge and strength He has given me to carry out this research work up to the end. All glory and honour be unto Him. Am grateful to my supervisors: Doctors Christine Wanjala and Benard Guyah for giving me all the advice, guidance and support that I needed enabling the success and completion of this research work. I sincerely acknowledge the children in Nairobi city, Kenya, together with their parents and legally accepted representatives, for their corporation and participation in the study.

May God bless you all.

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ABSTRACT

Statistics have shown that out of one billion childhood diarrhoea infections caused by enteric bacteria globally, 300 million occurred in Africa while 5 million occurred in Kenya in the year 2016. Epidemiology of enteric bacteria shows geographic uniqueness with changing patterns attributed to seasonal environmental changes and poor water, sanitation and hygiene practices. Furthermore, increasing level of enteric bacteria antimicrobial resistance is driven by seasonal changes in inappropriate use of antimicrobials in the management of flu-like illness. However, prevalence and seasonality of bacterial diarrhea and antibiotic resistance among children in Nairobi city, Kenya, remains unknown. Therefore, this cross-sectional study characterized enteric bacteria and antibiotic resistance among diarrheic children (n=374) younger than five years seeking treatment at Mbagathi hospital, Nairobi city, Kenya. Diarrheic children were recruited via systematic random sampling and their socio-demographic information collected using a questionnaire. Written informed consent was obtained from the parent/guardian of the study participants. Approximately 25 grams of stool samples were cultured and identified via gram staining and biochemical tests. Resistant phenotypes were determined by disk diffusion method. Polymerase chain reaction was used for genotyping. A total of 136 (36.4%) children were infected with diarrheagenic *E. coli* harbouring enteroaggregative *Escherichia coli* (EAEC), enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *Escherichia coli* (ETEC) or enteroinvasive *Escherichia coli* (EIEC) single and hybrid virulent gene. *Salmonella* species harbouring *invA, hilA, sopB* or *stn* virulent genes was detected in 9 (2.4%) children. There were 12 (3.2%) children infected with *Shigella* species which harboured *ipaH* virulent gene. *Campylobacter* and *Yersinia* species which harboured *cadF* and *Inv* virulent genes, was isolated from 6 (1.6%) and 5 (1.3%) children, respectively. *Aeromonas* species which harboured *aerA, hlyA, alt,* or *ast* virulent genes was detected in 4 (1.1%) children. There were 9 (2.4%) children co-infected with *Shigella* species and *E. coli*. Prevalence of *E. coli* in particular EAEC and ETEC virulent genotypes and that of *Shigella/E. coli* co-infection was higher during the dry season ($P<0.05$). Most of the phenotypic resistant isolates harboured *citm, bla CMY, aadA1, aac(3)-IV, qnr, catA1, ereA and tetA* genes associated with ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline resistance. Prevalence of phenotypic and genotypic resistant diarrheagenic *E. coli* to ampicillin and erythromycin was lower while that of ciprofloxacin was higher during the dry season ($P<0.005$). *Shigella* phenotypic and genotypic resistance rates to ampicillin, ceftriaxone, streptomycin and erythromycin were higher during the rainy season ($P<0.005$). This study demonstrated the seasonality and relevant contribution of different enteric bacteria in seasonal trends. Therefore, children in Nairobi should be provided with safe water, sanitation and hygiene.

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ACRONYMS AND ABREVIATION

AMR	Antimicrobial resistance
ASBA	Ampicillin Sheep Blood Agar
ATCC	American type culture collection
Bp	Base-pair
BPW	buffered peptone water
BSA	Bismuth Sulfiite Agar
CIN	Cefsulodin-Irgasan Novobiocin
CLSI	Clinical and Laboratory Standards Institute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
GBD	Global Burden of Disease
GEMS	Global Enteric Multicenter Study
HIV	human immunodeficiency virus

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IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
ITCZ	Intertropical Convergence Zone
KDHS	Kenya Demographic and Health Survey
KIA	Kligler iron agar
KOH	Potassium hydroxide
LAIA	Lysine arginine iron agar
LIA	Lysine iron agar
MAM	March April May
MCA,	Maconkey agar
mCCDA	modified charcoal-cefoperazone deoxycholate agar
MDR	Multidrug resistance
MgCL	Magnesium chloride
Min	Minute
MIO	Motility Indole Ornithine Medium
MR	Methyl Red
NaCL	Sodium Chloride
OND	October November December
PCR	Polymerase chain reaction
S	Second
SIM	Sulfide-Indole-Motility
STEC	Shiga toxin-producing *Escherichia coli*
Tris-HCl	Tris (hydroxymethyl) aminomethane (THAM) hydrochloride

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TSA	Trypticase soy agar
UNICEF	United Nations Children's Fund
USA	United States of America
USD	United States Dollar
VP	Voges-Proskauer broth,
WASH	Water, sanitation and hygiene
WHO	World Health Organization
WMA	World Medical Association
XLD	Xylose Lysine Deoxycholate
XLDA	Xylose Lysine Deoxycholate agar
µL	Microliter
µM	Micromolar

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OPERATIONAL TERMS

Dry season:	Dry seasons will be months of June, July, August, September and October.
Genotype:	The genetic constitution of an organism
Phenotype:	The observable physical properties of an organism
sp.:	An unspecified species of the genus.
spp.:	(plural) Indicates "several species".
Wet/rainy season:	Characterized as “long” rain season, occurring in March-April-May, and “short” rains, occurring in the months of October-November-December.

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CHAPTER ONE

INTRODUCTION

1.0 Introduction

This chapter introduces background information relevant to the study of seasonal phenotypes and genotypes of enteric bacteria and antibiotic resistance among diarrheic children. It also presents the problem statement and objectives that will guide the study. Finally, this chapter discusses the justification as to why the study is important and highlights the scope of the study.

1.1 Background Information

Globally, the burden of child deaths due to diarrhoea has already halved since 2000, reducing from 1.5 million to 0.9 million deaths, owing to improvements in coverage of preventive and treatment interventions (UNICEF, 2016). Majority of these deaths occur in resource-limited continents including Africa (GBD, 2018; UNICEF, 2016). While diarrhoea death rate in children younger than 5 years in Sub-Saharan Africa has been declining yearly by approximately 4% from 2000, morbidity and mortality is still high (GBD, 2018; UNICEF, 2016) with nearly 300 billion diarrhoea episodes and half million diarrhoea-associated deaths occurring in Sub-Saharan Africa (GBD, 2018).

Enteric bacterial pathogens accounts for more than 300 million episodes of diarrhoea and one million deaths among children globally, majority of which is caused by *Escherichia coli* and the remainder being *Campylobacter, Shigella, Vibrio* and *Salmonella species* (GBD, 2018). In Kenya, previous study demonstrated that *Shigella, E. coli,* and *Salmonella* species caused diarrhoea in Kilifi county (Njuguna

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et al., 2016), while another study in Kisii, Kisumu and Kericho counties found an association between *Shigella* but not *E. Coli* and Salmonella with diarrhoea (Swierczewski *et al.*, 2013) suggesting geographic uniqueness of gut microbiota in immunity towards enteric infection patterns (Bundgaard-Nielsen *et al.*, 2018; Gupta *et al.*, 2017; Singh *et al.*, 2015). Moreover, previous study found an association between *Campylobacter* and *Shigella* with HIV exposed infected in Kisumu (van Eijk *et al.*, 2010). However, no association was found in Kisii and Homabay counties (Pavlinac *et al.*, 2014). This conflicting finding suggest that HIV infection alters the gut microbiota profile hence immunity functional role towards enteric bacteria (Rinninella *et al.*, 2019; Zilberman-Schapira *et al.*, 2016). There is inconclusive information on enteric pathogens circulating among children in Nairobi. A more recent study in Nairobi city identified *Yersinia enterocolitica* as an aetiology of childhood diarrhoea (Njuguna *et al.*, 2016) while previous a study did not (Boru *et al.*, 2013; Sang *et al.*, 2012) indicating emergence and spread of new strains driven by poor water, sanitation and hygiene (WASH) practices in the city (Njuguna *et al.*, 2016).

Vectors and hosts transmitting enteric pathogenic bacteria are affected by seasonal climate changes, which together, influence vector, host, human and pathogen biology resulting in seasonal cycles of enteric bacterial diseases. For example, high incidence of some enteric bacterial infection in warm season is associated with rapid growth of and consequent mechanical transmision (Asadgol *et al.*, 2019; Bazalova & Dolezel, 2017; Geden *et al.*, 2019). On the other hand, low environmental temperatures reduces expression immune cells including B lymphocytes and immunological cytokines like IFN-γ, IL-10 and IL-1β, thus increasing host susceptibility to enteric

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bacterial pathogens infection (Asadgol *et al.*, 2019; Larsen *et al.*, 2018). Previous study in Pakistan demonstrated seasonal trends in infection with *Vibrio cholera*, *Campylobacter jejuni*, diarrhogenic *Escherichia coli*, *Salmonella* and *Shigella* spp. (Alam *et al.*, 2003) while a study in China did not (Qu *et al.*, 2016) suggesting a complex relationship between the micro-environment with bacterial growth and transmission (Grassly & Fraser, 2006). In Kenya, seasonal trends in enteric pathogens among diarrheic children has been demonstrated in central region (Shah *et al.*, 2016) with substantial seasonal variation across geographic regions (Shah *et al.*, 2017), indicating strong regional variation in seasonal transmission of enteropathogens (Lee *et al.*, 2016). However, there is no information on the seasonal variation of enteric pathogenic bacteria phenotypes and genotypes among children experiencing diarrhoea in Nairobi city, Kenya.

The discovery of antibiotics led to optimism that enteric bacterial infections can be controlled and prevented. However, antibiotic resistant enteric bacteria are still the leading cause of death in developing world including Kenya (Brogan & Mossialos, 2016). Information on enteric pathogens antimicrobial resistant phenotypes and genotypes among diarrheic children in Nairobi city, Kenya, is inconclusive. For instance, previous study reported resistance of *Shigella* and pathogenic *E. coli* isolates to chloramphenicol, streptomycin, trimethoprim/sulfamethoxazole, tetracycline and ampicillin (Njuguna *et al.*, 2016) while a recent study showed changing patterns with increasing resistance (Nyanga *et al.*, 2017). The increasing resistance has been linked to inappropriate antibiotic use in the country where clinicians over-prescribe or under-prescribe antibiotics for non-dysentery in children while drug stores dispense antibiotics without prescription (Muloi *et al.*, 2019; Rhee *et al.*, 2019). This selective

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pressure of antibiotics drives the emergence of antibiotics resistant genes (Munita and Arias, 2016).

What is sure is that antibiotic resistance in pathogenic bacteria is accelerated by inappropriate antimicrobial use. Seasonal changes influence the way antibiotics are used in a population and the consequent development of resistance genes (Suda *et al.*, 2014). For instance, previous studies reported peaks in enteric bacteria antibiotic resistance to antibiotics commonly prescribed for cold and flu during cold and rainy season (Sun *et al.*, 2012; van Hees *et al.*, 2007). This suggest that antibiotics use in bacteria uninfected individuals induces rapid expansion of gut microbiota's antibiotic resistant genes which can be horizontally transmitted to enteric pathogen during infection (Becattini *et al.*, 2016). Seasonal trends in antibiotic consumption has been reported in Nairobi city, Kenya, with high levels of antibiotic treatment for respiratory viral infections during rainy seasons (Omulo *et al.*, 2017). However, there is no information on seasonality of enteric pathogen antibiotic resistant phenotypes and genotypes in Kenya. As such, the present study examined seasonal variation of enteric bacteria phenotypes and genotypes and antibiotic resistance among children experiencing diarrhoea in Nairobi city, Kenya.

1.2 Statement problem

As reviewed above, diarrheal diseases is the second leading cause of mortality in children < 5 years accounting for 1.7 billion episodes and more than 0·9 million deaths globally, most of which occur in developing countries including Kenya. Diarrhoea, caused by enteric bacteria, is still a public health problem in Kenya including Nairobi city (Njuguna *et al.*, 2016). Prevalence of enteric bacteria shows

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geographic uniqueness with emergence and spread of new strains linked to poor water, sanitation and hygiene (WASH) further fuelling diarrhoea epidemiology (Njuguna *et al.*, 2016). Epidemiology of diarrhoea is further complicated by the seasonality of enteric bacteria associated with seasonal climate changes causing distinct seasonal patterns of diarrhoea across geographic regions. Furthermore, there is increasing level of resistance to commonly used antibiotics in the treatment of diarrhoea associated bacteria, in this era where there is a decline in new antibiotics development. This resistance is attributed to inappropriate antibiotic use as well as seasonal changes in antibiotics dispensation and prescription for viral respiratory diseases with peaks observed during the cold rainy season (Sun *et al*., 2012). However, there is scanty information on seasonality of enteric bacterial pathogens phenotypes and genotypes and antimicrobial resistance among children experiencing diarrhoea in Nairobi city, Kenya. This study investigated seasonal phenotypes and genotypes of enteric bacterial pathogens among diarrheic children seeking treatment at Mbagathi Hospital in Nairobi city, Kenya.

1.3 Objective

1.3.1 General objective

To determine the prevalence and seasonality of bacterial diarrhea and antimicrobial resistance among diarrheic children seeking treatment in Mbagathi hospital in Nairobi city, Kenya.

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1.3.2 Specific objectives

1. To determine enteric pathogenic bacteria phenotypes and virulent genotypes among diarrheic children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi City, Kenya.
2. To determine seasonal variation of enteric pathogenic bacteria phenotypes and virulent genotypes among diarrheic children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi city, Kenya.
3. To determine phenotypic and genotypic antibiotic resistance in enteric bacterial pathogens isolated in children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi City, Kenya.
4. To determine seasonal variation of enteric bacterial pathogens antibiotic resistant phenotypes and genotypes among diarrheic children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi, Kenya.

1.4 Research questions

1. What are the enteric bacterial pathogens phenotypes and virulent genotypes among diarrheic children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi City, Kenya?
2. What is the seasonality of enteric pathogenic bacteria phenotypes and virulent genotypes among diarrheic children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi, Kenya?
3. What is the status of phenotypic and genotypic antibiotic resistance of enteric bacterial pathogens in diarrheic children ≤ 5 years seeking treatment in Mbagathi hospital in Nairobi, Kenya?

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4. What is the seasonality of enteric bacterial pathogens antibiotic resistant phenotypes and genotypes among diarrheic children ≤5 years seeking treatment in Mbagathi hospital in Nairobi, Kenya?

1.5 Significance

The phenotypic and genotypic characterization of enteric bacterial pathogens and their antibiotic resistance guides epidemiological studies and public health prevention strategies. In addition, the recognition of seasonal trends in the phenotypes and genotypes of bacterial enteric infections and antimicrobial resistance guides diagnosis, empirical therapy, and enteric bacterial infection control measures. Moreover identifying the seasonal transmission of phenotypes and genotypes of enteric pathogens and antibiotic resistance provides information on how climate variability impact human health. This information is important to the ministry of health programs responsible for disease prevention and promotive. Finally, knowing of seasonal phenotypes and genotypes of enteric pathogens and antibiotic resistance guides clinical management of diarrheic patients.

1.6 Justification

There is limited information on seasonal phenotypes of enteric pathogenic bacteria in children experiencing diarrhoea in Kenya (Shah *et al.*, 2016). However, there is no information on seasonal enteric bacterial genotypes as well as phenotypic and genotypic antimicrobial resistance in Kenya. High prevalence of diarrhoea showing seasonality with clinicians prescribing antibiotics to treat diarrheic infection without secondary infection has been observed among diarrheic children treated at the outpatient department of Mabagathi hospital, Nairobi, Kenya (Njuguna *et al.*, 2016;

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Rhee *et al.*, 2019). Nevertheless, there is no information on seasonal phenotypes and genotypes of enteric bacterial pathogens and antimicrobial resistance among diarrheic children treated at Mbagathi hospital. Thus, it is important to determine the seasonal phenotypes and genotypes of enteric bacterial pathogens and antimicrobial resistance among children seeking treatment at Mbagathi hospital. This guides clinical management of diarrheic patients seeking treatment at Mbagathi hospital.

1.7 Theoretical framework

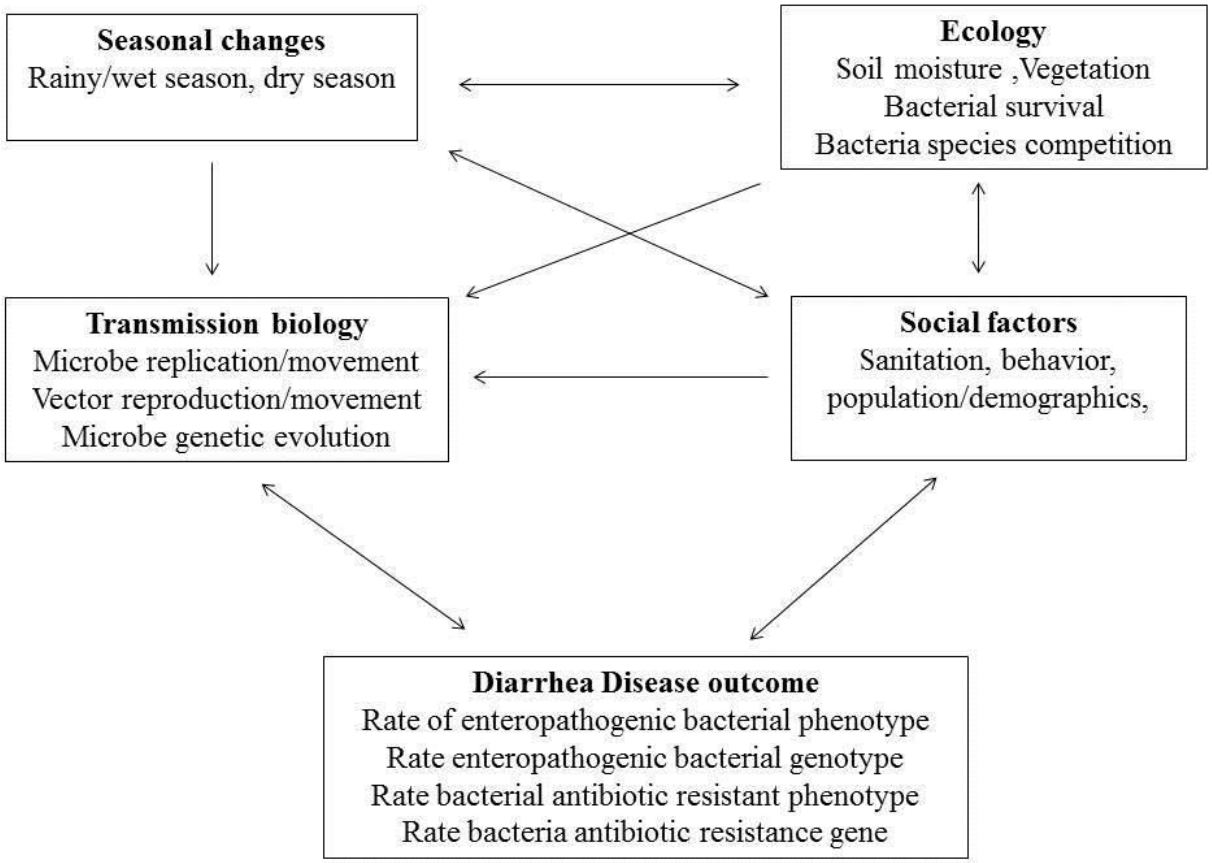

Figure 1.1: The relationship between seasonal changes, social factors and transmission of enteric bacteria phenotypes and genotypes

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There are three basic pathways by which seasonal change affects enteric bacterial pathogens (Figure 1.1). Seasonal change impact transmission of enteric bacteria through influencing bacterial reproduction, distribution and genetic evolution. Seasonal change affects ecosystem of enteric bacteria through influencing the habitat, environment, and competitors of pathogens. The change of environmental factors influence human susceptibility to infection, either as a result of seasonal changes on human biology (immune function) and behavior resulting in direct transmission via person to person contact and indirect transmission via contact with ecological/environmental bacterial reservoirs. As a result, not only the quantity of phenotype and genotypes of pathogens but also the seasonal distributions of pathogens may change.

