One Health -One City; the extent of Shiga-toxin producing *Escherichia coli* in Cape Town.

By

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Declaration

I, John Bosco Kalule, hereby declare that the work on which this thesis is based is my original work and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Table of Contents

List of Tables

List of Figures

List of abbreviations and acronyms

∘C	Degree Celcius
µg	Microgram
µl	Microliters
aat	anti-aggregation protein transporter gene
AC	Acetonitrile
Aeromonas sorbria	*A. sorbria*
AMC	Amoxicillin-clavulanate
AMK	Amikacin
AMP	Ampicillin
ANOVA	Analysis of Variance
APEC	Avian pathogenic *E. coli*
ATCC	American Type Culture Collection
BA	Blood Agar
BAM	Bacteriologic Analytical Methods
bfp	bundle forming pili
BiNGO	Biological Networks Gene Ontology
bp	Base pairs
Campylobacter jejuni	*C. jejuni*
CAZ	Ceftazidime

CCDA	Charcoal Cefoperazone Deoxycholate modified agar
CCFH	Codex Committee on Food Hygiene
CDC	Centre for Disease Control
CDEC	Cell detaching *E. coli*
CDT	Cytolethal Distending Toxin
CIA	Critically Important Antibiotics
CIN	Cefsulodin –Irgasan- Novobiocin
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
COL	Colistin
CTX	Cefotaxime
CXA	Cefuroxime Axetil
CXM	Cefuroxime
DAEC	Diffusely adherent *E. coli*
DALYs	Disability Adjusted Life Years
DEC	Diarrheic *E. coli*
DNA	Deoxyribonucleic Acid
eae	Intimin coding gene
EAggEC	Enteroaggregative *E. coli*
EAHEC	Enteroaggregative haemorrhagic *E. coli*
EDTA	Ethylenediaminetetraacetic acid

EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
Escherichia coli	*E. coli*
ETP	Ertapenem
FAO	Food and Agricultural Organisation
FDR	False Detection Rate
FEP	Cefepime
FERG	Foodborne Epidemiology Research Group
FOX	Cefoxitin
FSIS	Food Safety and Inspection Services
GEMS	Global Enteric Multi-centre Study
GEN	Gentamicin
GO	Gene ontology
GSH	Groote Schuur Hospital
HACCP	Hazard Analysis Critical Control Points
HIV	Human Immunodeficiency Virus
HPA	Health Protection Agency
HPLC	High-Performance Liquid Chromatography
HREC	Higher Research Ethics Committee
HUS	Haemolytic Uraemic Syndrome

ICMSF	International Commission for the Microbiological Specification of Foods
IFSAC	Interagency Food Safety and Analytics Collaboration
IMP	Imipenem
Ipa	Invasion plasmid antigen
LC-MS/MS	Liquid Chromatography- tandem Mass Spectrometry
LEE	Locus of enterocyte effacement
LFQ	Label Free Quantification
LR	Lotus River
LT	Heat-labile toxin
MEM	Meropenem
MIC	Minimum Inhibitory Concentration
MLVA	Multi-Locus Variable Number of Tandem Repeats Analysis
NCHC	Nyanga Community Health Centre
ND	Not detected
NHLS	National Health Laboratory Services
NIT	Nitrofurantoin
NM	Non-motile
NMEC	Neonatal Meningitis associated *E. coli*
NT	Non typeable
OmpA	Outer membrane protein A
OmpC	Outer membrane protein C

OmpF	Outer membrane protein F
OsO_4	Osmium tetroxide
PCR	Polymerase Chain Reaction
Plesiomonas shigelloides	*P. shigelloides*
r.p.m	revolutions per minute
RIS	Resistant Intermediate Susceptible
SABS	South Africa Bureau of Standards
Salmonella enterica	*S. enterica*
Shigella flexneri	*S. flexneri*
ST	Heat-stable toxin
STEC	Shiga-toxin producing *E. coli*
STEC	Shiga toxin producing *E. coli*
SXT	Trimethoprim- sulphamethoxazole
TCBS	Thiosulfate – Citrate – Bile - Salts
TE	Tris – EDTA buffer
TGC	Tigecycline
Tm	Melting temperature
TZP	Tazobactam-piperacillin
UPEC	Uropathogenic *E. coli*
Vibrio vulnificus	*V. vulnificus*
VNTR	Variable Number of Tandem Repeats