1.8 Conceptual framework

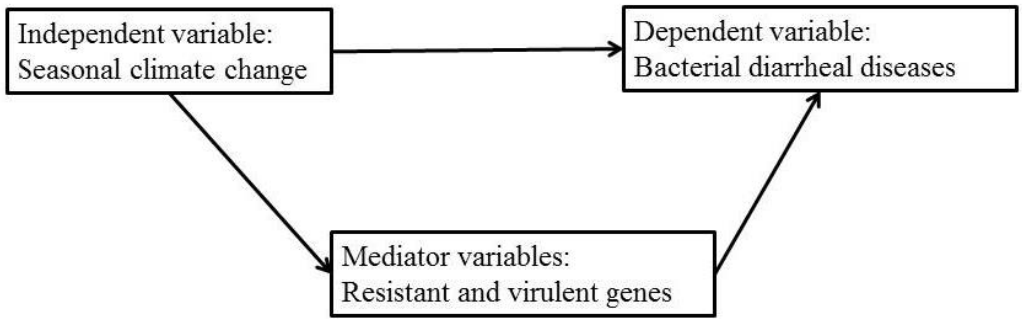

Figure 1.2: Conceptual framework showing the impact of climate change on bacterial disease

Bacterial diarrheal diseases is dependent on seasonal climate change. Climate change can affect human health via indirect pathways particularly via changes in biological and ecological process that influence bacterial disease transmission. Climate change impacts bacterial resistant and virulent genes which in turn impacts bacterial pathogenicity.

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1.9 Scope of the study

There are several ways to genotype bacteria, however, the present study examined the presence of virulent genotypes using polymerase chain reaction technique. There are two ways on how bacteria develop resistance; intrinsic and extrinsic (genetics) mechanism. For the purpose of this study, only extrinsic mechanism was assayed on all phenotypic resistant bacteria for the presence of single antibiotic resistant gene by polymerase chain reaction.

1.10 Limitation of the study

Other studies detected viral, bacterial and parasitic gastroenteritis among diarrheic Kenyan children (Swierczewski *et al.*, 2013; Njuguna *et al.*, 2016), thus it is possible that the bacterial pathogens reported in this study may not be sole cause of the diarrhoea. Study participants were recruited within the hospital hence the prevalence of enteric bacteria and antimicrobial resistance does not represent community prevalence. This study will not establish if the bacterial isolates are community or hospital acquired infections. All resistant and virulent genes were not investigated in this study. Diffusely adherent E. coli (DAEC), another less well-defined E. coli pathotype, was not diagnosed for the difficulties in its identification and classification. Chronic diarrheal as well as HIV infected cases were not identified in this study. Chronicity of diarrhoea was not determined in this study.

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CHAPTER TWO

REVIEW OF RELATED LITERATURE

2.0 Introduction

This chapter contains a review of some of the literature relevant to the study. The main topics reviewed include epidemiology of diarrhea, prevalence and seasonal variation of enteric bacteria and antibiotic resistance phenotypes and genotypes in children.

2.1 Epidemiology of diarrhea

In the year 2016, there were 4.4 billion episodes of diarrhoea globally, almost 1.2 and 3.6 billion, respectively, of those episodes involved children less than 5 years and adults older than 70 years signifying that ageing populations have created further challenges for the control of diarrheal disease (GBD, 2018). Diarrhoea accounted for more than 1.6 billion deaths making it the fifth leading cause of mortality among children less than 5 years (GBD, 2018). In Sub-Saharan Africa, there were more than 1.2 billion cases of diarrhoea, of which 371 million leading to at least 0.2 million deaths occurred in children younger than five years (GBD, 2018; UNICEF, 2016). *Shigella* was the leading aetiology among enteric bacterial infections, accounting for over 269 million episodes of diarrhea, 10.1 million of which occurred in children younger than five years (GBD, 2018). Also, *Aeromonas, Campylobacter, Vibrio, Clostridium,* pathogenic *E. coli* and Non-typhoidal *Salmonella* spp. contributed significantly to diarrhoea episodes and mortality at the global level. In Kenya, approximately 1,499,146 cases of diarrhoea were reported among children under five years annualy (UNICEF, 2016).

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2.2 Enteric bacterial pathogens phenotypes and genotypes

Globally, the highest rates of diarrhoea mortality tend to occur during early and late-life stages (GBD, 2018) and in particular childhood diarrhoea mortality is high in countries with the lowest gross national income (GNI) including Kenya (GBD, 2018; UNICEF, 2016). Enteric bacterial pathogens are the leading cause of diarrhoea and their distribution vary from region to region and even within the same country (Bublitz *et al.*, 2014). Therefore, it is important to determine the prevalence of enteric bacteria pathogens in Kenya children.

Several studies explored the aetiology of bacterial diarrheal diseases in Kenyan children. For example, previous studies showed that *Shigella* species was associated with acute diarrhoea in Siaya, Kericho and Kisumu counties (Shapiro *et al.*, 2001; Swierczewski *et al.*, 2013), whereas diarrhoea in Nairobi county was associated with *E. Coli* and *Salmonella* but not *Shigella* (Njuguna *et al.*, 2016). In Kilifi county, a study reported that *Shigella*, *E. Coli*, and *Salmonella* species were associated with diarrhoea (Njuguna *et al.*, 2016), while a study in Kisii, Kisumu and Kericho counties found an association between *Shigella* but not *E. Coli* and *Salmonella* spp. with diarrhoea (Swierczewski *et al.*, 2013). *Campylobacter* species has been associated with diarrhoea in Kisii county (Swierczewski *et al.*, 2013) but not Siaya County (Shapiro *et al.*, 2001). These variations are attributed to geographical populations' uniqueness of gut microbiota profile that plays an important role in immunomodulation altering the pattern of enteric bacterial infections severity (Bundgaard-Nielsen *et al.*, 2018; Gupta *et al.*, 2017; Singh *et al.*, 2015).

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Studies have also determined aetiology of diarrhoea in children with known HIV status. A study in Kisumu found an association between *Campylobacter* and *Shigella* with HIV exposed infected children (van Eijk *et al.*, 2010). However, no association was found in Kisii and Homabay counties (Pavlinac *et al.*, 2014) suggesting that HIV infection alters the immunity functional role of gut microbiota towards enteric bacteria (Rinninella *et al.*, 2019; Zilberman-Schapira *et al.*, 2016). Taken together, there is a substantial divergence of gut microbiome between HIV infected and uninfected individuals from different geographic regions in Kenya.

The age-associated trend of bacteria has been demonstrated in Kenya. For instance, diarrheagenic *E. coli*, and *C. jejuni* were found more often in children while *Shigella* was isolated more often from adults ≥20 years of age in Kisumu and Kericho counties (Swierczewski *et al.*, 2013). A study involving diarrheic patients in Siaya county, Kenya, reported that *Campylobacter* and *Shigella* predominate in children < 4 years while *Shigella* species predominates in persons ≥ 5 years (Brooks *et al.*, 2003). Another study in Siaya Kenya involving diarrheic patients reported that rates of campylobacteriosis infections were greatest among persons aged <5 years and decreased progressively with increasing age, whereas shigellosis rates were lowest among children and increased progressively with increasing age (Brooks *et al.*, 2006). These differences are attributed to age related changes in gut-microbiota (An *et al.*, 2018), which influences varying geographic patterns of enteric bacterial infection (Rinninella *et al.*, 2019). Taken together, the age-dependent changes in the incidence of enteric bacterial diseases suggest possible role of host-pathogen balance attributed to intestinal microbiota.

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Studies have reported regional disparities in the prevalence of enteric bacteria pathogens among diarrheic children in Kenya. For instance, significantly higher rates of EAEC and ETEC and lower rates of EPEC in Homa Bay County while rates of *Shigella*, *Salmonella* and *Aeromonas* were similar between Homa Bay and Kiambu counties (Shah *et al.*, 2017). Similar rates of *Campylobacter*, *Shigella*, and nontyphoidal *Salmonella* spp. were detected in Kisumu and Siaya counties within the lake region (Beatty *et al.*, 2009). A study reported the prevalence of *E. coli*, *Salmonella*, *Shigella*, and *Vibrio*, respectively, to be 10.4%, 3.0%, 2.2%, and 1.5% in Kisumu county, 12.0%, 2.3%, 2.3%, and 1.1% in Nairobi County, 5.0%, 6.4%, 3.5% and 0.0%, in Kilifi county, and 15.4%, 3.0%, 1.5% and 0.0% in Busia county (Sang *et al.*, 2012). The distinct geographical patterns of enteric bacteria disease appear to be driven by a combination of different ecological and pathogen associated risk factors in the country.

Information on prevalence of enteric bacteria among diarrheic children in Nairobi city, Kenya, is inconclusive. For example, previous study isolated *E. coli*, *Salmonella*, and *Shigella* (Boru *et al.*, 2013) while another study detected *vibrio* in addition to *E. coli*, *Salmonella*, and *Shigella* (Sang *et al.*, 2012). A more recent study isolated *Yersinia enterocolitica* infection associated with poor water, sanitation and hygiene (WASH) practices (Njuguna *et al.*, 2016) suggesting that newly virulent enteric pathogens are emerging in Kenya including Nairobi city. Therefore, this study investigated the prevalence of enteric bacteria among diarrheic children in Nairobi city, Kenya.

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2.3 Seasonal variation of enteric bacterial pathogens in children

Seasonal variation in the incidence of human infection can influence diagnosis and empiric treatment and has the potential to guide both the design and the evaluation of infection prevention interventions. The importance of seasonal variation in community-associated infection incidence is well recognized, particularly for influenza (Newman *et al.,* 2018). Each year, the emergence of seasonal influenza in cold, winter months drives heightened public health initiatives to prevent and control transmission, including surveillance for infections, vaccination of at-risk populations, and educational outreach to health care workers and the public at large (Newman *et al.,* 2018).

While certain hospital-associated pathogens, particularly enteric bacteria, are increasingly recognized as exhibiting seasonal trends in infection incidence among diarrheic children, the information is inconsistent. For instance, according to global enteric multisite study (GEMS) study, EPEC, and ETEC infection rates are high during the rainy season than dry season while *Campylobacter*, *shigella* and *V. cholerae*, show no seasonal associations in rainy and dry season (Chao *et al.*, 2019). A study in Tanzania reported higher infection rates of EAEC and *Shigella* with concomitant lower infection rates ETEC during dry season than rainy season while EPEC showed no seasonal infection trends (Vargas *et al.*, 2004). In Nigeria, ETEC and EPEC were higher during dry season than rainy season while STEC and EAEC were isolated with similar frequencies in both seasons (Onanuga *et al.*, 2014). In China, diarhhegenic *E. coli* and *Shigella*, was higher during summer compared to spring, summer, autumn and winter while *Salmonella* and *Vibrio parahaemolyticus* showed no seasonal variations (Qu *et al.*, 2016). A study in Pakistan reported that

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Vibrio cholera, *Campylobacter jejuni*, Enteropathogenic *Escherichia coli*, *Salmonella* and *Shigella* spp. showed distinct seasonal variation with summer predilection (Alam *et al.,* 2003). The distinctive seasonal pattern of *enteric bacteria* incidence between countries suggests that seasonal changes in the environment which vary geographically contribute to this pattern (Lee *et al.*, 2016). Taken together, seasonal cyclicity is a global feature of enteric bacterial diseases and each enteric bacterial disease has its own seasonal window of occurrence, which, importantly, may vary among geographic locations and differ from other diseases within the same location.

There is a strong variation in distribution of enteric bacterial infections across geographic distinct sub-regions of Kenya as demonstrated by a previous study. For example, a study conducted in Kiambu County reported higher infection rate of diarrhoea caused by EAEC, and *Shigella* during the dry season while infection caused by EPEC, ETEC, *Aeromonas, Salmonella, Yersinia enterocolitica* and *Providencia* species showed no seasonal variations (Shah *et al.*, 2016). Additional analysis revealed higher prevalence of EAEC, ETEC, and *Salmonella* and lower prevalence of EPEC during dry season while the prevalence of ETEC is higher and that of EPEC is lower during rainy season in Homabay County compared to Kiambu County (Shah *et al.*, 2017), suggesting a strong, direct, regional variations of environmental influence on pathogen epidemiology, pathogen reservoirs and transmission pathways or factors that affect frequency of pathogen-host interactions (Lee *et al.*, 2016). Nairobi city has a distinct seasonal patterns of acute diarrhoea (Njuguna *et al.*, 2016), which may be associated with specific enteric bacteria. However, seasonal variation of enteric bacteria pathogens among diarrheic children in Nairobi city, Kenya, remains

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unknown. Therefore, this study investigated seasonal variations of enteric bacterial pathogens among diarrheic children in Nairobi city, Kenya.

2.4 Prevalence of Antibiotic resistance in enteric pathogens among diarrheic children

Global antibiotic statistics suggested an increase of 35% in the antibiotic consumption between 2000 and 2010, and the current antibiotic industry stands at USD 39.8 billion (up to 2015) (Van Boeckel *et al.*, 2014). Russia, India, China, Brazil, and South Africa are major contributing countries, where 76% of the rise in antibiotic consumption has been estimated (Van Boeckel *et al.*, 2014). The changes experienced in the enhanced consumption of antibiotics over the past decade remain unprecedented, and this is chiefly a result of the emergence of new diseases (Khan *et al.*, 2019). The increase in antibiotic consumption and industrialization might be due to overuse or misuse of antibiotics recommended by physicians/self-medication at the time of infection (Muloi *et al.*, 2019; Rhee *et al.*, 2019). A world health organization (WHO) report on the casualties related to antibiotic resistance depicted an alarming 700,000 lives per year currently, and predicts a disturbing 10 million/year by 2050, ensuring that antibiotic resistance will be the most prevalent cause of death (Brogan and Mossialos, 2016). WHO also forewarns the severity of antibiotic resistance, stating that "it threatens the achievements of modern medicine, a post-antibiotic era, in which common infections and minor injuries can kill, is a very real possibility for the 21st century" (Brogan and Mossialos, 2016). Therefore, it is important to continuously monitor antibiotic resistance

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There is increasing information on antimicrobial susceptibility patterns among children with diarrhoea in Kenya. For instance, cross-sectional study in Kisii, Homa Bay, and Migori counties reported that *Shigella* and *Salmonella* spp. are non-susceptibile to cotrimoxazole, ampicillin and tetracycline (Brander *et al.*, 2017). A study in Kiambu reported that higher resistance of enteric bacterial pathogens to amoxicillin, ampicillin, erythromycin and trimethoprim/sulfamethoxazole (Shah *et al.*, 2016). ETEC, EPEC and EAEC, *Salmonella paratyphoid, Shigella flexineri* and *Shigella dysentriae* isolates have been reported to be resistant to amoxicillin, sulphinatozole, cotrimoxazole in Meru county (Karambu *et al.*, 2013). Previous study in Kericho and Kisumu counties reported that *Shigella, Salmonella* and diarrheagenic *E. coli* isolates were multidrug resistant to ampicillin, tetracycline and trimethoprim/sulfamethoxazole with several enteroaggregative and enterotoxigenic *E. coli* isolates producing extended-spectrum beta-lactamases (Swierczewski *et al.*, 2013). A study conducted concurrently in Kilifi, Busia, Kisumu, and Nairobi counties of Kenya reported the highest levels of resistance among the *E. coli* and *Shigella* isolates were observed in ampicillin, trimethoprim/sulphamethoxazole and tetracycline (Sang *et al.*, 2012). This variation in resistance patterns may be attributed to the incidence of antibiotic resistance genes which vary per health practioner geographic area because some Kenyan health practioners prefer empirical therapy to diagnostic antimicrobial resistance testing yet they have inadequate knowledge on local disease epidemiology and susceptibility patterns (Mitsakakis *et al.*, 2018; Odhiambo *et al.*, 2014). Taken together, there is emergence and spread of antimicrobial resistance strains across geographic regions in Kenya attributed to inappropriate use of antibiotics.

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Studies in Nairobi city, Kenya, have shown that enteropathogenic bacteria can develop antimicrobial resistance. A study reported that ETEC isolates were resistant to amoxicillin, ampicillin, erythromycin and tetracycline, *Shigella* and *Salmonella* spp. isolates were resistant to ceftriaxone, trimethroprim sulfamethaxazole, amoxicillin and erythromycin while *Shigella* and *salmonella* spp. were found to be resistant to tetracycline and chloramphenicol, respectively (Boru *et al.*, 2013). However, a more recent study reported that both *E. coli* pathotypes and *Shigella* species were resistant to ampicillin, trimethoprim/sulfamethoxazole, streptomycin, chloramphenicol and tetracycline while gentamycin and kanamycin resistance occurred in diarrheagenic *E. coli* only (Nyanga *et al.*, 2017) indicating that antimicrobial resistance can vary and change with time. It has been reported that human drug stores sell antibiotics to customers without prescription (Muloi *et al.*, 2019) while clinicians over-prescribe or under-prescribe antibiotics for non-dysentery in children (Rhee *et al.*, 2019) at this time when indiscriminate use of antibiotics drives antimicrobial resistance. However, information about prevalence of antimicrobial resistance among diarrheic children in Nairobi city, Kenya, is inconclusive. Therefore, this study investigated the prevalence of antibiotic resistance of enteric bacterial pathogens among diarrheic children in Nairobi city, Kenya.

2.5 Seasonal variations of antibiotic resistance of bacterial pathogens

Seasonal variation of antimicrobial resistance is an important public health problem. This seasonal variation is apparent for multiple antimicrobial classes including penicillin and cephalosporin (Alsan *et al.*, 2015). The rapidity with which resistance in certain organisms has been shown to respond to changes in antibiotic use, as well as the interaction between community antibiotic use and resistance in both the

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community and the hospital, suggests that strong seasonal variation in antibiotic use due to seasonal increases in respiratory diseases, such as influenza (Alsan *et al.*, 2015), may be associated with seasonal changes in drug resistance levels. Thus, it is important to understand seasonality of antibiotic resistance to allow implementation of future public health interventions.

Emerging evidence suggests that antimicrobial resistance (AMR) rates in bacteria show seasonal variation as a results of a dynamic interaction between host and environment, and antibiotic selective pressure. For instance, prescription rates of penicillins, cephalosporins and macrolides for upper respiratory tract infections increased by 75% and 100% in the winter compared to summer (Suda *et al.*, 2014), which was associated with winter-peaks of resistance in *S. pneumoniae* to penicillins and cephalosporins (Iroh Tam *et al.*, 2015), whereas AMR rates to macrolides showed no seasonal variation (Gherardi *et al.*, 2015). A study in the United States showed that the rates of penicillin-resistant *S. pneumoniae* were higher in spring than in winter (Hoberman *et al.*, 2005), while other studies in Spain showed higher resistance rates in both summer and winter (Baquero *et al.*, 1999; Marco *et al.*, 2000). On the other hand, a study done in Israel and Lituania reported higher resistance rates of multidrug-resistant *S. pneumoniae* in winter than in summer (Dagan *et al.*, 2008; Staceviciene *et al.*, 2016). Furthermore, resistant rates of *H. influenzae* to penicillins and macrolides tended to be higher in winter than in summer in a study done in Japan (Hashida *et al.*, 2008), while another study from Italy did not find significant differences in resistance rates between autumn and spring (Marchisio *et al.*, 2001). Taken together, the variability in seasonality of antimicrobial resistance rates is

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attributed to seasonal variation in patterns and levels of antibiotic prescription for flu-like illnesses across geographic regions (Suda *et al*, 2014).

Enteric bacterial pathogens also show seasonal variation in susceptibility to antibiotics. A study in Spain reported resistance of E. coli to ciprofloxacin and amoxicillin-clavulanic acid was significantly higher in autumn and winter (Asencio Egea *et al.*, 2018). However, In USA, a study reported resistant rates of Escherichia coli to ampicillins and ciprofloxacin was higher during winter season (Sun *et al.*, 2012) while another study reported no significant seasonality in resistance rates throughout the year (Ramsey *et al.*, 2019) suggesting geographic variation in flu-associated antibiotic use (Alsan *et al.*, 2015). Previous study reported seasonal variation in antibiotic consumption in Nairobi city, Kenya, with health facilities and pharmacies prescribing or dispensing antibiotic to patients with cold or influenza infections during rainy seasons (Omulo *et al.*, 2017) which is likely to drive antimicrobial resistance. However, there is no information on seasonal variation of antibiotic resistance in Kenya. Therefore this study will determine seasonal variation of antibiotic resistance of enteric bacterial pathogens among diarrheic children in Nairobi City, Kenya.

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CHAPTER THREE

METHODOLOGY

3.0 Introduction

This chapter describes the methods used to answer the research questions of the study. The main themes include description of the study area, study design, study subjects and sample, permission for data collection and ethical considerations, procedures for data collection, and data analysis.

3.1 Study site

This study was conducted at Mbagathi county Referral Hospital, Nairobi city, Kenya (Appendix 1). The city is situated at 1°09′S 36°39′E and 1°27′S 37°06′E and occupies 696 square kilometres. It borders Kiambu County to the North and West, Kajiado to the South and Machakos to the East. Among the three neighbouring counties, Kiambu County shares the longest boundary with Nairobi County. Nairobi has a tropical climate with two seasons in a year referred to as wet (rainy) and dry season. There are the two rainy seasons: the "long rains" from March to May, and the less intense "short rains" from October to December (Shilenje and Ogwang, 2015).

Mbagathi hospital was chosen because of the high prevalence of diarrhoea of 17% among children (MoH, 2015a). The high prevalence of diarrhoea in children visiting Mbagathi hospital is due to poor water, sanitation and hygiene practices in Nairobi city (Njuguna *et al.*, 2016). In addition, distinct seasonal patterns of diarrhoea, which may be associated with enteric bacteria, has been reported among patients seeking treatment at Mbagathi hospital in Nairobi city, Kenya (Njuguna *et al.*, 2016). Finally, there is variation in seasonal consumption of antibiotics with increasing prescription

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and dispensation for cold/flu treatment during the rainy seasons among patients seeking treatment at Mbagathi hospital (Omulo *et al.*, 2017) which is likely to drive distinct seasonal pattern in antimicrobial resistance. Thus, by making an impact in such an area, it's certain that the knowledge acquired from this population can be extrapolated to other regions of Kenya for improved public health.

3.2 Study population

Diarrhogenic children under five years of age seeking treatment for diarrheal illness at Mbagathi county Hospital, Nairobi County, Kenya.

3.2.1 Inclusion criteria

Diarrhogenic children under five years of age seeking treatment for diarrheal illness at Mbagathi county Hospital, Nairobi County, Kenya and whose parents or guardians gave consent were recruited in the study.

3.2.2 Exclusion criteria

Diarrheic children under five years who had previously taken antibiotics within 72 hours of the onset of symptoms were excluded from the study. In addition, Children of guardians who were previously interviewed for this study were excluded. Parents who did not give consent were excluded from the study.

3.3 Study design

This was a hospital based cross-sectional study. Diarrhoea was defined, according World Health Organization (WHO) guidelines as the occurrence of three or more loose, liquid, or watery stools in a 24-hour period (WHO, 2009).

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3.4 Sampling technique

Sample selection was done using the systematic random sampling where the first unit (case) was selected randomly among diarrheic children seeking treatment in the hospital. The k^{th} case after the starting point followed a systematic selection. The k^{th} case represents the sampling interval which was calculated by dividing the approximate population (N) of children with diarrhoea in Nairobi reported to be 1,920 (MoH, 2015a) by the sample size (n) of 374 (Raina, 2015). Therefore, every 5^{th} case of diarrhoea was selected until a sample size of 374 is reached.

3.5 Sample size determination

The sample size was calculated using prevalence of enteric bacteria reported to be 58.2%, in a study involving diarrheic children in Nairobi, Kilifi, Kisumu and Busia, counties in Kenya (Iijima *et al.*, 2017). The sample size was determined using the formula $n = Z^2pq/d^2$ (Zelelie *et al.*, 2019).

Where n = the sample size required,

z = 1.96: confidence level test statistic at the desired level of significance,

p = 58.2%: prevalence of enteropathogenic bacteria among diarrheic children,

q=1-p: proportion of diarrheic children without enteropathogenic bacteria infections,

d = 0.05: acceptable error willing to be committed.

$n= (1.96^2 \times 0.582 \times 0.418) \div 0.05^2$

The optimum sample size estimated was n = 374.

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3.6 Determination of Seasons

The Inter-tropical Convergence Zone (ITCZ) dictates the distribution and spread of the rain regime creating two distinct wet seasons annually in Kenya (Shilenje and Ogwang, 2015). The wet seasons are characterized as “long” rain season, occurring in March-April-May (MAM), and “short” rains, occurring in the months of October-November-December (OND) (Shilenje and Ogwang, 2015). Monthly meterological parameters (precipitation and temperature) was acquired from the the World Meteorological Organisation which provides official weather observations, weather forecasts and climatological information from the National Meteorological and Hydrological Services of Member states including Kenya (http://climexp.knmi.nl/start.cgi). Since Kenya has a tropical climate, a dry season month was defined as a month with an average precipitation level below 60 millimetres based on the Koppen climate classification system (Beck *et al.*, 2018).

3.7 Demographic and clinical data

A questionnaire was used to obtain information on age, gender, travel history, nausea, vomiting, abdominal pain, and diarrhoea history of the children from the parents/guardians (Appendix 4). Additional information on occupation of the guardian and water source and treatment was be recorded on questionnaire. Clinical features such as body temperature, nutritional status, and dehydration signs were collected by clinicians.

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3.8 Isolation, identification and virulotyping of enteric bacterial pathogens

3.8.1 Isolation, identification and virulotyping of *E. coli*

3.8.1.1 Isolation and identification of *E. coli*

Approximately 25g of stool sample was mixed with 5 ml of sterile peptone water and streaked on MacConkey Agar and Xylose Lysine Deoxycholate Agar. After 24 hours of incubation under aerobic atmosphere at 37°C. Non-mucoid rose red colonies on MacConkey Agar and yellow colonies on Xylose Lysine Deoxycholate Agar were identified as *E. coli* based on colony characteristics. Presumptive *E. coli* colonies were purified and maintained on Tryptic Soy Agar (TSA) slant for biochemical, virulotyping and drug sensitivity tests.

Presumptive *E. coli* colonies were confirmed with biochemical tests using Gram's stain, Sulfide Indole motility medium, Kligler iron agar, Lysine deoxycholate agar, Simmons citrate agar and Christensen urea agar. *E. coli* isolates were gram-negative, motile, hydrogen sulphide-negative, indole-positive, Lysin decarboxylase-positive, Simmons citrate-negative, dextrose-positive, lactose-positive, and urease-negative

3.8.1.2 Virulotyping of *E. coli*

DNA was extracted from 24 hour old colonies using the QIAamp DNA Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's instruction. Bacterial cells were emulsified in 250 µl lysis buffer (0.1 M NaCl, 50 mM disodium EDTA, 0.1 M Tris-HCl, pH 8) containing 0.5 % (w/v) sodium dodecyl sulfate 0.5% (w/v), proteinase K (0.5 mg/ml), and RNase (0.8 mg/ml) and incubated in a water bath at 60°C for 5 min followed by centrifugation at 1000 X g for 60 seconds. Two hundred microlitres of the supernatant was placed in a new eppendorf tube and overlaid with

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30 µl of 6 M NaCl and 2 volumes of room temperature 70 % ethanol. The tube was left to stand at room temperature for five minutes before centrifugation at 1000 X g for three minutes. The supernatant was discarded and the crude DNA rinsed with 70 % ethanol and dried out by leaving the eppendorf tubes open for a few minutes in the clean bench. Finally, the DNA was suspended in 200 µl of sterile water and stored at −20 °C until use in PCR.

The *E. coli* virulent genes were determined using multiplex polymerase chain reaction (PCR) with the primer sets in table 3.1. Multiplex PCR was performed using virulence genotype-specific primers to detect *aspU, aggR,* and *pcvd432* for EAEC, *est* and *elt* for ETEC, *eae* for EPEC, stx for EHEC, and *ipaH for* EIEC. PCR reaction was carried out with 2.5 µL of the template DNA added to 47.5 µL mix containing, DreamTaq Green PCR Master Mix, nuclease free water and 1.0 µMof each primer. The PCR amplification consisted of 2 min at 94ºC denaturing temperature, followed by 40 cycles of 30 sec at 92ºC denaturing temperature, 30 sec at 59ºC annealing temperature, and 30 sec at 72ºC extension temperature. The PCR products were visualized under ultraviolet light using a 2% agarose ethidium bromide-stained gel (appendix 2).

3.8.2 Isolation, identification and virulotyping of *Salmonella* species

3.8.2.1 Isolation and identification of *Salmonella* species

For identification of *Salmonella* species, about 25g samples were dissolved in about 200mL of sterilized buffered peptone water (BPW), and incubated at 37ºC for 16-20 hours. About 10mL from the incubated BPW culture was selectively enriched into the 100mL sterilized Selenite Cystine Broth and incubated again at 37ºC for 24-48 hours.

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After incubation, 1 loop full inoculum from the selective enrichment culture was streaked onto the pre-incubated Bismuth Sulfiite Agar (BSA) and Xylose Lysine Deoxycholate (XLD) agar plate. Black colonies on Bismuth Sulfiite Agar (BSA) and red to pink (since the background is red) with black centre on Xylose Lysine Deoxycholate (XLD) agar were identified as *Salmonella* species. Presumptive *Salmonella* colonies were purified and maintained on Tryptic Soy Agar (TSA) slant for biochemical, virulotyping and drug sensitivity tests.

Biochemical tests performed to confirm *Salmonella* species using Gram's stain, nutrient broth, lysine iron agar, Methyl Red (MR) and Voges-Proskauer (VP) broth, Simmons citrate agar, Kligler iron agar (KIA), Sulfide-Indole-Motility (SIM), Christensen urea agar and Motility Indole Ornithine Medium (MIO). *Salmonella* species were gram-negative, motile, lysine decarboxylase-positive, Methyl Red-positive, Voges-Proskauer-negative, citrate-negative, hydrogen sulphide-positive, dextrose-postive, lactose negative, indole-negative, urease-negative and Ornithine decarboxylase-negative. Further confirmation of biochemical reactive cultures was done by agglutination test with Salmonella polyvalent (O) somatic antisera. Isolates with a typical biochemical profile, which agglutinate with both H and O antisera were identified as *Salmonella* spp.

3.8.2.2 Virulotyping of *Salmonella* species

DNA was extracted from 24 hour old *Salmonella* culture as described above. All *Salmonella* isolates were screened for the presence or absence of *invA, hilA, sopB,* and *Stn* virulent genes. The primers and the size in base pairs of the respective amplification products of the four virulence genes are presented in Table 1. The

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optimized multiplex PCR reaction mixture (25 μL) contained 2 μL of DNA template, 5 μL of 5 × PCR buffer, 2.5 μL of 25 mM MgCl2, 0.5 μL of 10 mM deoxynucleotide triphosphate (dNTP), 0.5 μL of 1.2 μM primer mix and 14.2 μL of deionized water. The mixture was then treated with 0.3 μL (1.5 U) Taq DNA polymerase. PCR amplification was performed with the following conditions: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were electrophoresed in a 2% agarose gel and visualised under ultraviolet light (appendix 2).

3.8.3 Isolation, identification and virulotyping of *Shigella* species

3.8.3.1 Isolation and identification of *Shigella* species

About 25g of the stool sample was transferred to Selenite F broth and incubated at 37°C for 24 hrs. Simultaneously, a loop full of stool sample suspension was streaked on MacConkey Agar and xylose lysine deoxycholate agar (XLDA) and then incubated at 37°C for 24 hours. Furthermore, culture negative specimens on MacConkey agar and xylose lysine deoxycholate agar (XLDA) media were sub-cultured from Selenite F broth enrichment broth to MacConkey Agar (MCA) and xylose lysine deoxycholate agar (XLDA) plates to improve recovery of the isolates. Growth of *Shigella* species were detected by their typical red colony characteristic on xylose lysine deoxycholate agar (XLDA) and convex and colourless on MacConkey agar (MCA). Pure presumptive *Shigella* isolates were maintained in Tryptic Soy Agar (TSA) slant for biochemical, virulotyping and drug sensitivity tests.

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Biochemical test of *Shigella* isolates was performed using Gram's stain, motility agar, Kligler iron agar (KIA), urea agar, lysine iron agar (LIA), citrate medium and indole media. Shigella isolates were non-motile, gram-negative, urease-negative, hydrogen sulphide-negative, indole-variable, lactose fermenting-negative and citrate negative. *Shigella* biochemical reactive cultures were further confirmed by slide agglutination test using polyvalent somatic (O) antigen grouping sera.

3.8.3.2 Virulotyping of *Shigella* species

DNA was extracted from 24 hours old cultures as described above. Primer sequences used in amplification of *ipaH* gene and product size are presented in table 3.1. PCR reaction comprised of 25 µl reaction volume, containing 1X Buffer, 2.0 mM MgCl, 0.5 U Taq polymerase (Promega), 5% DMSO (Sigma Aldrich) 0.2 mM each dNTPs, 0.004 mM *ipaH* primers, 0.016 mM ial primers and 10 µl of DNA template. The reaction mixture was placed in a thermocycler for 30 cycles, 10 cycles in PCR touch down (each cycle consisting of 1 min at 94ºC, 30 s at 65ºC decreasing to 55ºC, and 1 min at 72ºC) and last 20 cycles in normal PCR (1 min at 94ºC, 30 s at 55ºC and 1 min at 72ºC). An additional step at 5 min at 72ºC for primer extension was added at the end of the reaction. The PCR products were detected by 2% agarose gel electrophoresis (appendix 2).

3.8.4 Isolation, identification and virulotyping of *Yersinia* species

3.8.4.1 Isolation and identification of *Yersinia* species

About 25g of stool sample was aseptically transferred into 225ml Peptone sorbitol bile broth (PSBB), homogenized for 30 seconds and incubated immediately at 10°C for 10 days. The enrichment broth was removed from the incubator and mixed well.

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One loop-full of enrichment broth was transferred to 0.1 ml 0.5% KOH in 0.5% saline and mixed for 3 seconds. One loop full was then streaked on MacConkey plate and another to Cefsulodin-Irgasan Novobiocin (CIN) plate and both were incubated at 30ºC for 24-48 hours. *Yersinia* species colonies on Cefsulodin-Irgasan Novobiocin (CIN) had deep red center with sharp border surrounded by clear colorless zone with entire edge. *Yersinia* species was characterised by flat, colorless, or pale pink colonies on MacConkey agar. Biochemical test was done using Gram's stain, oxidase test reagent, motility test medium, Lysine arginine iron agar (LAIA) slant, Christensen's urea agar plate, and Bile Esculin agar plate by stabbing with inoculation needle and incubated for 48 hours at room temperature. Isolates giving alkaline slant and acid butt, no gas and no hydrogen sulphide reaction in Lysine arginine iron agar (LAIA), which are also urease-positive, motile, gram negative are presumptive *Yersinia*.

3.8.4.2 Virulotyping of *Yersinia* species

DNA was extracted from 48 hours old cultures as described above. Primers specific to the *inv* gene of Yersinia species is listed in table 3.1. PCR reactions were performed in 50-µL volumes containing 5 µL of DNA template, 0.2 mM concentrations of deoxynucleoside triphosphates, 5 µL of 10× PCR buffer, 3 mM MgCl2, 1 µM concentrations of each forward and reverse primer, 1.25 U of Taq DNA polymerase. The thermal cycling conditions were as follows: 1 cycle of denaturation at 95°C for 10 min; 25 cycles of melting at 95°C for 45 s, annealing at 60°C for 60 s, elongation at 72°C for 70 s; and a final extension at 72°C for 10 min. Amplified samples were analyzed by electrophoresis (120 V, 208 mA) in 2% agarose gel and stained by ethidium bromide. A molecular weight marker with 100-bp increments (100-bp ladder) was used as the size standard (appendix 2).

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3.8.5 Isolation, identification and virulotyping of *Aeromonas* species

3.8.5.1 Isolation and identification of *Aeromonas* species

About 25g of stool samples were inoculated in alkaline peptone water and incubated at 25ºC for 18 hours. One loop full of the enriched inoculum from the alkaline peptone water culture was streaked on Ampicillin Sheep Blood Agar (ASBA) and incubated at 35 ºC for 24 hours. *Aeromonas* appeared as smooth, convex, grayish colonies on Ampicillin Sheep Blood Agar (ASBA). Isolates that were Gram-negative rods and cytochrome oxidase positive were tested for motility, indole, oxidase, urease, Voges Proskauer, arginine dehydrolase, esculin hydrolysis, ornithine decarboxylase-negative, catalase, Lysine Decarboxylase, citrate and gas production from glucose. *Aeromonas* isolates were motile, indole-postive, oxidase-positive, urease-negative, Voges Proskauer-positive, arginine dehydrolase-negative, esculin hydrolysis-positive, ornithine decarboxylase-negative, catalase-positive, lysine decarboxylase-positive, citrate-positive and produced gas from glucose.

3.8.5.2 Virulotyping of *Aeromonas* species

DNA was isolated using commercially available kit as described above. The primer sequence used to amplify *aerA, hlyA, alt,* and *Ast* genes and the PCR product size are presented in table 3.1. DNA samples (5 ng per reaction mixture) were amplified in a 25-µl reaction mixture consisting of 50 mM potassium chloride; 10 mM Tris chloride; 1.25 mM magnesium chloride; 200 µM (each) dATP, dCTP, dGTP, and dTTP; 2.0 µM of each primer; and 1.25 U of Taq DNA polymerase. Parameters for the amplification included an initial denaturation at 95°C for 5 minute, followed by 50 cycles of denaturation at 95°C for 0.5 minute, annealing of the primers at 59°C for 0.5

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min, and primer extension at 72°C for 0.5 minute. A final extension at 72°C for 7 minute was used. Eight microliters of PCR product mixed with 5x gel loading dye was loaded onto 2% agar gel in 0.5x Tris-Borate-EDTA buffer, and a 100 bp DNA ladder was used as a molecular weight marker. Gels were visualized by a UV transilluminator (appendix 2).

3.8.6 Isolation, identification and virulotyping of *Campylobacter* species

3.8.6.1 Isolation and identification of *Campylobacter* species

About 25g of stool samples were homogenized in sterile thioglycollate broth. Broth samples were incubated at 42 °C for 48 hours in a microaerophilic atmosphere (10% CO_2, 5% O_2 and 85% N_2). A loopful of enrichment broth was streaked onto modified charcoal-cefoperazone deoxycholate agar (mCCDA) and 5% blood agar and incubated under microaerophilic conditions at 42 °C for 48 hours. Colonies from the plates were checked for gram stain (slender, curved, "gull wing" shaped Gram-negative rod), motility, oxidase and catalase tests, microaerobic growth at 25 and 42°C, and indoxyl acetate hydrolysis. The recognized colonies were stored at -70 °C in thioglycolate broth containing 15% glycerol for further validation using molecular methods.

3.8.6.2 Vurolotyping of *Campylobacter* species

DNA was extracted from cultured isolates as described above. The primer sequence used in the amplification of *cadF* gene of *Campylobacter* species is presented in table 3.1. The PCR was carried out in a 25-µL reaction mixture, containing 10ng of DNA template extracted by the boiling method, 2.5 µL PCR buffer 10X, 200 µM dNTP, 5 mM MgCl2, 0.1 µM of each primer, 1 unit of Taq DNA polymerase, and

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sterile deionized water. Amplification conditions were 95°C for three minutes (one cycle), then denaturation at 94°C for 30 seconds, annealing at 43°C for 30 seconds and extension at 72°C for 30 seconds for 32 cycles in a thermocycler. Finally, an additional extension step (five minutes, 72°C) was carried out. ven in table 1) for 1 min and extension at 720C for 1 min. The PCR product was analysed by electrophoresis on 2% agarose gel. DNA bands were stained with ethidium bromide and visualized (appendix 2).