WGS	Whole Genome Sequencing
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate
Yersinia enterocolitica	*Y. enterocolitica*

Abstract

Introduction

The estimated global burden of STEC (Shiga toxin producing *Escherichia coli*) is 2,481,511 illnesses, 269 deaths, and 26,827 DALYs with 48% of these being foodborne. This thesis provides information on STEC diagnostic strategy, undetected STEC in a tertiary referral hospital in Cape Town, and the virulence and antimicrobial resistance properties of tellurite resistant diarrheic *E. coli* isolated on CHROMagar™STEC (CHROMagar Microbiology, Paris, France). Deploying the One – Health surveillance approach to study selected diarrheic bacterial pathogens in an informal settlement setting, this study sheds light on the extent of bacterial foodborne pathogens in human and non-human sources.

Methods

The performance of CHROMagar™STEC for screening for STEC in stool, river water, and meat was assessed relative to an in-house developed duplex real-time PCR assay (for detection of *stx*$_1$ and *stx*$_2$) as gold standard. Stool samples presented to the stool culture bench at a tertiary referral hospital between September 2014 and May 2015 were screened for STEC using CHROMagar™STEC with and without enrichment in Tryptic Soy Broth. Not all specimen were enriched so as to evaluate the performance of the medium in either of the culture conditions. The enrichments were screened for *stx* using the duplex real-time PCR assay. Mauve colonies on CHROMagar™STEC that were confirmed to be *E. coli* were tested for possession of the *aat*, *daaC*, *eaeA*, *ipa,* and *bfp* using gel-based PCR and for *stx*$_1$ and *stx*$_2$ using the duplex real – time PCR assay. All the diarrheic *E. coli* were characterised by antibiotic susceptibility testing, serotyping and immunochromatography. Using quantitative tandem mass spectrometry, the virulence and antimicrobial resistance associated proteins of three selected drug resistant EPEC/STEC strains were studied. Differential expression of cell envelope proteins was further investigated using Transmission Electron Microscopy.

Between September 2015 and May 2016, stool from children under 12 years, surface water from the Lotus River, and meat from the informal abattoirs were collected and processed using

standardised methods. Clinical and epidemiological data was collected using a questionnaire. Following enrichment in TSB, samples were tested for virulence genes (using the in-house developed duplex real-time PCR assay) and serotype specific marker genes *rfbE* and *wbdI* (using gel-based PCR). Enrichments were then screened for STEC and other diarrheic *E. coli, Salmonella enterica (S. enterica)*, *Shigella spp.*, *Campylobacter spp.*, *Aeromonas spp.*, *Plesiomonas spp.*, and *Vibrio spp.* using standardised methods. The antibiograms, serotypes (for *S. enterica* and diarrheic *E. coli*), and virulence genes carried (by the diarrheic *E. coli*) were determined. The total coliform count per ml of Lotus River surface water was also determined.

Multi- Locus Variable Number of Tandem-repeats Analysis (MLVA) was conducted on diarrheic *E. coli* from human and non-human sources targeting seven genomic VNTR loci. Two multiplexes and one singleplex PCR was done using dye-labeled primers and capillary electrophoresis done to determine fragment sizes. The electrophoregram files were directly imported into the BioNumerics v7.6 software and the number of tandem repeats per locus (allelic number) determined. A dendrogram was then constructed using categorical coefficients and the ward algorithm.

Results

The mean melting temperature on melt-curve analysis for detection of *stx*$_1$ was 58.2℃ and for *stx*$_2$ was 65.3℃; detection of both targets could therefore occur in the same run. The limit of detection of the duplex real-time PCR assay was 5.3 target copies/µl of broth, 116 ±4.03 CFU per 25g of meat and 144 ±6.4 CFU/100ml of Lotus River surface water for both targets. For stool, the sensitivity, specificity, negative predictive value and positive predictive value of the CHROMagar™STEC were 33.3%, 77.4%, 95.3% and 11.3% respectively. For water, the sensitivity, specificity, negative predictive value and positive predictive value of the CHROMagar™STEC were 5.26%, 66.7%, 62.5% and 6.25%, respectively. For meat, the sensitivity, specificity and negative predictive value of the CHROMagar™STEC were 0%, 52.94% and 66.7%, respectively.