Table 3.1: Genotyping primers

Primer sequences	**Genotype**	**Species**	**Fragment size**
F: CCCGAATTCGGCACAAGCATAAGC R: CCCGGATCCGTCTCGCCAGTATTCG	EPEC	*E. coli*	881bp
F: GAGCGAAATAATTTATTATGTG R: TGATGATGGCAATTCAGTAT	EHEC	*E. coli*	518bp
F: TTAATAGCACCCGGTACAAGCAGG R: CCTGACTCTTCAAAAGAGAAAATTAC	ETEC	*E. coli*	147bp
F: TCTCTATGTGCATACGGAGC R: CCATACTGATTGCCCGCAAT	ETEC	*E. coli*	322bp
F: GTTCCCTTGACCGCCTTTCCGATACCGTC R: GCCGGTCAGCCACCCTCTGAGAGTAC	EIEC,	*E. coli*	600bp
F: GTATACACAAAAGAAGGAAGC R: ACAGAATCGTCAGCATCAGC	EAEC	*E. coli*	254bp
F: AGACTCTGGCGAAAGACTGTATC R: ATGGCTGTCTGTAATAGATGAGAAC	EAEC	*E. coli*	194 bp
F: GCCTTTGCGGGTGGTAGCGG R: AACCCATTCGGTTAGAGCAC	EAEC	*E. coli*	282 bp
F: GTTCCCTTGACCGCCTTTCCGATACCGTC R: GCCGGTCAGCCACCCTCTGAGAGTAC	*IpaH*	*Shigella*	600bp
F: ACCACGCTCTTTCGTCTGG R: GAACTGACTACGTAGACGCTC	*invA,*	*Salmonella*	942bp
F: TGTTTCCGGGCTTGTGCT R: CAGGGCATTTGCTGATTCTTCC	*hilA,*	*Salmonella*	854bp
F: -AGCATCTCTAAACGCTACTG R: GCTTCTATCACTCAGCTTCA	*sopB,*	*Salmonella*	470bp
F: ATTGAGCGCTTTAATCTCCT R: GCTGTTGAATCTGTACCTGA	*Stn*	*Salmonella*	543bp
F: TGGAGGGTAATTTAGATATG R: CTAATACCTAAAGTTGAAAC	*cadF*	*Campylobacter*	400bp
F: CTGTGGGGAGAGTGGGGAAGTTTGG R: GAACTGCTTGAATCCCTGAAAACCG	*Inv*	*Yersinia*	570bp
F: CCTATGGCCTGAGCGAGAAG	*aerA,*	*Aeromonas*	309bp

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R: 59-CCAGTTCCAGTCCCACCACT			
F: GGCCGGTGGCCCGAAGATACGGG	*hlyA,*	*Aeromonas*	900bp
R: GGCGGCGCCGGACGAGACGGG			
F: TGACCCAGTCCTGGCACGGC	*alt*	*Aeromonas*	148bp
R: GGTGATCGATCACCACCAGC			
F: TCTCCATGCTTCCCTTCCACT	*Ast*	*Aeromonas*	331bp
R: GTGTAGGGATTGAAGAAGCCG			

EPEC, enteropathogenic *E. coli*. ETEC, enterotoxigenic *E. coli*. EAEC, enteroaggregative *E. coli*. EIEC, enteroinvasive *E. coli*. EHEC, enterohaemoragic *E. coli*. Bp, base pairs.

3.9 Antimicrobial resistance phenotyping

Antibiotic susceptibility was performed using Kirby-Bauer disk diffusion method on Mueller Hinton agar by incubating at 37°C for 18 hours (Humphries *et al.*, 2018). Antibiotic discs of ampicillin (10µg), ceftriaxone (30µg), streptomycin (10µg), gentamycin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (15 µg), and tetracycline (30 µg) were used. Broth turbidity was made to match with 0.5 McFarland standards. According to the size of the zone of inhibition, the organisms were classified as sensitive, intermediately sensitive, or resistant to each antibiotic using Clinical Laboratory Standard Institute interpretation guideline (Humphries *et al.*, 2018). *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeroginosa* ATCC 27853 were used as quality control strains.

3.10 Genotyping antimicrobial Resistance

The isolates were grouped on the basis of resistance phenotype and determined for the presence of corresponding antibiotic resistance genes. The presence of resistance genes to ampicillin: *citm*, ceftriaxone: *bla CMY*, streptomycin: aadA1, gentamycin: *aac(3)-IV*, ciprofloxacin: *qnrA1*, chloramphenicol: *catA1*, erythromycin: *ere(A)* and tetracycline: *tet(A)* were detected by single PCR using

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primers in table 3.2. All the reactions were prepared by using 2 µl template DNA, 12.5 µl 2X PCR master mix (0.04 U/µl Taq DNA polymerase, PCR buffer, 3 mM MgCl2, 0.4 mM of each dNTP), and 0.4 µM of each primer in a volume of 25 µl. Amplification reactions were carried out as follows: Five min at 95°C, 35 cycles each consisting of 1 min at 94°C, 30 s at ~55°C and 1 min at 72°C, followed by a final extension step of 5 min at 72°C. Amplified samples were analyzed by electrophoresis in 2% agarose gel and stained by ethidium bromide (appendix 3).

Table 3.2: Antimicrobial resistance primers and amplicon size

Antibiotic type	**Antibiotic resistant gene**	**Primer sequence**	**Amplicon size**
Ampicillin	*Citm*	F: TGG CCAGAACTGACAGGCAAA R: TTT CTC CTG AAC GTG GCT GGC	462bp
Ceftriaxone	*bla CMY*	F: CAATGTGTGAGAAGCAGTC R: CGCATGGGATTTTCCTTGCTG	462bp
Streptomycin	*aadA1*	F: TATCCAGCTAAGCGCGAACT R: ATTTGCCGACTACCTTGGTC	447bp
Gentamycin	*aac(3)-IV*	F: CTTCAGGATGGCAAGTTGGT R: TCATCTCGTTCTCCGCTCAT	286bp
Ciprofloxacin	*Qnr*	R: GGGTATGGATATTATTGATAAAG R: CTAATCCGGCAGCACTATTTA	670bp
Chloramphenicol	*catA1*	F: AGTTGCTCAATGTACCTATAACC R: TTGTAATTCATTAAGCATTCTGCC	547bp
Erythromycin	*(ere(A))*	F: GCCGGTGCTCATGAACTTGAG R: CGACTCTATTCGATCAGAGGC	419bp
Tetracycline	*(tet(A))*	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	577bp

A, adenine. T, thymine. C, cytosine. G,guanosine. Bp, base pair.

3.11 Data analysis

Data collected was entered into excel, cleaned, coded and exported into SPSS. Statistical analyses was performed using SPSS version 19.0 for Windows (IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.). Descriptive

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statistics, namely frequencies and percentages, was used to present demographic and clinical data, phenotypes and genotypes of enteric bacterial pathogens and antimicrobial resistance. Chi-Square test was used to compare frequencies of enteric phenotypes and virulent genotypes, resistant phenotypes and genotypes between the rainy and dry seasons. Statistical analysis was set at $P < 0.05$.

3.12 Ethical consideration

This study was ethically approved by Masinde Muliro University of Science and Technology Institutional Ethical Review Committee (appendix 5). Research permit was obtained from National Commision for Sience technology and Innovation (appendix 6). This study was conducted according to Helsinki declarations (WMA, 2014). A consent form was read and signed by either parent or guardian of each child (Appendix 4). Study participants benefitted from free health edition and bacterial diagnosis. The findings of this research were disseminated through publications and presentations to the Ministry of health. All children were attended to by qualified health practitioners. Diarrheic children were treated by clinicians according to World Health Organization guidelines for treatment of diarrhea in children (WHO, 2005). All study participants' information and test results remained confidentially throughout the study period. All study forms were stored in lockable carbinets with access limited only to the study investigators.

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CHAPTER FOUR

RESULTS

4.0 Introduction

This chapter presents the meteorological factors of the study area during the study period. Also, the results of the study objectives are presented.

4.1 Meteorological factors

In Nairobi, monthly average temperature varied between 18.0 °C and 23.2 °C with a mean of 20.2 °C (Figure 4.1). Monthly maximum temperature ranged from 23.1°C to 29.8°C with a mean of 25.7 °C. Monthly minimum temperature ranged from 12.2 °C to 16.9 °C with a mean of 14.7 °C. Monthly precipitation level varied between 0 mm and 233.6 mm during the study period (Figure 4.2). Nairobi experienced dry and wet seasons, dry seasons were in the months of February to March and June to October, 2016 (precipitation < 60.0 mm) with the rest of the months being wet (precipitation > 60.0 mm). Long rain seasons were between April to May, 2016 and the short rains occurred in November, 2015 and 2016, corresponding with the country's historical climate profile of the "long rains" season from March to May, and the less intense "short rains" from October to December (Shilenje and Ogwang, 2015).

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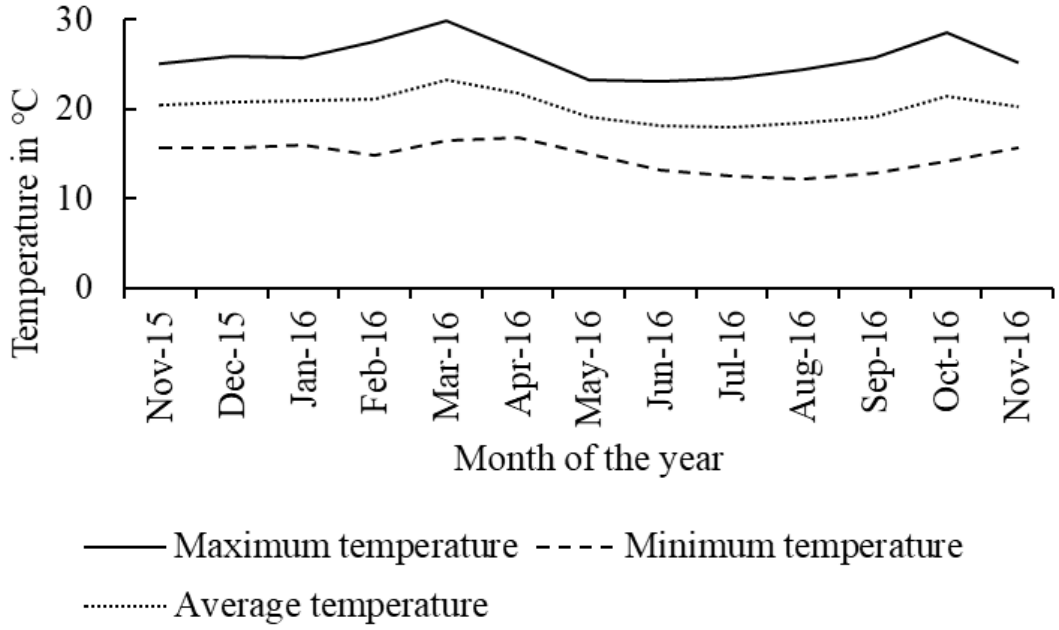

Figure 4.1: Maximum, minimum and average temperatures between November 2015 to November 2016.

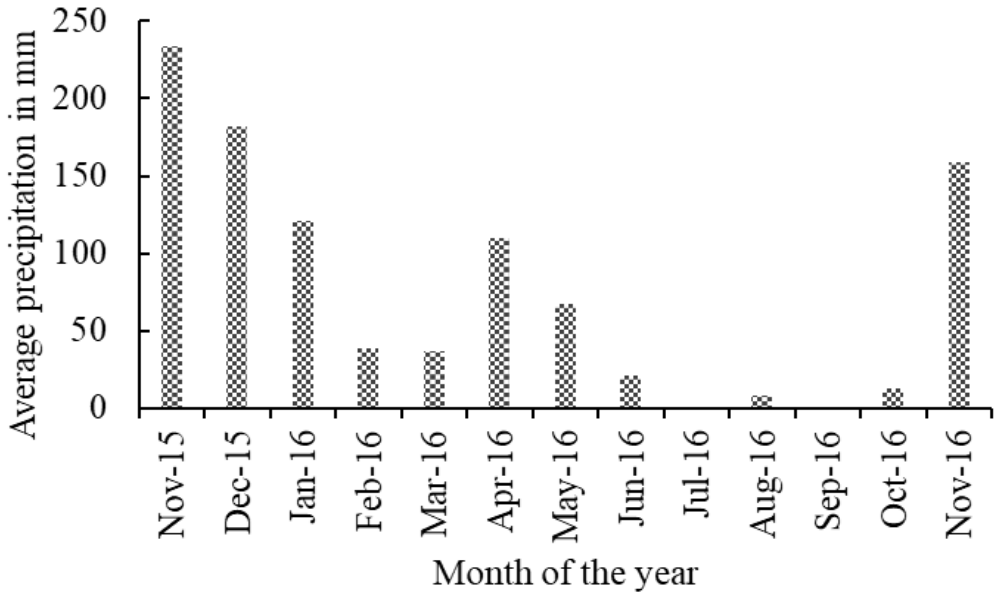

Figure 4.2: Monthly precipitation between November 2015 to November 2016.

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4.2 Demographic and clinical information of diarrheic children

The demographic and clinical information of the diarrheic children under five years of age in Nairobi, Kenya, is presented in table 4.1. A total of 374 children were recruited, 218 and 156, were recruited during the dry and rainy season, respectively. The age distribution showed that, out of the total study subjects, 242 (70.0%) were within the age group between 1 and 36 months and 112 (30.0%) children were between 37 and 60 months. The overall gender distribution was 181 (48%) females and 193 (52%) males. Guardians of 371 (99.2%) and 3 (0.8%) reported using piped and borehole water, respectively. In addition, 220 (58.8%) reported treating drinking water. Occupation distribution showed that 2 (0.5%), 17 (4.5%), 5 (1.3%), 14 (3.7%), and 178 (47.6%) of the guardians were in the health care practitioner, office administrative support, construction/installation/repair, education/training, and sales, respectively, while 158 (42.2%) were unemployed.

Temperature of <38.0°C and ≥ 38.0°C was recorded in 58 (15.5%) and 316 (84.5%) children, respectively. In this study, 308 (82.4%), 35 (9.4%) and 31 (8.3%), respectively, reported having diarrhea for 1-3, 4-6 and ≥7 days. Vomiting was evidenced in 298 (79.7%) patients, fever in 310 (82.9%), abdominal cramp in 251 (67.1%), headache in 12 (3.2%), nausea in 50 (13.4%), and appetite loss in 345 (92.2%) children. Clinical diagnosis of dehydration revealed that 311 (83.2%) had sunken eyeballs, 117 (31.3%) children had dry tongue and 192 (51.3%) had reduced skin elasticity.

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Table 4.1: Demographic and clinical information of study participant

Characteristics	Number (%)
Diarrheal cases	
Dry season	218 (58.3)
Rainy Season	156 (41.7)
Age in months	
1-36	242 (70.0)
37-60	112 (30.0)
Gender	
Female	181 (48)
Male	193 (52)
Source of water	
Piped water	371 (99.2)
Borehole	3 (0.8)
Water treatment	220 (58.8)
Occupation of guardian	
Health care practitioner	2 (0.5)
Office/administrative/support	17 (4.5)
Construction/installation/repair	5 (1.3)
Education/ training	14 (3.7)
Sales	178 (47.6)
Unemployed	158 (42.2)
Body temperature in °C	
<38.0	58 (15.5)
≥ 38.0	316 (84.5)
Duration of diarrhea in days	
1-3	308 (82.4)
4-6	35 (9.4)
≥7	31 (8.3)
Symptoms	
Vomiting	298 (79.7)
Fever	310 (82.9)
Abdominal cramp	251 (67.1)
Headache	12 (3.2)
Nausea	50 (13.4)
Appetite loss	345 (92.2)
Sunken eyeball	311 (83.2)
Dry tongue	117 (31.3)
Reduced skin elasticity	192 (51.3)

Data are presented as number and proportions (%) of study participants. ≤, less than or equal to. <, less than. ≥, greater than or equal to. >, greater than. Health care practitioner (Nurse, Clinical officer). Office administrative support (secretary, clerical officer, social worker, driver, househelp, caretaker). Construction/installation/repair (welder, carpenter, mason, tailor). Education/ training (teacher). Sales (saloonist, hawkers and small scale business, sales agents).

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4.3 Enteric bacterial pathogens phenotypes and genotypes among diarrheic children in Nairobi City, Kenya

The incidence of enteric bacterial pathogens phenotypes and genotypes is presented in table 4.2. A total of 136 (36.4%) children were infected with diarrheagenic *E. coli*. Pathogen genotyping showed that 78 (57.4%), 15 (11.0%), 38 (27.9%), and 2 (1.5%) of the diarrheagenic *E. coli* isolates harboured EAEC, EPEC, ETEC and EIEC virulent genes, respectively, while mixed virulent gene was detected in 1 (0.7%) for EAEC/EPEC/ETEC and 2 (1.5%) for EAEC/ETEC. *Salmonella* species was detected in stool samples of 9 (2.4%) children. Virulotyping showed that 9 (100.0%), 7 (77.8%), 9 (100.0%), and 6 (77.8%) of the *Salmonella* isolates harboured *invA, hilA, sopB,* and *stn* virulent genes, respectively. There were 12 (3.2%) children infected with *Shigella* species, of which 2 (0.5%), 1 (0.3%), 7 (1.9%), and 2 (0.5%) were infected with *Shigella boydii, Shigella dysentriae, Shigella flexneri* and *Shigella sonnei,* respectively. All (100%) the *Shigella* isolates harboured *ipaH* virulent gene. There were 6 (1.6%) and 5 (1.3%) children infected with *Campylobacter* and *Yersinia* species which all (100.0%) harboured *cadF* and *Inv* virulent genes, respectively. *Aeromonas* species was detected in stool samples of 4 (1.1%) children. Virulent gene typing revealed that 3 (75.0%), 1 (25.0%), 1 (25.0%) and 1 (25.0%) of the *Aeromonas* isolates harboured *aerA, hlyA, alt,* and *ast* virulent genes, respectively. There were 9 (2.4%) children infected with both *Shigella* species and *E. coli,* of which, 4 (44.4%) harboured EAEC/*ipaH* mixed virulent gene, 2 (22.2%) were EAEC/*ipaH* mixed virulent gene, while one (11.1%) case was reported for EAEC/*ipaH*, EPEC/*ipaH* and ETEC/*ipaH* mixed virulent genes.

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Table 4.2: Phenotype and virulent genotype of bacterial pathogens among diarrheic children in Nairobi City, Kenya

Enteropathogenic bacteria	Phenotype number n (%)	Virulent genotype	Genotype number n (%)
Diarrheagenic *E. coli*	136 (36.4)	EAEC	78 (57.4)
		EPEC	15 (11.0)
		ETEC	38 (27.9)
		EIEC	2 (1.5)
		EAEC/EPEC/ETEC	1 (0.7)
		EAEC/ETEC	2 (1.5)
		EHEC	0 (0.0)
Salmonella species	9 (2.4)	*invA*	9 (100.0)
		Hila	7 (77.8)
		sopB	9 (100.0)
		Stn	6 (77.8)
Shigella species	12 (3.2)	*ipaH*	12 (100.0)
Shigella boydii	2 (0.5)	*ipaH*	2 (100.0)
Shigella dysentriae	1 (0.3)	*ipaH*	1 (100.0)
Shigella flexneri	7 (1.9)	*ipaH*	7 (100.0)
Shigella sonnei	2 (0.5)	*ipaH*	2 (100.0)
Campylobacter species	6 (1.6)	*cadF*	6 (100.0)
Yersinia species	5 (1.3)	*Inv*	5 (100.0)
Aeromonas species	4 (1.1)	*aerA*	3 (75.0)
		hlyA	1 (25.0)
		alt	1 (25.0)
		Ast	1 (25.0)
Shigella species/*E. coli* co-infection	9 (2.4)	EAEC/*ipaH*	4 (44.4)
		EAEC/*ipaH*	2 (22.2)
		EAEC/ *ipaH*	1 (11.1)
		EPEC/ *ipaH*	1 (11.1)
		ETEC/ *ipaH*	1 (11.1)

Data are presented as number and proportions (%) of enteric bacteria and virulent genotypes. *E. coli*, *Escherichia coli*. EPEC, enteropathogenic *E. coli*. ETEC, enterotoxigenic *E. coli*. EAEC, enteroaggregative E. coli. EIEC, enteroinvasive *E. coli*. EAEC (*aspU*, *aggR*, and *pcvd432* virulent genes). EPEC (*eae* virulent gene). ETEC (*est* and *elt* virulent genes). EIEC (*ipaH* virulent gene). EHEC (*stx* virulent gene).

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4.4 Seasonal variation of enteric bacterial pathogens phenotype and genotype among diarrheic children in Nairobi city, Kenya

Seasonal variation of enteric bacterial pathogens phenotype and genotype isolated from diarrheic children in Nairobi city, Kenya, is presented in table 4.3. The incidence of diarrheagenic *E. coli* was significantly higher during the dry season compared to rainy season (48.6% vs. 19.2%; *P*<0.0001). Consisted with higher incidence of diarrheagenic *E. coli*, the frequency of EAEC (28.9% vs. 9.6%; *P*<0.0001) and ETEC (13.8% vs. 5.1%; *P*=0.006) *E. coli* genotype was significantly higher during the dry season relative to rainy season. However, the incidence of EPEC (4.65 vs. 3.2%; *P*=0.502) and EIEC (0.9% vs. 0.0%; *P*= 0.512) *E. coli* genotype infection and that of EAEC/EPEC/ETEC (0.0% vs. 0.6%; *P*=0.417) and EAEC/ETEC (0.5% vs. 0.6%; *P*=1.000) mixed *E. coli* genotype infection was similar between the dry and rainy seasons.

The incidence of *Salmonella* phenotype (2.8% vs. 1.9%; *P*=0.740) and *Salmonella* virulent genotypes *invA* (2.8 vs. 1.9; *P*=0.740), *hilA* (1.4 vs. 0.6; *P*=0.644), *sopB* (0.9 vs. 1.9; P=0.653) and *Stn* (0.5 vs. 1.3; *P*=0.573) did not differ significantly between the seasons. The incidence of *Shigella* species (3.2% vs. 3.2%; *P*=0.997) and its *ipah* virulent gene was comparable between the seasons. Likewise, the incidence of *Shigella boydii* (0.9% vs. 0.0%; *P*=0.512), *Shigella dysentriae* (0.5% vs. 0.0%; *P*=1.000), *Shigella flexneri* (1.8% vs. 1.9%; *P*=1.000), and *Shigella sonnei* (0.0% vs. 1.3%; *P*=0.173) was similar between the dry and rainy seasons. The incidence of *Shigella/E. coli* co-infection was significantly higher during the dry season compared to rainy season (4.1 vs. 0.0%; *P*=0.012). However, the incidence of EAEC/*ipaH* (1.8% vs. 0.0%; *P*=0.144), EAEC/*ipaH* (0.9% vs. 0.0%; *P*=0.512), EAEC/*ipaH* (0.5%

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vs. 0.0%; *P*=1.000), EPEC/*ipaH* (0.5% vs. 0.0%; *P*=1.000), and ETEC/*ipaH* (0.5% vs. 0.0%; *P*=1.000) mixed *E. coli/ Shigella* virulent genotype was similar between the seasons.

The incidence of *Campylobacter* phenotype (1.8% vs. 1.3%; *P*=1.000) and *Campylobacter cadF* genotype (1.8 vs. 1.3%; *P*=1.000) and that of *Yersinia* phenotype (1.8% vs. 0.6%; *P*=0.406) and *Yersinia inv* virulent genotype (1.8 vs. 0.6; *P*=0.406) was similar between the seasons. *Aeromonas* (0.9% vs.1.3%) species infection rates did not differ significantly across the season. Likewise, *Aeromonas* virulent genotypes *aerA* (0.9 vs. 0.6; P=1.000), *hlyA* (0.0 vs. 0.6; *P*=0.417), *alt* (0.0 vs. 0.6; *P*=0.417) and *Ast* (0.0 vs. 0.6; *P*=0.417) was detected at similar rates between the seasons.

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Table 4.3. Seasonal variation of enteric bacterial pathogens phenotype and genotype among diarrheic children in Nairobi, Kenya

Bacteria phenotype	Dry (n=218)	Rainy (n=156)	*P*-value	Virulent genotype	Dry (n=218)	Rainy (n=156)	*P*-value
Diarrheagenic *E. coli*	106 (48.6)	30 (19.2)	**<0.0001**[a]	EAEC	63 (28.9)	15 (9.6)	**<0.0001**[a]
				EPEC	10 (4.6)	5 (3.2)	0.502 [a]
				ETEC	30 (13.8)	8 (5.1)	**0.006** [a]
				EIEC	2 (0.9)	0 (0.0)	0.512[b]
				EAEC/EPEC/ETEC	0 (0.0)	1 (0.6)	0.417 [b]
				EAEC/ETEC	1 (0.5)	1 (0.6)	1.000 [b]
Salmonella species	6 (2.8)	3 (1.9)	0.740[b]	*invA*	6 (2.8)	3 (1.9)	0.740 [b]
				Hila	3 (1.4)	1 (0.6)	0.644[b]
				sopB	2 (0.9)	3 (1.9)	0.653[b]
				Stn	1 (0.5)	2 (1.3)	0.573[b]
Shigella species	7 (3.2)	5 (3.2)	0.997[a]	*IpaH*	7 (3.2)	5 (3.2)	0.997 [a]
Shigella boydii	2 (0.9)	0 (0.0)	0.512[b]	*IpaH*	2 (0.9)	0 (0.0)	0.512 [b]
Shigella dysentriae	1 (0.5)	0 (0.0)	1.000 [b]	*IpaH*	1 (0.5)	0 (0.0)	1.000 [b]
Shigella flexneri	4 (1.8)	3 (1.9)	1.000 [b]	*IpaH*	4 (1.8)	3 (1.9)	1.000 [b]
Shigella sonnei	0 (0.0)	2 (1.3)	0.173 [b]	*IpaH*	0 (0.0)	2 (1.3)	0.173 [b]
Campylobacter	4 (1.8)	2 (1.3)	1.000 [b]	*cadF*	4 (1.8)	2 (1.3)	1.000 [b]
Yersinia	4 (1.8)	1 (0.6)	0.406 [b]	*Inv*	4 (1.8)	1 (0.6)	0.406 [b]
Shigella/E. coli	9 (4.1)	0 (0.0)	**0.012** [b]	EAEC/*ipaH*	4 (1.8)	0 (0.0)	0.144 [b]
				EAEC/*ipaH*	2 (0.9)	0 (0.0)	0.512 [b]
				EAEC/*ipaH*	1 (0.5)	0 (0.0)	1.000 [b]
				EPEC/*ipaH*	1 (0.5)	0 (0.0)	1.000 [b]
				ETEC/*ipaH*	1 (0.5)	0 (0.0)	1.000 [b]
Aeromonas	2 (0.9)	2 (1.3)	1.000 [b]	*aerA*	2 (0.9)	1 (0.6)	1.000 [b]
				hlyA	0 (0.0)	1 (0.6)	0.417 [b]
				alt	0 (0.0)	1 (0.6)	0.417 [b]
				Ast	0 (0.0)	1 (0.6)	0.417 [b]

Data are presented as number and proportions (%) of enteric bacteria and virulent genotypes. *E. coli*, *Escherichia coli*. EPEC, enteropathogenic *E. coli*. ETEC, enterotoxigenic *E. coli*. EAEC, enteroaggregative *E. coli*. EIEC, enteroinvasive *E. coli*. [a] Chi-Square test. [b] Fisher's exact test.

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4.5 Phenotypic and genotypic antibiotic resistance of enteric bacterial pathogens among diarrheic children in Nairobi City, Kenya

The phenotypic and genotypic antimicrobial resistance of the enteric bacterial pathogens isolated from diarrheic children in Nairobi city, Kenya, is presented in table 4.4. A total of 80 (55.2%), 18 (100.0%), 91 (76.6%), 69 (47.6%), 41 (28.3%), 90 (62.1%), 12 (8.3%) and 120 (82.8%) of diarrheagenic *E. coli* were resistant to ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline, respectively. Genotypic antimicrobial resistance analysis revealed that, 79 (98.8%), 16 (88.9%), 85 (93.4%), 69 (100.0%), 39 (95.1%), 87 (96.7%), 11 (91.7%) and 120 (100.0%) of *E. coli* phenotypic resistant to ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline harbored ampicillin *(citm),* ceftriaxone *(bla CMY),* streptomycin *(aadA1),* gentamycin *(aac(3)-IV),* ciprofloxacin *(qnr),* chloramphenicol *(catA1),* erythromycin (*ereA)* and tetracycline *(tetA)* resistant gene, respectively.

Although none of the *Salmonella* isolate was resistant to gentamycin, 7 (77.8%), 7 (77.8%), 9 (100.0%), 8 (88.9%), 7 (77.8%), 6 (66.7%) and 5 (55.6%) of *Salmonella* species were resistant to ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline, respectively. Additionally, ampicillin *(citm),* ceftriaxone *(bla CMY),* streptomycin *(aadA1),* gentamycin *(aac(3)-IV),* ciprofloxacin *(qnr),* chloramphenicol *(catA1),* erythromycin (*ereA)* and tetracycline *(tetA)* resistant gene was detected in 6 (85.7%), 6 (85.7%), 9 (100.0%), 8 (100.0%), 6 (85.7%), 6 (100.0%), and 5 (100.0%) of *Salmonella* isolates that were phenotypic resistant to ampicillin, ceftriaxone, streptomycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline, respectively.

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Phenotypic antibiotic analysis showed that 13 (28.6%), 10 (47.6%), 12 (57.1%), 2 (9.5%), 4 (19.0%), 12 (57.1%), 2 (9.5%) and 14 (66.7%) of *Shigella* isolates were resistant to ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline, respectively. Most importantly, 13 (100.0%), 10 (100.0%), 12 (100.0%), 2 (100.0%), 4 (100.0%), 11 (100.0%), 2 (91.7%) and 14 (100.0%) of *Shigella* isolates that were phenotypic resistant to ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline harbored ampicillin *(citm)*, ceftriaxone *(bla CMY)*, streptomycin *(aadA1)*, gentamycin *(aac(3)-IV)*, ciprofloxacin *(qnr)*, chloramphenicol *(catA1)*, erythromycin (*ereA)* and tetracycline *(tetA)* resistant gene, respectively.

While *Campylobacter* species was not resistant to streptomycin and ciprofloxacin, 3 (50.0%), 1 (16.7%), 1 (16.7%), 4 (66.7%), 3 (50.0%) and 5 (83.3%) of the *Campylobacter* isolates were resistant to ampicillin, ceftriaxone, gentamycin, chloramphenicol, erythromycin and tetracycline, respectively. Additionally, 3 (100.0%), 1 (100.0%), 1 (100.0%), 4 (100.0%), 2 (66.7%) and 5 (100.0%) of *Campylobacter* isolates resistant to ampicillin, ceftriaxone, gentamycin, chloramphenicol, erythromycin and tetracycline haboured ampicillin *(citm)*, ceftriaxone *(bla CMY)*, gentamycin *(aac(3)-IV)*, chloramphenicol *(catA1)*, erythromycin (*ereA)* and tetracycline *(tetA)* resistant gene, respectively .

Even though *Yersinia* was not resistant to streptomycin and ciprofloxacin, 5 (100.0%), 1 (20.0%), 5 (100.0%), 4 (80.0%), 1 (20.0%) and 3 (60.0%) of *Yersinia* species were resistant to ampicillin, ceftriaxone, gentamycin, chloramphenicol,

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erythromycin and tetracycline, respectively. Moreover, ceftriaxone *(bla CMY)*, gentamycin *(aac(3)-IV)* and erythromycin (*ereA)* resistant genes was not detected while ampicillin *(citm)*, chloramphenicol *(catA1)*, and tetracycline *(tetA)* resistant gene was detected in each case of phenotypic resistant *Yersinia* isolate. While *Aeromonas* isolates was not resistant to ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin and erythromycin, 2 (50.0%) and 3 (75.0%) of *Aeromonas* isolates were resistant to cchloramphenicol, and tetracycline, respectively. Most importantly, none of cchloramphenicol resistant *Aeromonas* isolate harbored chloramphenicol *(catA1)* resistant gene while tetracycline (*tetA*) resistant gene was detected in only 1 (33.3%) of the *Aeromonas that was* phenotypic resistant to tetracycline.

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Table 4.4: Antimicrobial susceptibility patterns of enteric bacterial pathogens among diarrheic children in Nairobi city, Kenya

Enteropathogenic bacteria	Antibiotic	Phenotypic resistance			Genotypic resistance	
		Sensitive	Intermediate	Resistant	Genotype	Number (%)
Diarrheagenic *E. coli*	Ampicillin	22 (15.2)	43 (29.7)	80 (55.2)	*Citm*	79 (98.8)
	Ceftriaxone	106 (73.1)	21 (14.5)	18 (12.4)	*bla CMY*	18 (100.0)
	Streptomycin	34 (23.4)	20 (13.8)	91 (76.6)	*aadA1*	85 (93.4)
	Gentamycin	48 (33.1)	28 (19.3)	69 (47.6)	*aac(3)-IV*	69 (100.0)
	Ciprofloxacin	94 (64.8)	10 (6.9)	41 (28.3)	*Qnr*	39 (95.1)
	Chloramphenicol	14 (9.7)	41 (28.3)	90 (62.1)	*catA1*	87 (96.7)
	Erythromycin	81 (55.9)	52 (35.9)	12 (8.3)	*ere(A)*	11 (91.7)
	Tetracycline	15 (10.3)	10 (6.9)	120 (82.8)	*tet(A)*	120 (100.0)
Salmonella species	Ampicillin	0 (0.0)	2 (22.2)	7 (77.8)	*Citm*	6 (85.7)
	Ceftriaxone	2 (22.2)	0 (0.0)	7 (77.8)	*bla CMY*	6 (85.7)
	Streptomycin	0 (0.0)	0 (0.0)	9 (100.0)	*aadA1*	9 (100.0)
	Gentamycin	9 (100.0)	0 (0.0)	0 (0.0)	*aac(3)-IV*	-
	Ciprofloxacin	0 (0.0)	1 (11.1)	8 (88.9)	*Qnr*	8 (100.0)
	Chloramphenicol	2 (22.2)	0 (0.0)	7 (77.8)	*catA1*	6 (85.7)
	Erythromycin	1 (11.1)	2 (22.2)	6 (66.7)	*ere(A)*	6 (100.0)
	Tetracycline	3 (33.3)	1 (11.1)	5 (55.6)	*tet(A)*	5 (100.0)
Shigella species	Ampicillin	2 (9.5)	6 (28.6)	13 (28.6)	*Citm*	13 (100.0)
	Ceftriaxone	11 (52.4)	0 (0.0)	10 (47.6)	*bla CMY*	10 (100.0)
	Streptomycin	2 (9.5)	7 (33.3)	12 (57.1)	*aadA1*	12 (100.0)
	Gentamycin	18 (85.7)	1 (4.8)	2 (9.5)	*aac(3)-IV*	2 (100.0)
	Ciprofloxacin	11 (52.4)	6 (28.6)	4 (19.0)	*Qnr*	4 (100.0)
	Chloramphenicol	5 (23.8)	4 (19.0)	12 (57.1)	*catA1*	11 (100.0)
	Erythromycin	5 (23.8)	14 (66.7)	2 (9.5)	*ere(A)*	2 (91.7)
	Tetracycline	4 (19.0)	3 (14.3)	14 (66.7)	*tet(A)*	14 (100.0)
Campylobacter	Ampicillin	2 (33.3)	1 (16.7)	3 (50.0)	*Citm*	3 (100.0)

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Enteropathogenic bacteria	Antibiotic	Phenotypic resistance			Genotypic resistance	
		Sensitive	Intermediate	Resistant	Genotype	Number (%)
	Ceftriaxone	4 (66.7)	1 (16.7)	1 (16.7)	*bla CMY*	1 (100.0)
	Streptomycin	5 (83.3)	1 (16.7)	0 (0.0)	*aadA1*	-
	Gentamycin	5 (83.3)	0 (0.0)	1 (16.7)	*aac(3)-IV*	1 (100.0)
	Ciprofloxacin	6 (100.0)	0 (0.0)	0 (0.0)	*Qnr*	-
	Chloramphenicol	0 (0.0)	1 (33.3)	4 (66.7)	*catA1*	4 (100.0)
	Erythromycin	1 (16.7)	2 (33.3)	3 (50.0)	*ere(A)*	2 (66.7)
	Tetracycline	1 (16.7)	0 (0.0)	5 (83.3)	*tet(A)*	5 (100.0)
Yersinia	Ampicillin	0 (0.0)	0 (0.0)	5 (100.0)	*Citm*	1 (20.0)
	Ceftriaxone	0 (0.0)	4 (80.0)	1 (20.0)	*bla CMY*	0 (0.0)
	Streptomycin	0 (0.0)	5 (100.0)	0 (0.0)	*aadA1*	-
	Gentamycin	0 (0.0)	0 (0.0)	5 (100.0)	*aac(3)-IV*	0 (0.0)
	Ciprofloxacin	5 (100.0)	0 (0.0)	0 (0.0)	*Qnr*	-
	Chloramphenicol	0 (0.0)	1 (20.0)	4 (80.0)	*catA1*	1 (25.0)
	Erythromycin	0 (0.0)	4 (80.0)	1 (20.0)	*ere(A)*	0 (0.0)
	Tetracycline	1 (20.0)	1 (20.0)	3 (60.0)	*tet(A)*	1 (33.0)
Aeromonas	Ampicillin	0 (0.0)	4 (100.0)	0 (0.0)	*Citm*	-
	Ceftriaxone	4 (100.0)	0 (0.0)	0 (0.0)	*bla CMY*	-
	Streptomycin	2 (50.0)	2 (50.0)	0 (0.0)	*aadA1*	-
	Gentamycin	4 (100.0)	0 (0.0)	0 (0.0)	*aac(3)-IV*	-
	Ciprofloxacin	4 (100.0)	0 (0.0)	0 (0.0)	*Qnr*	-
	Chloramphenicol	0 (0.0)	2 (50.0)	2 (50.0)	*catA1*	0 (0.0)
	Erythromycin	1 (25.0)	3 (75.0)	0 (0.0)	*ere(A)*	-
	Tetracycline	0 (0.0)	1 (25.0)	3 (75.0)	*tet(A)*	1 (33.3)

Data are presented as number and proportions (%) of sensitive, intermediate and phnotypic resistant bacterial isolates and antibiotic resistant genes.