A total of 733 stool samples (226 were enriched while 507 were not) were processed in Groote Schuur Hospital. Of the 226 stool specimens screened with enrichment, real-time PCR detected

stx in 14 specimens (6.2%, 95% Confidence Interval=3.43%-10.18%), comprising eight *stx*$_1$, five *stx*$_2$ and one with both *stx*$_1$ and *stx*$_2$. Of the 33 diarrheic *E. coli* isolates, 15% (5/33, 95% CI=6.7 – 30.1) were STEC, 45% (15/33, 95%CI=29.84 – 62.01) were EAggEC, 18% (6/33, 95%CI=8.6 – 34.4) were atypical EPEC, 15% (5/33, 95% CI=6.7 – 30.1) were typical EPEC, and 3% (1/33, 95%CI=0.54 – 15.32) were DAEC. Serotypes O104 (5/33, 15%) and O55 (6/33, 18%) were the dominant diarrheic *E. coli* serotypes. Fifty-five percent of the 33 diarrheic *E. coli* (95% CI = 40.8 – 72.7) showed resistance to ampicillin while all the isolates showed resistance to trimethoprim-sulphamethoxazole.

The proteomes of three selected EPEC/STEC isolates showed an abundance of virulence and antimicrobial resistance proteins. The ciprofloxacin-resistant strain showed greater cell envelope thickness compared to the ciprofloxacin susceptible EPEC and STEC strains.

In the Nyanga township, 66, 64, and 85 stool, water, and meat samples were tested. The *rfbE* and *wbdl* were detected in 2% (1/66) and 2% (1/66) of stool, 6% and 0% of meat, and 14% and 8% of water respectively. Of the 66 stool samples tested, the bacterial pathogens detected were: *S. enterica* (6%, 4/66), *Shigella flexneri* (17%, 11/66), *Plesiomonas shigelloides* (9%, 6/66), *Aeromonas sorbria* (3%, 2/66), *Campylobacter jejuni* (5%, 3/66), DAEC (12%, 8/66), EAggEC (3%, 2/66), STEC (2%, 1/66), EPEC (2%, 1/66), and EIEC (2%, 1/66). Of the 64 water specimens screened, real-time PCR detected *stx* (*stx*$_1$ only) in 19 samples (30%, 95% Confidence Interval= 18.91%-42.42%). The bacterial pathogens detected were: *Shigella flexneri* (5%, 3/64), *Plesiomonas shigelloides* (17%, 11/64), *Aeromonas sorbria* (6%, 4/64), DAEC (3%, 2/64), EAggEC (2%, 1/64), STEC (2%, 1/64), and non-choleare-*Vibrio* (9%, 6/64). Of the 85 meat specimens, real-time PCR detected *stx* in 21 samples (17 *stx*$_1$ and 4 *stx*$_2$) (25%, 95% CI= 13.06%-31.39%). The bacterial pathogens detected were: *Salmonella enterica* (5%, 4/85), *Plesiomonas shigelloides* (1%, 1/85), *Aeromonas sorbria* (3%, 3/85), *Campylobacter jejuni* (7%, 6/85), DAEC (2%, 2/85), and EPEC (1%, 1/85). For all sample types, the number of diarrheic *E. coli* virulence genes detected in the TSB enrichments was higher than the actual number of diarrheic *E. coli* isolated There was no significant difference in the prevalence of resistance to selected antibiotics among human and environmental diarrheic *E. coli* isolated in Nyanga. Water from the Lotus River surface water possessed a high number of faecal coliforms (mean CFU/ml =1.11E+06 -2.74E+05) indicating faecal contamination.