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4.6 Seasonal variation of phenotypic and genotypic antibiotic resistance of enteric bacterial pathogens among diarrheic children in Nairobi city, Kenya

Seasonal variation of antibiotic resistance of enteric bacterial pathogens isolated from diarrheic children in Nairobi city, Kenya, is presented in table 4.5. The incidence of diarrheagenic *E. coli* resistant to ampicillin was significantly lower during the dry season compared to rainy season (50.4% vs. 73.3%; *P*=0.025). Although ceftriaxone (11.3% vs. 16.7%; *P*=0.428), streptomycin (63.5% vs. 60.0%; *P*=0.726), gentamycin (50.4% vs. 36.7%; *P*=0.179), chloramphenicol (63.5% vs. 56.7%; *P*=0.493) and tetracycline (81.7% vs. 86.7%; *P*=0.525) antimicrobial resistant rates of diarrheagenic *E. coli* did not differ significantly between the seasons, significantly higher resistant rates of diarrheagenic *E. coli* to ciprofloxacin (32.2% vs. 13.3%; *P*=0.043) was significantly higher while that of erythromycin (5.2% vs. 20.0%; *P*=0.009) was lower during the dry season compared to rainy season. Antibiotic resistant gene analysis revealed that rates of ampicillin (*citm*) (49.6 vs. 73.3%; *P*= 0.020) and erythromycin (ereA) (4.3 vs. 20.0; *P*=0.004) resistant genes were high while ciprofloxacin (*qnr*) (31.3% vs. 10.0%; *P*=0.021) resistant gene was lower during the rainy season relative to dry season. The distribution of ceftriaxone (*bla CMY*) (11.3% vs. 16.7%; *P*=0.428), streptomycin (*aadA1*) (61.7% vs. 46.7%; *P*=0.135), gentamycin (*aac(3)-IV*) (50.4% vs. 36.7%; *P*= 0.179), chloramphenicol (*catA1*) (60.9% vs. 56.7%; *P*=0.676) and tetracycline (*tetA*) (81.7% vs. 86.7%; *P*=0.525) resistant genes were similar between seasons.

There were no seasonal differences in phenotypic antimicrobial resistant rates of *Salmonella* species to ampicillin (66.7% vs. 100.0%; *P*=0.500) and ceftriaxone (83.3 vs. 66.7%; *P*=1.000). All *Salmonella* species (100.0%) isolated from diarrheic

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children during the dry and rainy season were resistant to streptomycin. Seasonal trends in *Salmonella* phenotypic antimicrobial resistance was not reported in ciprofloxacin (100.0% vs. 66.7%; *P*=0.333), Chloramphenicol (100.0% vs. 33.3%; *P*=0.083), erythromycin (50.0% vs. 100.0%; *P*=0.464) and tetracycline (33.3% vs. 100.0%; *P*=0.167). Genotyping antimicrobial resistant genes revealed that rates of ampicillin (*citm*) (50.0% vs. 100.0%; *P*=0.464), ceftriaxone (*bla CMY*) (66.7% vs. 66.7%; P= 1.000), ciprofloxacin (*qnr*) (100.0% vs. 66.7%; *P*=0.333), chloramphenicol (*catA1*) (83.3% vs. 33.3%; *P*=0.226), erythromycin (*ereA*) (50.0% vs. 100.0%; *P*=0.464), tetracycline (*tetA*) (33.3% vs. 100.0%; *P*=0.167) were similar between seasons. In addition, all (100.0%) *Salmonella* isolated during the dry and rainy season harboured streptomycin resistant gene.

Even though there was no seasonal difference in antimicrobial resistant rates of *Shigella* isolates to gentamycin (12.5% vs. 0.0%; *P*=1.000), Ciprofloxacin (25.0% vs. 0.0%; *P*=0.532), chloramphenicol (50.0% vs. 80.0%; *P*=0.338) and tetracycline (62.5% vs. 80.0%; *P*=0.624), resistant rates to ampicillin (100.0% vs. 50.0%; *P*=0.044), ceftriaxone (31.2% vs. 100.0%; *P*=0.012), streptomycin (43.8% vs. 100.0%; *P*=0.027), erythromycin (0.0% vs. 40.0%; *P*=0.048) were significantly higher during the rainy season compared to dry season. Antibiotic resistant gene analysis revealed that ampicillin (citm) (50.0% vs. 100.0%; *P*=0.044), ceftriaxone (*bla CMY*) (31.2% vs. 100.0%; *P*=0.012), streptomycin (*aadAI)* (43.8% vs. 100.0%; *P*=0.027), erythromycin (ereA) (0.0% vs. 40.0%; P=0.048) resistant genes were higher during the rainy season while distribution of gentamycin (*aac(3)-IV*) (12.5% vs. 0.0%; *P*=1.000), ciprofloxacin (*qnr*) (25.0% vs. 0.0%; *P*=0.532), chloramphenicol

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(*catA1*) (43.8% vs. 80.0%; *P*=0.311) and tetracycline (tetA) (62.5% vs. 80.0%; *P*=0.624) was similar between seasons.

Campylobacter species showed no seasonal trends in antimicrobial resistance to Ampicillin (50.0% vs. 50.0%; *P*=1.000), ceftriaxone (25.0% vs. 0.0%; *P*=1.000), Gentamycin (0.0% vs. 50.0%; *P*=0.333), chloramphenicol (50.0% vs. 100.0%; *P*=0.467), Erythromycin (75.0% vs. 0.0%; *P*=0.400), tetracycline (75.0% vs. 100.0%; *P*= 1.000). Likewise, there was no seasonal difference in distribution of ampicillin (*citm*) (50.0% vs. 50.0%; *P*=1.000), ceftriaxone (25.0% vs. 0.0%; *P*=1.000), gentamycin (*aac(3)-IV*) (0.0% vs. 50.0%; *P*=0.333), chloramphenicol (*catA1*) (50.0% vs. 100.0%; *P*=0.467), erythromycin (*ereA*) (50.0% vs. 0.0%; *P*=0.467) and tetracycline (*tetA*) (75.0% vs. 100.0%; *P*=1.000) resistant genes.

All (100.0%) *Yersinia* species isolated during the dry and rainy season were resistant to ampicillin. The incidence of *Yersinia* isolates resistant to ceftriaxone (0.0% vs. 100.0%; *P*=0.200), chloramphenicol (75.0% vs. 100.0%; P=1.000), erythromycin (25.0% vs. 0.0%; P=1.000) and tetracycline (75.0% vs. 100.0%; P=1.000) was comparable between the seasons. All (100.00%) the Yersinia isolate that were phenotypic resistant to ampicillin, chloramphenicol and tetracycline during the dry season harboured ampicillin (*citm*) , chloramphenicol (*catA1*) and tetracycline (*tetA*) resistant gene.

There was no seasonal difference in incidence of *Aeromonas* species resistant to chloramphenicol (0.0% vs. 100.0%; P= 0.333) and tetracycline (50.0% vs. 100.0%;

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P=1.000). One isolate that was phenotypic resistant to tetracycline during the dry season expression *tetA* gene.

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Table 4.5: Seasonal variation of antibiotic resistance of enteric bacterial pathogens

Enterobacteria	Antibiotic	Dry	Rainy	*P*-value	Resistant Gene	Dry	Rainy	*P*-value
Diarrheagenic *E. coli*	Ampicillin	58 (50.4)	22 (73.3)	**0.025**[a]	*Citm*	57 (49.6)	22 (73.3)	**0.020**[a]
	Ceftriaxone	13 (11.3)	5 (16.7)	0.428[a]	*bla CMY*	13 (11.3)	5 (16.7)	0.428[a]
	Streptomycin	73 (63.5)	18 (60.0)	0.726[a]	*aadA1*	71 (61.7)	14 (46.7)	0.135[a]
	Gentamycin	58(50.4)	11 (36.7)	0.179[a]	*aac(3)-IV*	58(50.4)	11 (36.7)	0.179[a]
	Ciprofloxacin	37 (32.2)	4 (13.3)	**0.043**[b]	*Qnr*	36 (31.3)	3 (10.0)	**0.021**[b]
	Chloramphenicol	73 (63.5)	17 (56.7)	0.493[a]	*catA1*	70 (60.9)	17 (56.7)	0.676[a]
	Erythromycin	6 (5.2)	6 (20.0)	**0.009**[a]	*ere(A)*	5 (4.3)	6 (20.0)	**0.004**[a]
	Tetracycline	94 (81.7)	26 (86.7)	0.525[a]	*tet(A)*	94 (81.7)	26 (86.7)	0.525[a]
Salmonella species	Ampicillin	4 (66.7)	3 (100.0)	0.500[b]	*Citm*	3 (50.0)	3 (100.0)	0.464[b]
	Ceftriaxone	5 (83.3)	2 (66.7)	1.000[b]	*bla CMY*	4 (66.7)	2 (66.7)	1.000[b]
	Streptomycin	6 (100.0)	3 (100.0)	-	*aadA1*	6 (100.0)	3 (100.0)	-
	Gentamycin	0 (0.0)	0(0.0)	-	*aac(3)-IV*	0 (0.0)	0(0.0)	-
	Ciprofloxacin	6 (100.0)	2 (66.7)	0.333[b]	*Qnr*	6 (100.0)	2 (66.7)	0.333[b]
	Chloramphenicol	6 (100.0)	1 (33.3)	0.083[b]	*catA1*	5 (83.3)	1 (33.3)	0.226[b]
	Erythromycin	3 (50.0)	3 (100.0)	0.464[b]	*ere(A)*	3 (50.0)	3 (100.0)	0.464[b]
	Tetracycline	2 (33.3)	3 (100.0)	0.167[b]	*tet(A)*	2 (33.3)	3 (100.0)	0.167[b]
Shigella species	Ampicillin	8 (50.0)	5 (100.0)	**0.044**[a]	*Citm*	8 (50.0)	5 (100.0)	**0.044**[a]
	Ceftriaxone	5 (31.2)	5 (100.0)	**0.012**[a]	*bla CMY*	5 (31.2)	5 (100.0)	**0.012**[a]
	Streptomycin	7 (43.8)	5 (100.0)	**0.027**[a]	*aadA1*	7 (43.8)	5 (100.0)	**0.027**[a]
	Gentamycin	2 (12.5)	0 (0.0)	1.000[b]	*aac(3)-IV*	2 (12.5)	0 (0.0)	1.000[b]
	Ciprofloxacin	4 (25.0)	0 (0.0)	0.532[b]	*Qnr*	4 (25.0)	0 (0.0)	0.532[b]
	Chloramphenicol	8 (50.0)	4 (80.0)	0.338[b]	*catA1*	7 (43.8)	4 (80.0)	0.311[b]
	Erythromycin	0 (0.0)	2 (40.0)	**0.048**[b]	*ere(A)*	0 (0.0)	2 (40.0)	**0.048**[b]
	Tetracycline	10 (62.5)	4 (80.0)	0.624[b]	*tet(A)*	10 (62.5)	4 (80.0)	0.624[b]
Campylobacter	Ampicillin	2 (50.0)	1 (50.0)	1.000[b]	*Citm*	2 (50.0)	1 (50.0)	1.000[b]

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Enterobacteria	Antibiotic	Dry	Rainy	*P*-value	Resistant Gene	Dry	Rainy	*P*-value
	Ceftriaxone	1 (25.0)	0 (0.0)	1.000 [b]	*bla CMY*	1 (25.0)	0 (0.0)	1.000 [b]
	Streptomycin	0 (0.0)	0 (0.0)	-	*aadA1*	-	-	-
	Gentamycin	0 (0.0)	1 (50.0)	0.333 [b]	*aac(3)-IV*	0 (0.0)	1 (50.0)	0.333 [b]
	Ciprofloxacin	0 (0.0)	0 (0.0)	-	*Qnr*	0 (0.0)	0 (0.0)	-
	Chloramphenicol	2 (50.0)	2 (100.0)	0.467 [b]	*catA1*	2 (50.0)	2 (100.0)	0.467 [b]
	Erythromycin	3 (75.0)	0 (0.0)	0.400 [b]	*ere(A)*	2 (50.0)	0 (0.0)	0.467 [b]
	Tetracycline	3 (75.0)	2 (100.0)	1.000 [b]	*tet(A)*	3 (75.0)	2 (100.0)	1.000 [b]
Yersinia	Ampicillin	4 (100.0)	1 (100.0)	-	*Citm*	0 (0.0)	1 (100.0)	-
	Ceftriaxone	0 (0.0)	1 (100.0)	0.200 [b]	*bla CMY*	0 (0.0)	0 (0.0)	-
	Streptomycin	0 (0.0)	0 (0.0)	-	*aadA1*	0 (0.0)	0 (0.0)	-
	Gentamycin	0 (0.0)	0 (0.0)	-	*aac(3)-IV*	0 (0.0)	0 (0.0)	-
	Ciprofloxacin	0 (0.0)	0 (0.0)	-	*Qnr*	0 (0.0)	0 (0.0)	-
	Chloramphenicol	3 (75.0)	1 (100.0)	1.000 [b]	*catA1*	0 (0.0)	1 (100.0)	-
	Erythromycin	1 (25.0)	0 (0.0)	1.000 [b]	*ere(A)*	0 (0.0)	0 (0.0)	-
	Tetracycline	2 (75.0)	1 (100.0)	1.000 [b]	*tet(A)*	0 (0.0)	1 (100.0)	-
Aeromonas	Ampicillin	0 (0.0)	0 (0.0)	-	*Citm*	0 (0.0)	0 (0.0)	-
	Ceftriaxone	0 (0.0)	0 (0.0)	-	*bla CMY*	0 (0.0)	0 (0.0)	-
	Streptomycin	0 (0.0)	0 (0.0)	-	*aadA1*	0 (0.0)	0 (0.0)	-
	Gentamycin	0 (0.0)	0 (0.0)	-	*aac(3)-IV*	0 (0.0)	0 (0.0)	-
	Ciprofloxacin	0 (0.0)	0 (0.0)	-	*Qnr*	0 (0.0)	0 (0.0)	-
	Chloramphenicol	0 (0.0)	2 (100.0)	0.333 [b]	*catA1*	0 (0.0)	0 (0.0)	-
	Erythromycin	0 (0.0)	0 (0.0)	-	*ere(A)*	0 (0.0)	0 (0.0)	-
	Tetracycline	1 (50.0)	2 (100.0)	1.00 [b]	*tet(A)*	1 (50.0)	0 (0.0)	-

Data are presented as number and proportions (%) of phnotypic resistant bacterial isolates and antibiotic resistant genes. *E. coli*, *Escherichia coli*. *bla CMY*, ceftriaxone resistant gene. *aadA1*, streptomycin resistant gene. *aac(3)-IV*, gentamycin resistant gene. *qnrA1*, ciprofloxacin: resistant gene. *catA1*, chloramphenicol resistant gene. *ere(A)*, erythromycin resistant gene. *tet(A)*, tetracycline resistant gene. [b]Chi-square test. [b]Fisher's exact test. -, statistical analysis was not performed because either the entire row or column had zero counts.

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CHAPTER FIVE

DISCUSSION

5.0 Introduction

This chapter presents the discussion of the study findings. The chapter is devided into five sections namely introduction and four other sections discussing the obectives of the study.

5.1 Enteric pathogenic bacteria phenotypes and virulent genotypes among diarrheic children in Nairobi City, Kenya

Diarrheagenic *E. coli* predominates in this study with the remainder being *Shigella, Salmonella, Campylobacter, Yersinia* and *Aeromonus* species, in that order, highlighting the prominent role of *diarrhegenic E. coli* in enterobacteria associated diarrhoea among children in Nairobi city, Kenya. These findings are consistent with previous study in involving diarrheic children in Kiambu (Shah *et al.*, 2016) and Homa Bay counties (Shah *et al.*, 2017) in Kenya. As reviewed by (Chekabab *et al.*, 2013), diarrhegenic *E. coli* persist longer in natural ecological niches like soil and water supporting the hypothesis of *E. coli* transmission via contaminated child hands as children frequently touch soil and water (Parvez *et al.*, 2019; Pickering *et al.*, 2018). However, the findings of this study are inconsistent with previous study that demonstrated the dominancy of *Shigella* species, followed by diarrhegenic *E. coli, Salmonella* and *Yersinia* species among diarrheic children in Nairobi city, Kenya (Njuguna *et al.*, 2016). Maybe because of proved diarrhegenic *E. coli* in particular EAEC genome heterogeneity (Gomes *et al.*, 2016), using two virulence genes for EAEC detection decreased the rate of isolation in this previous study (Njuguna *et al.*, 2016). Most importantly, this study detected higher incidence of *Shigella* mono-

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infection (3.2%) than *Shigella/E. coli* co-infection (2.4%) suggesting that co-infections alters epidemiological dynamics due pathogen synergism worsening disease severity and consequently mortality (Olivo *et al.*, 2016), and this observation is consistent with previous study among diarrheic children in Kiambu county (Kenya) (Shah *et al.*, 2017), China (Qu *et al.*, 2016), Zanzibar and Rwanda (Andersson *et al.*, 2018). Therefore, diarrhegenic *E. coli, Shigella, Salmonella, Campylobacter, Yersinia* and *Aeromonas* are important causes of childhood bacteria associated diarrhoea justifying access to safe water, sanitation and hygiene among children in Nairobi city, Kenya.

Virulence genes help bacteria to invade the host, cause disease, and evade host defences (Andersson *et al.*, 2018). It was found that EAEC, EPEC, ETEC, EIEC, and EHEC virulent genotypes were frequently detected in diarrheagenic *E. coli*. This is consistent with previous study that detected EAEC, EPEC, ETEC, EIEC, and EHEC in diarrheagenic *E. coli* isolated from children with diarrhoea in Kiambu County, Kenya (Shah *et al.*, 2016). However, a study in Homa Bay county could not detect EIEC and EHEC in diarrheic children (Shah *et al.*, 2017), indicating that there could be possible ecological barriers in the transmission of these virulent genotypes (Jang *et al.*, 2017; Onanuga *et al.*, 2014). Combinations of EAEC/EPEC/ETEC and EAEC/ETEC virulent genotypes were also detected in diarrheagenic *E. coli*, which is in agreement with a previous study that detected hybrids of EAEC/EPEC/ETEC and EAEC/ETEC in DEC among diarrheic children seeking treatment at Mbagathi Hospital in Nairobi, Malindi District Hospital in Malindi, New Nyanza Provincial Hospital in Kisumu, and Alupe Leprosy Hospital in Busia, Kenya (Iijima *et al.*, 2017). Increasing trends in the hybrid phenomenon between EAEC and other virulent

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genotypes has been documented in Kiambu and Hombay counties of Kenya (Shah *et al.,* 2017) implying that hybrid strains which acquire mixed virulence genes via horizontal gene transfer (Nyholm *et al.,* 2015) are emerging as potential outbreak agents in the country and may cause high morbidity and mortalities (Karch *et al.,* 2012). All *Salmonella* virulence genes (*InvA, hilA, sopB* and *Stn*) studied here were detected with varying frequencies, which is consistent with previous study that analysed and detected *invA, stn, sopB* and *hilA* in *Salmonella* species isolated from stool samples of children in China (Yue *et al.,* 2020). All *Shigella, Campylobacter* and *Yersinia* isolates harboured *ipaH, CadF* and *Inv* virulence genes, respectively, a finding that is consistent with previous study that reported 100% detection of *IpaH* (Yaghoubi *et al.,* 2017) and *CadF* gene (Ghorbanalizadgan *et al.,* 2014) in isolates from Iranian diarrheic children and *Inv* gene in isolates from Chinese adult and children with diarrhoea (Zheng *et al.,* 2008). Genes such as *inv*A, *sop*B, and *hilA* of *Salmonella (Thung et al., 2017), ipaH* of *Shigella* (Mattock and Blocker, 2017), *CadF* of *Campylobacter* (Konkel *et al.,* 2020) and *Inv* of *Yersinia* (Bancerz-Kisiel *et al., 2018)* are involved in epithelial cell adhesion and invasion, a prerequisite for infection, may explain the abundance of these genes observed in this study. The occurrence of genes encoding hemolytic, cytotonic, cytotoxic, and enterotoxic activities (*aerA, hlyA, alt, ast*) may contribute to diarrheal-related virulence in *Aeromanas* infection (Roges *et al.,* 2020). In the present study, *aerA, hlyA, alt* and *Ast* were detected and *aerA* was the most prevalent. *Aeromonas aerA* gene is essential for adherence and invasion of intestinal mucosa (Roges *et al.,* 2020). Results from a study in Canada targeting patients with sporadic enteritis (Wang *et al.,* 2003) support the distribution of *Aeromonas* virulence genes observed in this study. Therefore, there is diversity in virulence profiles of enteric bacterial pathogen

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circulating in diarrheic children in Nairobi city, Kenya, indicating that the pathogenic strains may vary in their degree of virulence.

5.2 Seasonal variation of enteric pathogenic bacteria phenotypes and virulent genotypes among diarrheic children in Nairobi city, Kenya

Seasonal variation was found in diarrheagenic *E. coli* infection, especially in the EAEC and ETEC virulent genotypes, which occurred most frequently in the dry season compared to rainy season, suggesting that season impacts diarrheagenic *E. coli* pathotype persistence and transmission. These findings partly corroborate previous study involving diarrheic children in Homa Bay County, Kenya, (Shah *et al.*, 2016) that demonstrated peaks of EAEC but not ETEC infections and another study in Nigeria (Onanuga *et al.*, 2014) that showed peaks in ETEC but not EAEC during the dry season. There is an emerging consensus that diarrheagenic *E. coli*, particularly ETEC and EAEC, infections correlate with increasing environmental temperatures (Paredes-Paredes *et al.*, 2011). Thus warmer climates may encourage propagation and survival of *E. coli* and increased virulence in the natural environment, resulting in greater transmission (Abia *et al.*, 2016; van Elsas *et al.*, 2011). However, the findings of this study are inconsistent with previous study in Mexico, which showed seasonal trend in EAEC and ETEC infections with peaks during the rainy seasons among diarrheic children (Patzi-Vargas *et al.*, 2015), possibly as a result of local meteorological patterns (Cavicchioli *et al.*, 2019). Even though *Shigella* species mono-infection showed no seasonal variation, the high *Shigella*/diarrheagenic *E. coli* co-infections during the dry season suggest that diarrheagenic *E. coli* may compliment *Shigella* transmission. In any case, the two pathogens have synergism and the pathogenic potential of each organism appears to be enhanced during co-infection

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hence co-infection will aggravate diarrhoea facilitating their rapid dissemination into the environment and, consequently, the infection of new hosts (Andersson *et al.*, 2018). Taken together, dry season impacts presence, abundance, persistence, survival and transmission of *E. coli* and co-infection among diarrheic children in Nairobi city, Kenya.

5.3 Phenotypic and genotypic antibiotic resistance in enteric bacterial pathogens associated with childhood diarrhea in Nairobi City, Kenya

This study observed high antimicrobial resistance rates indicating rapid and ongoing spread of antimicrobial-resistant organisms. Specifically, more than half of diarrheagenic *E. coli* isolates were resistant to ampicillin, trimethoprim/sulfamethoxazole, streptomycin, kanamycin, chloramphenicol and tetracycline which is in agreement with a study in Nairobi city (Sang *et al.*, 2012) and Meru county (Karambu *et al.*, 2013) but inconsistent with previous study among the Maasai community of Narok and Kajiado counties that reported increased susceptibility to these antibiotics (Sang *et al.*, 2011), possibly due to reduced antimicrobials use among the Maasai community who mostly practice traditional medicine (Kimondo *et al.*, 2015). Over half of the *Shigella* isolates were resistant to Streptomycin, Chloramphenicol and Tetracycline while resistance to ampicillin, ceftriaxone, amoxicillin-clavulanic acid, kanamycin, ciprofloxacin, and erythromycin occurred in more than half *Salmonella* isolates, which partly agrees with a multisite study conducted in Kisii, Homa Bay and Migori counties, Kenya (Brander *et al.*, 2017) but conflicts a study in Zambia (Chiyangi *et al.*, 2017). Over half of *Campylobacter* isolates were resistant to ampicillin, chloramphenicol, and erythromycin which is consistent with findings in Ethiopia (Tafa *et al.*, 2014) and

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disagrees with study in China (Tian *et al.*, 2016). *Yersinia* were resistant to ampicillin, streptomycin, gentamycin and tetracycline which mirrors a study in Ethiopia (Andualem and Geyid, 2005) but disagrees with a study in Mexico (Novoa-Farias *et al.*, 2017). At least half of *Aeromonas* isolates were resistant to tetracycline and chloramphenicol which is in agreement with a study in Brazil (Prediger *et al.*, 2012) and disagrees with study in China (Tian *et al.*, 2016). Variations in resistance profiles worldwide indicate that some countries are overusing and misusing antibiotics facilitated by absence or inadequacies in laws, policies and regulations pertaining to antibiotics use (Van Boeckel *et al.*, 2014). For instance, even though Kenya has guidelines in place to control the use of antibiotics in animals (GoK, 2018), antibiotics are used as growth promoters and not for treatment of infections of farm animals (Caudell *et al.*, 2020). At the same time, although the purchase of antibiotics from retail pharmacies without a prescription is forbidden by Kenya's Pharmacy and Poisons Board (GoK, 2012), over-the-counter sale of antimicrobials without a prescription is possible (Muloi *et al.*, 2019). Such overuse and misuse of antibiotics drives the development of drug resistant pathogens in Kenya.

Molecular investigations on the underlying resistance mechanisms showed that most resistant phenotypes harboured *citm, blaCMY, aadA1, aac(3)-IV, Qnr, catA1, ere(A),* and *tet(A)* associated with ampicillin, ceftriaxone, streptomycin, gentamycin, ciprofloxacin, chloramphenicol, erythromycin and tetracycline resistance, respectively. However, some isolates were fully resistant but did not show the presence of these resistant genes indicating that resistance might be due to intrinsic resistance or different antibiotic resistant genes that were not evaluated in this study. Therefore, antimicrobial stewardship programs have to be developed to influence

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antibiotic use and prescribing behaviour to ensure long-term availability of effective treatment for bacterial infections in Kenya.

5.4 Seasonal variation of enteric bacterial pathogens antibiotic resistant phenotypes and genotypes among diarrheic children in Nairobi, Kenya

The present study illustrated seasonal variation in exposure to different antimicrobials in Kenya. The higher phenotypic and genotypic antimicrobial resistance rates of diarrheagenic *E. coli* to ampicillin, amoxicillin-clavulanic acid and erythromycin and *Shigella* species to ampicillin, ceftriaxone, and streptomycin during the rainy season partly mirrors a previous study in the USA (Ramsey *et al.*, 2019). Penicillin, cephalosporin and macrolides are among the most frequently prescribed antibiotics in Kenya with seasonal variations in ambulatory prescription and community use in Kenya (Momanyi *et al.*, 2019; Muloi *et al.*, 2019; Omulo *et al.*, 2017). It is likely that high consumption of oral penicillin and macrolides mostly for upper respiratory tract infections during the cold and flu season contribute to colonisation and subsequent infection with antimicrobial-resistant isolates (Alsan *et al.*, 2015; Sun *et al.*, 2012). This likely explains the seasonal variations in phenotypic and genotypic antimicrobial resistance rates of diarrheagenic *E. coli* to ampicillin, amoxicillin-clavulanic acid and erythromycin and *Shigella* species to ampicillin, ceftriaxone, and streptomycin. The higher phenotypic and genotypic antimicrobial resistance rate of diarrhegenic *E. coli* to ciprofloxacin during the dry season may be attributed to inappropriate use of ciprofloxacin in the management of non-bacterial diarrheal infection. It is important to note that majority of diarrhoea cases in this study occurred during the dry season reinforcing the findings of a previous study in Nairobi city, Kenya (Njuguna *et al.*, 2016). While the ministry of Health (MoH) in Kenya recommends ciprofloxacin for

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the management of bacterial dysentery (MoH, 2015b), clinicians' and physicians' prescribe this antibiotic (ciprofloxacin) for diarrheal illnesses without clinical criteria for secondary bacterial infections (Rhee *et al.*, 2019) driving rapid expansion of hosts' commensals antimicrobial resistance genes permitting pathogenic *E. coli* acquisition of antibiotic resistant-associated plasmids via horizontal gene transfer (Peterson and Kaur, 2018), may explain the higher diarrhegenic *E. coli* antimicrobial resistance rate during the dry season. Therefore, changes in antimicrobial use and prescription over a relatively short period of time during the year may impact antimicrobial resistance rates in Kenya.

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CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.0 Introduction

This chapter presents a summary of the study, the major findings, conclusions and pertinent recommendations with the respect to the main findings of the study.

6.1 Summary of findings

In this study, diarrhegenic *E. coli, Shigella, Salmonella, Campylobacter, Yersinia* and *Aeromonas* species harbouring virulence and antibiotic resistant genes were important aetiologies of diarrhoea in children under five years in Nairobi city, Kenya. Most importantly, the incidence of diarrheagenic *E. coli*, particularly EAEC and ETEC, and co-infection with *Shigella* species was higher during the dry season. In addition, there was higher diarrheagenic *E. coli* phenotypic and genotypic antimicrobial resistance to ampicillin, amoxicillin/clavulanic acid and erythromycin with concomitant lower ciprofloxacin resistant during the rainy season. *Shigella* antimicrobial resistance to ampicillin, ceftriaxone, streptomycin, and erythromycin was higher during the rainy season.

6.2 Conclusion

E. coli, Shigella, Salmonella, Campylobacter, Yersinia and *Aeromonas* species habouring virulent genes were aetiologies of diarrhea in children. There was seasonal variation in diarrheagenic *E. coli*, particularly EAEC and ETEC genotypes, and co-infection with *Shigella* species. Most of the bacterial isolates habour resistant genes to commonly prescribed antibiotics. Diarrheagenic *E. coli* and *Shigella* species showed seasonal trend in resistance to four commonly prescribed antibiotics.

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6.3 Recommendations of study

1) Public health interventions should be evaluated because there was a substantial burden of bacterial diarrheal diseases in children
2) There should be constant seasonal surveillance because there was seasonal peak in some pathogenic bacteria causing diarrhea.
3) There should be continuous antibiotic surveillance because the bacterial pathogens were highly resistant to various antimicrobials.
4) Identification of seasonally-dependent antibiotic resistant can be used to characterize the local epidemiology and inform the empirical treatment of symptomatic children.

6.4 Recommendations for future study

1) Future studies should be conducted at community level to determine the etiologies of childhood diarrhea.
2) Future studies should investigate why *E. coli* and *Shigella* species infections follow seasonal patterns.
3) Future studies should investigate if these are hospital acquired or community acquired antimicrobial resistance.
4) Future studies are required to better understand the factors underlying the seasonality of antimicrobial rates such as the seasonal variation of antibiotic use and its temporal association with antimicrobial resistance in enteric bacterial pathogens.
5) Future studies should investigate the presence of other antibiotic reistance and virulent genes in enteric bacteria isolated from Kenyan children with diarrhea.

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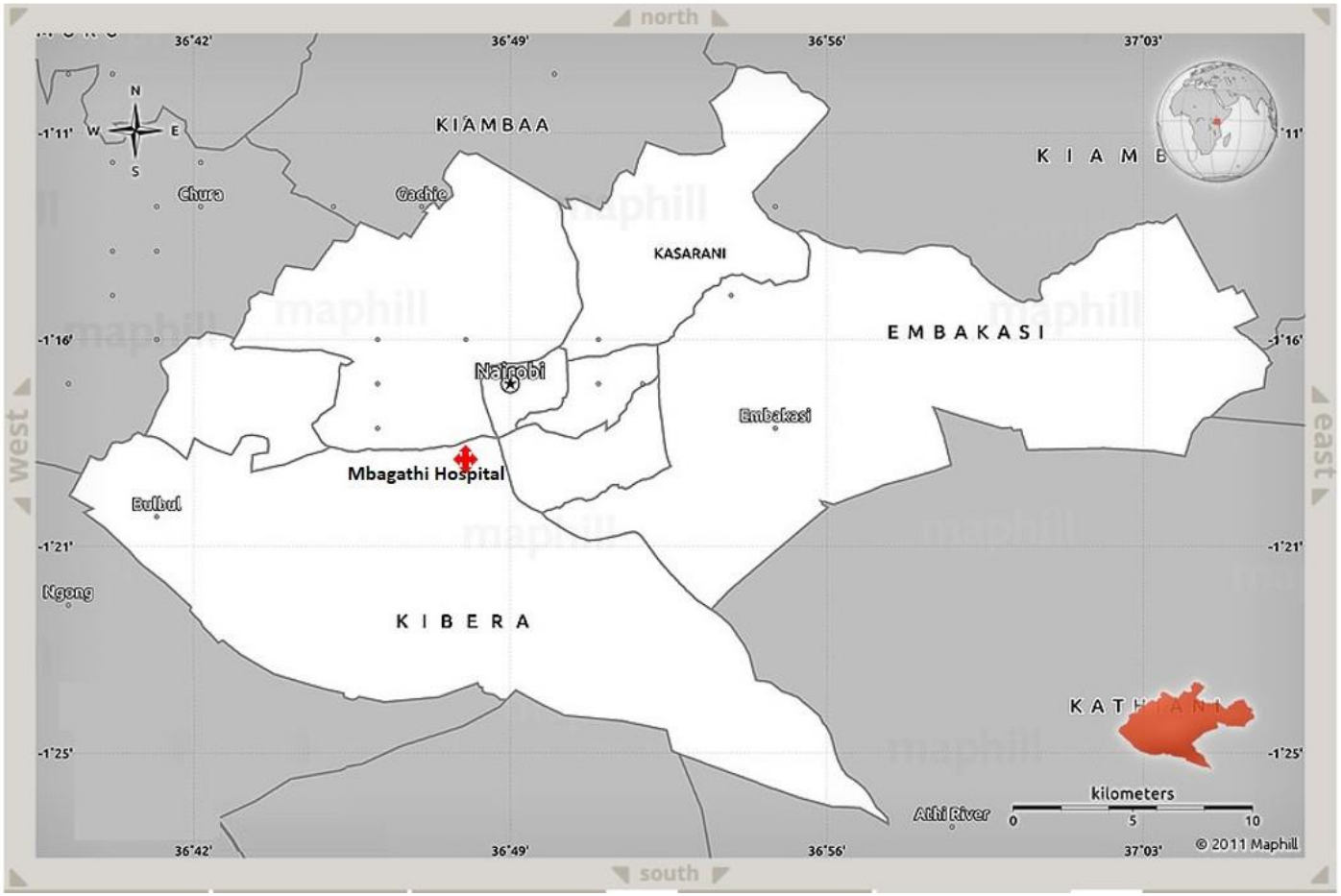
APPENDICES

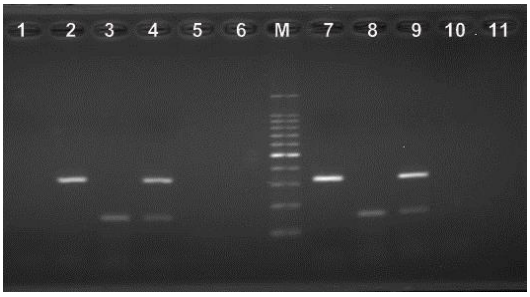
Appendix 1: Map of Nairobi county



Map of Nairobi county showing location of Mbagathi Couty Hospital. Adopted and modified from google maps.

Appendix 2: PCR bands for enteric bacteria virulent genes

A



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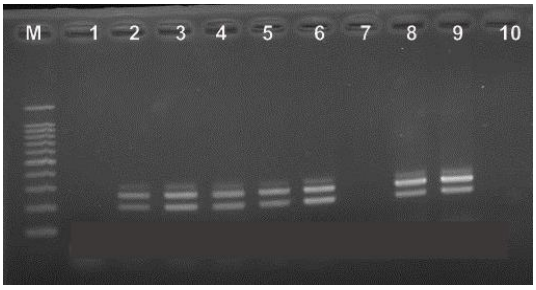
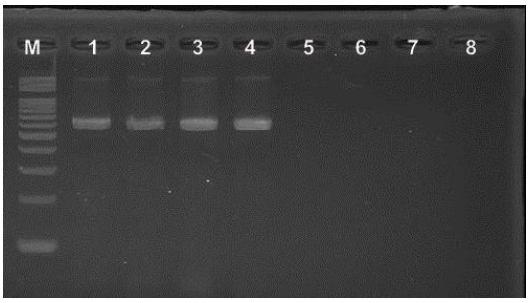
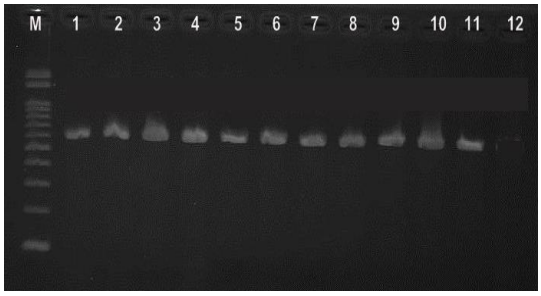