Conclusions

These studies demonstrate the importance of a One – Health surveillance approach for foodborne disease, and the need for broader adoption of similar surveillance approaches in South Africa. Specific gaps in this work, which should be addressed by further studies include the following: (1) using alternative culture media that allow growth of tellurite susceptible STEC to determine the extent of tellurite susceptible STEC, (2) comprehensive microbiological testing and strain characterization on patients presenting with HUS to determine strains associated with severe disease, (3) a larger scale case-control study over at least 12 months needs to be carried out in both primary health care and the community to determine pathogens associated with diarrhoea in children under 12 years, (4) Larger scale studies on food from informal abattoirs and river surface water are needed to determine the generalizability of our findings, (4) there is need to screen for antimicrobial resistance genes in the human and non-human samples in addition to determining the antibiograms of specific foodborne pathogens isolated, so as to assess the true magnitude of foodborne antimicrobial resistance (5) the linkage between ciprofloxacin resistance in STEC/EPEC strains and cell envelope thickness (as determined by transmission electron micrography) should be further examined using, a larger sample size and carefully controlled experimental design.

Chapter One

1 General Introduction and Thesis Outline

1.1 General Introduction

Shiga toxin producing *E. coli* (STEC) is one of the emerging foodborne pathogens that can lead to acute renal failure associated with haemolytic-uraemic syndrome (HUS) and haemorrhagic colitis[1] in infected patients as well as enormous economic losses in the food industry.[2] Several outbreaks of HUS due to STEC have been reported globally, especially in the developed countries[3-6] and only a few have been reported in Africa.[7,8] In many of these outbreaks, the pathogen mainly reported has been the non-sorbitol fermenting STEC O157:H7. [3-6] More recently, the non-O157 STEC-related outbreaks have increased, and the number of non-O157 STEC serotypes causing severe human disease is increasing.[9,10] However, in South Africa, since the 1990 outbreak that was caused by *E. coli* O157: NM, no *E. coli* O157 related outbreak has been reported. At the National Institute of Communicable Disease (NICD), research carried out showed that of the 2378 diarrheic *E. coli* collected between 2006 and 2009, 14 were STEC and only one was STEC O157:H7. In the NICD study, the 14 STEC were a co-incidental finding because they initially had been presented to the NICD as enteropathogenic *E. coli* (EPEC) given that they also carried the *eae* gene - a virulence marker for EPEC.[11] Despite the low STEC detection levels, there have been reports of high rates of acute renal failure associated with typical HUS for example at the Pretoria Academic Hospital.[12,13] In February 2017, another outbreak of typical HUS (un published work in which the PhD candidate participated) was reported at the Red Cross War Memorial Hospital involving four children. In this epidemic, biltong and a few other meat products were suspected reservoirs of the causative STEC pathogen. The non-human (environmental) reservoirs of STEC in Cape Town are not known. There is no published data on the prevalence of STEC in the non-human environment. Even though South Africa has an excellent public health system with functional food inspection systems at the established abattoirs, meat from the informal slaughter setting in the informal settlements such as Nyanga is not inspected.

In a bid to improve storm water quality in the City of Cape Town, a private firm called Mott MacDonald PDNA (Cape Town, South Africa) was hired to investigate the contributing factors to the pollution of storm water- a significant component of the City of Cape Town aquatic systems. The outcomes of this investigation showed that the informal abattoirs, which are open

spaces in the townships are a primary source of blood, gastro-intestinal contents, salts from hide curing, and other post slaughter waste that gets washed into the storm water channels such as the Lotus River. The gastro-intestinal contents are a rich source of foodborne pathogens including STEC.[14-16] And yet the informal abattoirs are the primary source of animal protein for people from the low to middle-income areas of Cape Town.

At the Groote Schuur Hospital, a tertiary referral hospital in Cape Town, the stool is screened for STEC on physician request only. Screening is done only for *E. coli* O157:H7 basing on the non-sorbitol fermentation property. As is the case in the developed countries and as recommended the Centre for Disease Control (CDC, U.S.A)[17], all stool samples from patients with acute diarrhoea should be screened for STEC.[18] Previous research on STEC at the Groote Schuur Hospital by Kullin *et*. al, focused on the presence of *stx* in DNA directly extracted from stool samples.[19] This research therefore never attempted to isolate viable STEC from the stool and did not provide data on the serotypes, and virulence characteristics of STEC.