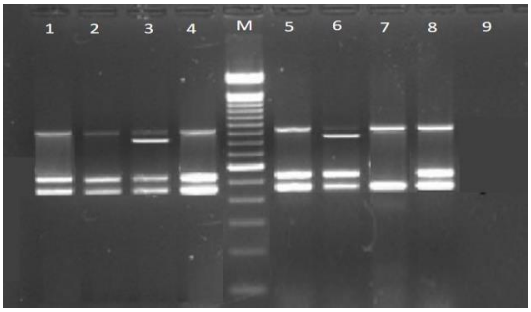
C



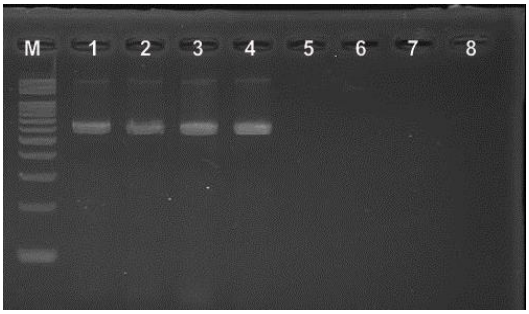
D



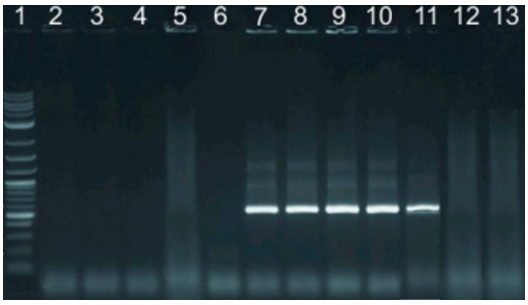
E



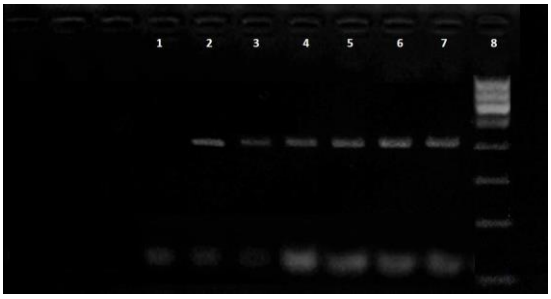
F

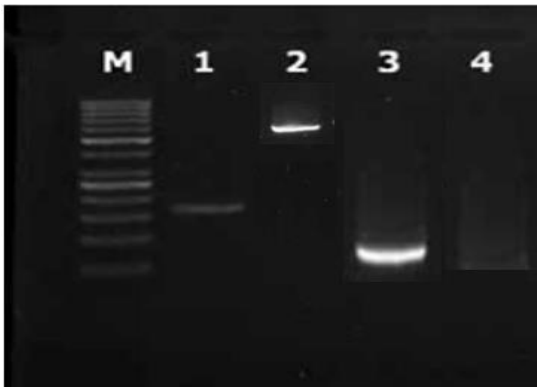


G



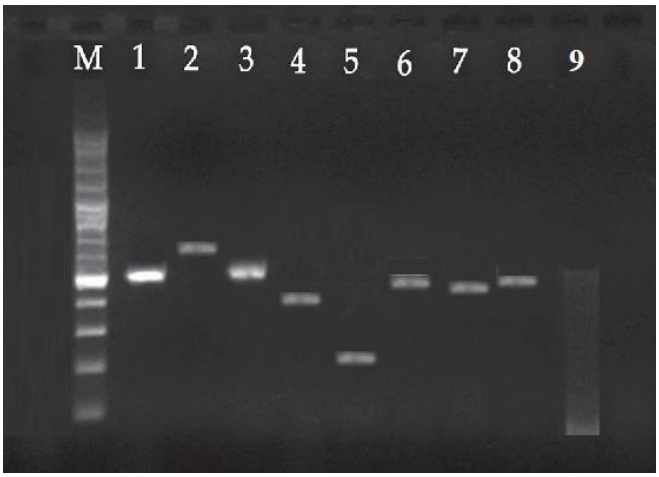
H





I PCR band size. **(A)** Lane M: molecular weight marker M, 100 bp ladder. Lanes 1 and 11: Negative control. Lane 2 and 7: e*lt* (322 bp). Lanes 3 and 8: *est* (147 bp). Lanes 4 and 9: e*lt* (322 bp) and *est* (147 bp). Lane 5, 6 and 10: *elt* and *est*-gene negative *E. coli* isolates. **(B)** Multiplex PCR for detecting EAEC, EIEC, and EPEC pathotypes. Lane M: molecular weight marker M, 100 bp ladder. Lanes 2,3,4,5,6,8 and 9: EAEC-*aggR* (254 bp) and *aspU* (282 bp). Lane M: molecular weight marker M, 100 bp ladder. Lanes: 1 and 7: *aggR, ipaH, and aspU*-gene negative samples. Lane 10: Negative control. **(C)** Multiplex PCR for detecting EAEC, EIEC, and EPEC pathotypes. Lane M: Molecular weight marker M, 100bp ladder. Lanes 1, 2, 3, 4: EIEC-*ipaH*-gene (600 bp). Lane 5,6 and 7: *ipaH*-gene negative sample. Lane 8: Negative control. **(D)** Singleplex PCR for detecting *shigella ipaH*-gene. Lane M: Molecular weight marker M, 100bp ladder. Lanes 1,2,3,4,5,6,7, 8, 9, 10 and 11: *ipaH* (619 bp). Lane 12: Negative control. **(E)** Singleplex PCR for detecting *Salmonella* virulent genes. Lane M: molecular weight marker M, 100 bp ladder; lanes 1, 2, 3, 4, 5, 6, 7, 8: *sopB* gene (470bp); lanes 1, 2, 3, 4, 5, 6, 8: *stn* gene (543bp); lanes 3,6: *hilA* gene (854bp); lanes 1, 2, 3, 4, 5, 6, 7, 8: *invA* gene (942bp). **(F)** PCR for detecting *Shigella* virulent gene. Lane M: molecular weight marker M, 100 bp ladder. Lanes 1, 2, 3, 4: *ipaH*-gene (600 bp). **(G)** PCR for detecting *Yersinia* virulent genes. Lanes 7, 8, 9, and 10: *inv* gene (570bp). **(H)** PCR for detecting *Campylobacter* virulent genes. Lane M: molecular weight marker M, 100 bp ladder. Lanes 2, 3, 4, 5, 6, and 7: *cadF* gene (400bp). **(I)** PCR for detecting *Aeromonas* virulent genes. Lane M: molecular weight marker M, 100 bp ladder. Lane 1: *aerA* gene (309bp); lane 2: *hylA* gene (900bp); lane 3: *alt* gene (148bp); lane 4: negative control.

Appendix 3: PCR bands for antimicrobial resistant genes



PCR for detecting antibiotic resistant genes. Lane M: molecular weight marker M, 100 bp ladder. Lane 1: Chloramphenicol *catA1* resistant gene (547bp); lane 2: Ciprofloxacin *Qnr* resistant gene (670bp); Lane 3: tetracycline *tetA* resistant gene (577bp); lane 4: erythromycin *ereA* resistant gene (419bp); lane 5: gentamycin *aac(3)-IV* resistant gene (286bp); lane 6: ceftriaxone *bla CMY* resistant gene (462bp); lane 7: streptomycin *aadA1* resistant gene (447bp); lane 8: ampicillin *Citm* gene (462bp); lane 9: negative control.

Appendix 4. Questionnaire

Questionnaire to be completed by medical providers at Mbagathi hospital			
Study Number			
Visit Date	_____/ _____/ _____ dd mm yyyy		
Date of onset of symptoms	_____/ _____ / _____ dd mm yyyy		
Age:	Sex ☐ Male ☐ Female		
Patient Information			
Place of resident	Village	District	Province
Have you visited another area in the last 2 weeks?	☐ Yes	☐ No	
Place visited last 2 weeks	Village	District	Province
Occupation:			
CLINICAL SYMPTOMS			

Diarrhea	Duration in days ☐ 1-2 ☐ 3-4 ☐ 5-6 ☐ >7

When did the diarrhea begin? ☐ 1-3 days ago ☐ 4-6 days ago ☐ >6days

Mucus in stool	☐ Yes	☐ No		
Any bloody stool	☐ Yes	☐ No		
Vomiting	☐ Yes	☐ No		
Fever	☐ Yes	☐ No		
Abdominal cramp	☐ Yes	☐ No		
Other symptoms	Headache ☐	Nausea ☐	Loss of appetite ☐	
List any other symptoms:				
Type of water source	Municipal ☐	Well ☐	Rain Water ☐	Borehole ☐
Do you treat the your drinking water? If yes……	☐ Yes	☐ No		

PHYSICAL EXAMINATION			
General condition	Stable (W[illegible]ng unsupported)	Sick looking (Require support) ☐	Very ☐ sick (Unconscious/confused)
Temperature			
Eyeballs	☐ Normal	☐ Sunken	

Tongue	☐ Normal	☐ Dry		
Skin elasticity	☐ Normal	☐ Reduced		
Other evidence of dehydration				
Is the child malnourished	☐ Yes	☐ No		
Treatment (s)				

Name of person completing form:_____

Signature _____ Date: _____

Appendix 5: Ethical approval

MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY
Tel: 056-31375
Fax: 056-30153
E-mail: ierc@mmust.ac.ke
Website: www.mmust.ac.ke
P. O. Box 190-50100
Kakamega, Kenya

Institutional Ethics Review Committee (IERC)

Ref: MMU/COR: 403012 Vol 3 (01) **Date:** 03rd December, 2020

Mark Kilongosi,
Masinde Muliro University of Science and Technology,
P.O. Box 190-50100,
Kakamega.

Dear Mr. Kilongosi,

RE: Seasonal phenotypes and genotypes of enteric bacteria and antibiotic resistance among children seeking treatment at Mbagathi hospital, Nairobi city, Kenya- MMUST/IERC/138/2020

Thank you for submitting your proposal entitled as above for initial review. This is to inform you that the committee conducted the initial review and approved (with no further revisions) the above Referenced application for one year.
This approval is valid from **03rd December, 2020** through to **03rd December, 2021**. Please note that authorization to conduct this study will automatically expire on **03rd November, 2021.** If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the MMUST IERC by **03rd November, 2021.**

Approval for continuation of the study will be subject to submission and review of an annual report that must reach the MMUST IERC Secretariat by **03rd November, 2021.** You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to MMUST IERC prior to implementation.

Please note that any unanticipated problems or adverse effects/event resulting from the conduct of this study must be reported to MMUST IERC. Also note that you are required to seek for research permit from NACOSTI prior to the initiation of the study.

Yours faithfully,

Dr. Gordon Nguka (PhD)
Chairman, Institutional Ethics Review Committee

Copy to:
- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)

Appendix 6: Research permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 330768

Date of Issue: 06/January/202

RESEARCH LICENSE

This is to Certify that Mr.. mark Kilongosi Webale of Masinde Muliro University of Science and Technology, has been licensed to conduct research in Nairobi on the topic: Seasonal phenotypes and genotypes of enteric bacteria and antibiotic resistance among children seeking treatment at Mbagathi hospital, Nairobi city, Kenya for the period ending : 06/January/2022.

License No: NACOSTI/P/21/8147

330768

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application

Appendix 7: Informed consent

Informed consent: English version

TITLE OF STUDY: Prevalence and Characterization of clinical isolates of diarrheagenic bacteria and Antimicrobial profiling in children under five years at Mbagathi District hospital Nairobi County, Kenya

LOCATIONS: Mbagathi District Hospital

INVESTIGATOR: Mark Webale

INCLUSION CRITERIA: All consenting parents of children under five years of age in outpatients and inpatients seeking treatment for diarrheal illness at Mbagathi District Hospital, Nairobi County, Kenya.

EXCLUSION CRITERIA: Children under five years who had taken antibiotics within 72hrs of the onset of symptoms and those whose parents are unwilling to participate or give stool samples.

INFORMED CONSENT

Your child is being asked to participate in a research study conducted by the investigators listed above. Your child's participation is entirely voluntary. If you do not want to participate, there will be no penalty. Your child will be treated medically by the doctor or clinical officer in the normal way. You may stop your child's participation at any time. If you choose for your child to leave the study, you should return to the study doctor and inform him or her.

You should read the information below and ask questions about anything you do not understand before deciding whether or not to participate. You will be given a copy of the consent form to keep.

PURPOSE OF THE STUDY

We are interested in finding out more about the germs that cause diarrhea in Kenya. We will also do laboratory tests to determine the effective drugs to treat diarrhea.

PROCEDURES

If you volunteer to participate in this study, we would ask you to do the following things:

STUDY TESTS

During this Study, your child will be asked to provide stool in a cup.

QUESTIONNAIRE

If you participate or allow your child to participate, we will ask you some questions. These include questions about your child's age, your job, where you live, how many times you/your child had diarrhea before coming to the hospital, recent medications your child has received, source of water, other illness your child had with the diarrhea such as headache or vomiting. You do not have to answer any question that makes you uncomfortable.

POTENTIAL RISKS AND DISCOMFORTS

There is no risk from providing stool. Participation in this study will not delay medical care your child would normally receive.

BENEFITS

The benefit of taking part in the study is that you/your child will have tests done, free of charge, to determine the cause of diarrhea and to determine the best way to treat it. In

addition, a better understanding of diarrheal illnesses may eventually lead to better treatments and preventive measures.

COMPENSATION

There is no direct compensation to volunteers for their participation.

CONFIDENTIALITY

All information collected about your child will remain confidential. Your child's name will not appear on any publication or report from this or other studies. Your child's name will not be kept in the computerized research database. All samples will be labeled only with a unique study number, not his/her names. Questionnaires and consent forms will be kept in a locked file at the National public Health laboratories storage facility for 10 years following completion of the study. Laboratory data sheets will be made available only to Principal Investigator, clinical personnel who require this information to treat you/your child, or to members of the Ministry of Health and who require this information for legal reasons or to study an outbreak.

PARTICIPATION

Your participation in this study is entirely voluntary. You are free to withdraw from this study at any time.

MEDICAL CARE FOR RESEARCH RELATED INJURY

Should your child be injured as a direct result of participating in this research project, your child will be provided medical care, at no cost for that injury. Your child will not receive any injury compensation, only medical care. You should also understand that this is not a waiver or release of your child's legal rights. You should discuss this issue thoroughly with the principal investigator before you enroll in this study.

YOUR RIGHTS AS A RESEARCH VOLUNTEER

If you have any questions about the study, your rights as a research volunteer, or a research-related injury, you should contact Mr.Peter Lokamar Nyanga at P.O. Box 20781 Nairobi, Kenya, telephone 0722447176, or the doctor on duty at the study site. The Chairman of the National Bioethics Committee ,Nairobi ,Kenya

Signature---------- Date ----------

WRITTEN CONSENT

I, _____(Name) having attained my ____ birthday and full capacity to consent for my child named: _____(Subject's name), do hereby consent to his/her participation in the research study:

"Surveillance of common circulating enteric pathogens and antimicrobial susceptibility patterns in Kenya, The methods and means by which the study will be conducted and the risks which may be reasonably expected have been explained to me by _____. I have been given the opportunity to ask questions concerning this research study, and any such questions have been answered to my full and complete satisfaction.

I understand that I may at any time during the course of this study cancel this consent agreement and withdraw my child from the study without prejudice.

SIGNATURE OF RESEARCH SUBJECT

Name of Subject: _____

Subject's/Fingerprint: _____Date: _____

Guardian's Signature: _____Date:_____
Permanent Address: _____
SIGNATURE OF WITNESS
My signature as witness certifies that the subject signed this consent form in my presence as his or her voluntary act.
Witness's Name: _____
Witness's Signature: _____ Date: _____
Study Number: _____

Informed consent: Kiswahili version

UCHUNGUZI HUU NI JUU YA: Elimu kuhusu magonjwa ya kuhara katika kaunti ya Nairobi, nchini Kenya
MAENEO: Hospitali kuu la Mbagathi District Hospital, kaunti ya Nairobi
MCHUNGUZI MKUU: Mark Webale
KANUNI KWA WANAOSHIRIKI: Watoto wote wa umri wa chini ya miaka mitano ambao wamelazwa/hawajalazwa hospitalini, wa umri huo huo, wanaotafutamatibabu ya magonjwa yakuharakatika hospitalikatika mahabaraya kukaguliwakatika Hospitali ya Mbagathi Kenya.
KANUNI KWA WASIOSHIRIKI: Watoto wagonjwa chini ya miaka mitanowaliokuwa wamemezaantibioticsndani ya masaa 72yamwanzo wa dalili,na wale wasiokuwana nia yakushirikiau kutoasampuli zakinyesi.
MAELEZO KUHUZU RIDHAA AU RUHUSA: Mwanzo anaombwa kushiriki kwenye utafiti huu wa uchunguzi unaoongozwa na mchunguzi ambaye majina yake yameorodheswa hapo juu.
Ushiriki wa mwanao ni wa kujitolea. Iwapo hutaki kushiriki, hutapata adhabu yoyote, mwanao atatibiwa na daktari au afisa wa utabibu kwa njia ya kawaida. Unaweza kumwondoa mwanao katika mradi huu wakati wowote. Ukiamua kumwondoa mwanao kwenye mradi huu unapaswa kurudi kwa daktari wa mradi ili uweze kumwaarifu.
Unapaswa kusoma taarifa ifuatayo na uulize maswali yoyote kuhusu jambo lolote ambalo hujalifahamu kabisa kabla ya kuamua kushiriki au kutoshiriki. Utapewa fomu ili uweze kuweka.

KUSUDI LA UCHUNGUZLU:
Tuna hamu sana ya kufanya uchunguzi kuhusu vijidudu vinavyosababisha ugonjwa wa kuhara katika nchi ya Kenya.
Pia tutafanya uchunguzi katika maabara kudhihiriha dawa zinazofaa zaidi kwa kutibu ugonjwa huu wa kuhara.
TARATIBU:
Iwapo umejitolea kushiriki katika uchunguzi huu, tungependa kuwaomba mfanye yafwatayo:
UCHUNGUZI:
Wakati wa uchunguzi,mtoto wako ataombwa kuleta kinyesi au choo. Mtoto wako atahitaji kutia choo kwenye kikombe atakachopewa.
MASWALI:
Ukirumhusu mwanao kushiriki, tutakuuliza maswali yafuatayo: Umri wa mtoto wako, kazi yako, mahali unapoishi, mtoto wako ameharisha mara ngapi kabla ya kumpeleka hospitalini na madawa alizopokea, chemichemi ya maji mnayotumia au mahali mnapotoa maji, magojwa mengine ambayo yanambatana na ugonjwa wa kuhara kama kwa mfano kuumwa na kichwa au kutapika. Unaweza kuliacha swali lolote ambalo halikupendezi kujibu.
HATARI ZISIZODHIHIRIKA NA PIA HALI ISIYO NA RAHA.
Hakuna hatari yoyote inayoweza kutokana na utoaji wa kinyesi au choo. Kushiriki kwenye uchunguzi huu hakutayachelewesha matumizi ya madawa kwa mwanao. mwanao atatumia madawa jinsi inavyotakikana.

FAIDA.

Faida inayotokana na kushiki kwenye uchunguzi huu ni kwamba: mtoto wako atachunguzwa bila malipo yoyote kudhihirisha chanzo cha ugonjwa na pia kutafuta njia bora zaidi ya kuutibu ugonjwa huu. Zaidi ya hayo, kuelewa kinaganaga kwa ugonjwa huu kunaweza kutuongoza kwa utafiti unaofaa wa kuzuia ugonjwa huu.

FIDIA

Hakuna fidia yoyote wajitoleaji wataipokea baada ya kushiriki.

SIRI.

Habari zote ambazo zitakusanywa kuhusu mwanao zitakuwa za siri daima. Jina la mwanao halitaonyeshwa kwenye tangazo au ripoti yoyote kutokana na uchunguzi huu au mwingine. Jina la mwanao halitawekwa kwenye kompyuta ya utafiti au database. Sampuli zote zitabandikwa tu tarakimu au nambari maalum wala sio jina la mwanao. Msimamizi anastahili kuchunguza rekodi za uchunguzi kwa ajili hii ndiyo moja wapo ya majukumu yake kutunza majina ya watu wanaoshiriki kwenye uchunguzi huu. Maswali na fomu za ruhusa zitawekwa katika faili ambazo zitahifadhiwa National Public Health Laboratories. Fomu hizi zitahifadhiwa kwa miaka kumi hadi wakati uchunguzi utakapokamilika.

Karatasi ya mambo yasiyokubaliwa kuwa hakika ya maabara (lab data sheet) zitapatikana tu kwa mchunguzi , madaktari au wauuguzi wa kliniki ambao watahitaji habari hii kwa matibabu ya mwanao, au mwanachama wa wizara ya afya ambaye atahitahi habari hii kwa sababu halali au kuchunguza mlipuko.

USHIRIKI

Ushiriki wako katika uchunguzi huu ni wa kujitolea. Lazima mtu ajitolee kwa hiari. Uko huru kujiondoa kwenye uchunguzi huu wakati wowote.

UTUNZAJI WA UTABIBU DHITI YA MADHARA YOYOTE KUTOKANA NA UTAFITI.

Mtoto wako akijeruhiwa wakati huu atapewa matibabu ya bure. Mwanao atapokea fidia yoyote au malipo yoyote. Atapokea tu matibabu. Wewe unastahili kufahamu kuwa, utafiti huu si haki halali kwa mwanao. Unapaswa kujadili jambo hili kabisa na Mchunguzi au mshauri kabla ya kujiandikisha kwenye uchunguzi huu.

HAKI YAKO KAMA MJITOLEAJI WA UTAFITI.

Iwapo una swali lolote kuhusu uchunguzi huu, haki yako kama mjitoleaji wa utafiti au kuhusu madhara yoyote kutokana na utafiti, unapaswa kuwasiliana na Mr Peter Lokamar katika sanduku la posta 20781, Nairobi, Kenya; nambari ya simu 0722447176 au Daktari wa zamu katika kliniki. Mwenye kiti wa National Bio-Ethical Committee,Nairobi,Kemya.

MAANDIKO YA RIDHIA. (UTOAJI WA RUHUSA).

Mimi_____(Jina) mwenye umri wa miaka_____ nimetoa ruhusa kwa mwanangu_____ nimekubali kushiriki katika utafiti wa uchunguzi kuhusu magonjwa ya kuhara katika nchi ya Kenya. Mbinu na njia ambazo uchunguzi huu utafanywa na hatari yoyote inayoweza kutokana , tayari nimeshaelezewa na _____.

Nimeshapewa idhini ya kuuliza maswali kuhusu utafiti huu na tayari hayo maswali nimeniridhika na majibu yote. Nafahamu kwamba ninaweza kuondoka wakati wowote au kumuondoa mtoto wangu bila dhuluma yoyote.

MUHURI (SAHIHI) YA MSHIRIKI WA UCHUNGUZI:

Jina la mshiriki_____ Tarehe:_____
Alama ya kidole ya mshiriki_____Tarehe_____
Muhuri (sahihi) ya mlezi _____
Anwani ya kudumu_____

MUHURI YA SHAHIDI

Muhuri yangu kama shahidi inathibitisha ya kwamba mtu huyu alikubali kutia fomu hii sahihi mbele yangu kama kitendo cha kujitolea.

Jina la shahidi _____
Muhuri ya shahidi _____
Tarehe _____
Nambari ya uchunguzi _____