CHROMagar™STEC is a chromogenic medium that allows tellurite resistant *E. coli* to form mauve colonies and has previously been tested for clinical and public health use in the developed countries. It showed excellent sensitivity for O157 and the top six non-O157 STEC serotypes. For this reason, it would be a good alternative to the use of sorbitol MacConkey that targets only *E. coli* O157:H7 given the increasing number of non-O157 STEC that are involved in outbreaks. This medium allows the formation of mauve colonies by tellurite resistant STEC. However, not all STEC are tellurite resistant, and so the tellurite susceptible STEC is missed on this medium.[20,21] Additionally, other pathotypes of diarrheic *E. coli* are tellurite resistant and form mauve colonies on CHROMagar™STEC.[22] This medium has been shown to favour the growth of the *stx*-negative derivatives of the major STEC serotypes (EHEC-like).[23] The shades of mauve displayed by tellurite resistant strains in this media also vary. From the currently published literature, no study in Africa has evaluated the use of this medium for isolation of STEC in clinical and public health settings. In this study, we report on only the tellurite resistant STEC and diarrheic *E. coli* (formed mauve on CHROMagar™STEC) in Cape Town. We focused on three sample types likely to be involved in the transmission cycle of STEC in this setting namely; stool from children less than

12 years attending the Nyanga Community Health Centre (NCHC), meat from the informal abattoirs in Nyanga, surface water from the Lotus River, and stool samples (irrespective of age and clinical data) from the Groote Schuur Hospital (GSH). Firstly, we validated an in-house developed hybridization probe-based real-time PCR assay able to simultaneously detect both *stx*$_1$ and *stx*$_2$ targets, for screening stool, meat and surface water for STEC. Secondly, we used the in-house validated real-time PCR assay as the gold standard to evaluate the performance of CHROMagar™STEC medium for screening meat from the informal abattoirs in Nyanga, surface water in Lotus River, and stool at the Groote Schuur Hospital. Thirdly, we characterised would be undetected STEC at GSH, and then used quantitative proteomics to assess the virulence and antimicrobial resistance proteins of three EPEC/STEC strains that formed mauve colonies on CHROMagar™STEC. This was aimed to predict the pathogenic potential and clinical relevance of diarrheic *E. coli* that formed mauve on this medium and to assess production of tellurite resistance proteins by these strains. Fourthly, we sought to determine the prevalence of STEC, diarrheic *E. coli,* and other foodborne pathogens in the stool of children less than 12 years attending the Nyanga Community Health Centre, meat from the informal abattoir settings, and the surface water from the Lotus River (section draining the Nyanga Township). Lastly, we used Multi-Locus Variable Number Tandem Repeats Analysis to determine the MLVA genotypes of diarrheic *E. coli* from human and non-human sources in Nyanga, as a means of source tracing and identifying the reservoirs of STEC in the Nyanga Township. The One Health approach was used to define the prevalence and characteristics of STEC at the human-environment interface in Cape Town.

1.2 Aims of the study

Aim 1: Enhanced surveillance of STEC and other diarrheic *E. coli* from human and non-human sources in Cape Town, South Africa.

Objective 1.1: To validate a real-time PCR test and compare it to culture on CHROMagar™STEC for detection of Shiga-toxin-producing *E. coli* in stool, water from the Lotus River, and meat samples from the informal abattoirs.

Objective 1.2: To detect and characterise undiagnosed Shiga-toxin-producing *E. coli* and other diarrheic *E. coli* in stool processed at a tertiary referral hospital, Cape Town, South Africa.

Objective 1.3: To compare the proteomes of three clinical diarrhoeagenic drug-resistant *E. coli* isolates grown on CHROMagar™STEC media.

Aim 2: Characterise the 2015/16 epidemiology of circulating STEC and other foodborne bacterial pathogens isolated from stool, meat and the Lotus River surface water, Nyanga, Cape Town.

Objective 2.1: To determine the prevalence and characteristics of STEC and other bacterial enteric foodborne pathogens from children with diarrhoea, meat from the informal abattoirs and Lotus River surface water in Nyanga Township, Cape Town, South Africa.

Objective 2.2: To determine the Multi-Locus Variable Number of Tandem Repeats Analysis (MLVA) genotypes of STEC and other diarrheic *E. coli* from the stool, meat from the informal abattoirs and Lotus River surface water in Nyanga Township, Cape Town.

1.3 Study design and population

1.3.1 Study design

This was a cross-sectional study conducted in Cape Town to investigate STEC in human and non-human sources. This study was conducted at the Groote Schuur Hospital and the Nyanga Township. In the Nyanga Township, participants were recruited at the Nyanga Community Health Centre. The environmental aspect of the study was conducted in Nyanga Township and involved sampling meat from the informal abattoirs and surface water from the Lotus River (the section traversing the Nyanga Township).

1.3.2 Study population

1.3.2.1 Groote Schuur Hospital

Between September 2014 and May 2015, we collected residual stool (submitted for bacterial pathogen culture) after routine testing from 733 consecutive stool specimens (irrespective of age or related clinical data) from the National Health Laboratory Services located at the Groote Schuur Hospital.

1.3.2.2 *Diarrhoea patients at the Nyanga Community Health Centre*

We consecutively collected 66 stool samples from children less than twelve years of age with diarrhoea that attended the NCHC on Monday, Tuesday, and Wednesday of every week during the study period (For six months between October 2015 and April 2016).

Inclusion criteria: Only children whose guardians consented to participation in this study and attended the Nyanga Community Health Centre on Monday, Tuesday, or Wednesday during the study period were included.

Exclusion criteria: Children whose guardians did not consent to participation in the study were excluded.

1.3.2.3 *Environmental sampling*

Between October 2015 and May 2016, we used random sampling to collect Forty-nine (49) raw and 36 ready – to - eat meat samples from 53 randomly selected local stalls along main access roads in the Nyanga Township.

Sixty-four (64) surface water samples were consecutively collected over a period of ten months (between 7 a.m and 12 noon) from July 2015 to April 2016 (**Figure 1:1**).

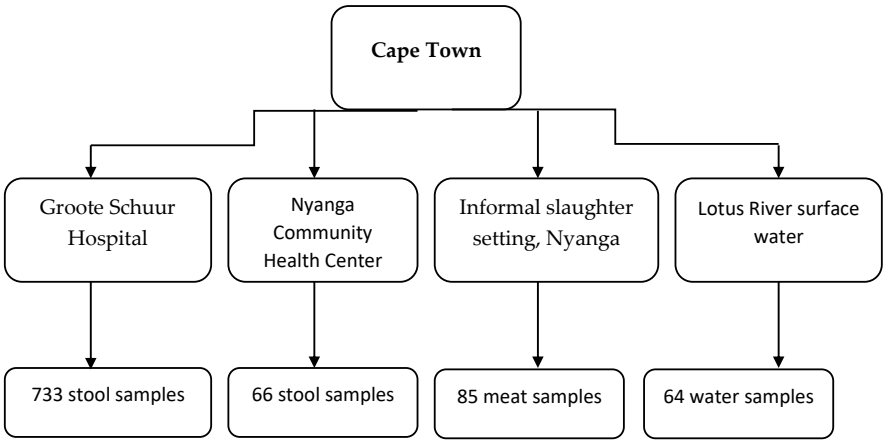

Figure 1:1 Summary of the study population

1.4 Ethics consideration

Ethical clearance to conduct research at the Groote Schuur Hospital was obtained from the Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (HREC REF: 015/2014).

To conduct research in Nyanga, ethical approval was obtained from the Research and Ethics Committee of the Faculty of Health Sciences, University of Cape Town, South Africa; HREC 140/2015. Also, consent was received from the ethics committee of the City of Cape Town (Identification number: 10516/6547).

1.5 Overview of the laboratory work flow

1.5.1 Laboratory work flow for stool samples from the Groote Schuur Hospital

Of the 733 stool specimens (based on convinience), 507 stool samples were processed by direct streaking onto CHROMagar™STEC, while 226 stool samples were enriched in Tryptic Soy Broth before streaking on CHROMagar™STEC. (**Figure 1:2**). Isolates confirmed as *E. coli* were then tested for possession of virulence genes by real-time and gel-based PCR. Only the *E. coli* isolates that carried virulence genes were serotyped, tested for sorbitol fermentation, tested for Shiga toxin production using immunochromatography, and tested for susceptibility to antibiotics using VITEK 2 automated system (biomerieux, USA) and broth microdilution.

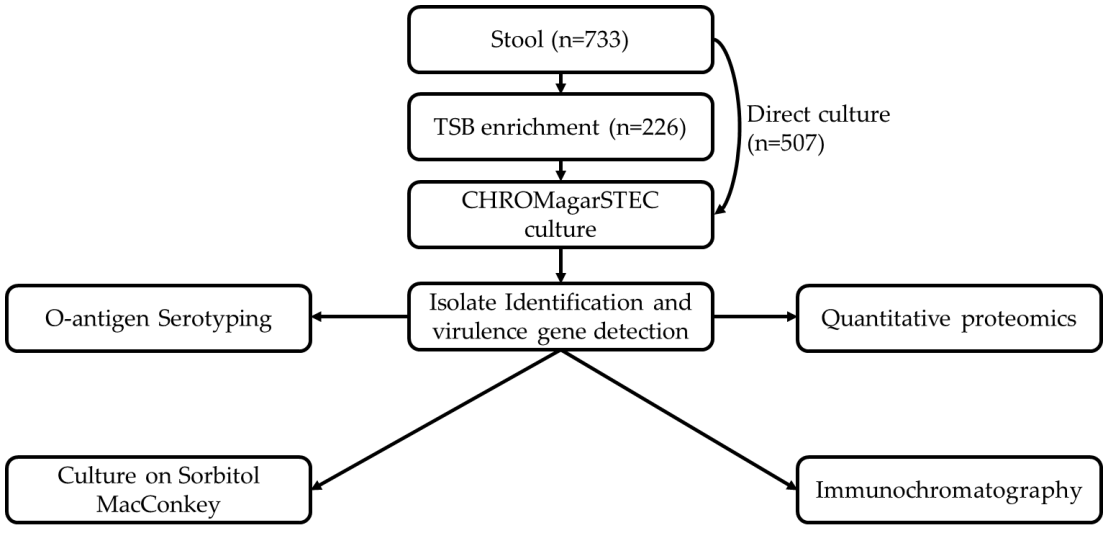

Figure 1:2 Laboratory work flow for stool samples from the Groote Schuur Hospital, Cape Town.

1.5.2 Laboratory work flow for stool, meat, and surface water samples collected in Nyanga.

Stool samples were enriched in TSB for 24h before streaking on CHROMagar™STEC. The meat was pummeled in a sterile stomacher bag and 25g of tissue enriched in 225ml of TSB for 24h before streaking on CHROMagar™STEC. 100ml of water was filtered through a 0.45µm nitrocellulose membrane and then membrane placed in TSB with agitation before incubation for 24h. The medium provides presumptive *E. coli* isolates (Mauve colonies) which were confirmed to be *E. coli* using the VITEK® 2 automated microbial identification system (biomerieux, USA, St. Louis, MO). *E. coli* isolates were then tested for possession of virulence genes using real-time PCR or gel-based PCR. Only those *E. coli* isolates that tested positive for virulence genes were then serotyped and tested for susceptibility to antibiotics using the VITEK® 2 automated system (biomerieux, USA, St. Louis, MO) and the broth microdilution method . From the TSB enrichment (or directly from stool or processed sample), we tested for other foodborne diarrhoea pathogens including *Salmonella*, *Shigella*, *Plesiomonas*, *Aeromonas*, *Campylobacter*, *Vibrio*, and *Yersinia* using selective culture media. The enrichments were also tested for serotype specific marker genes *rfbE* and *wbdl* (**Figure 1:3**).

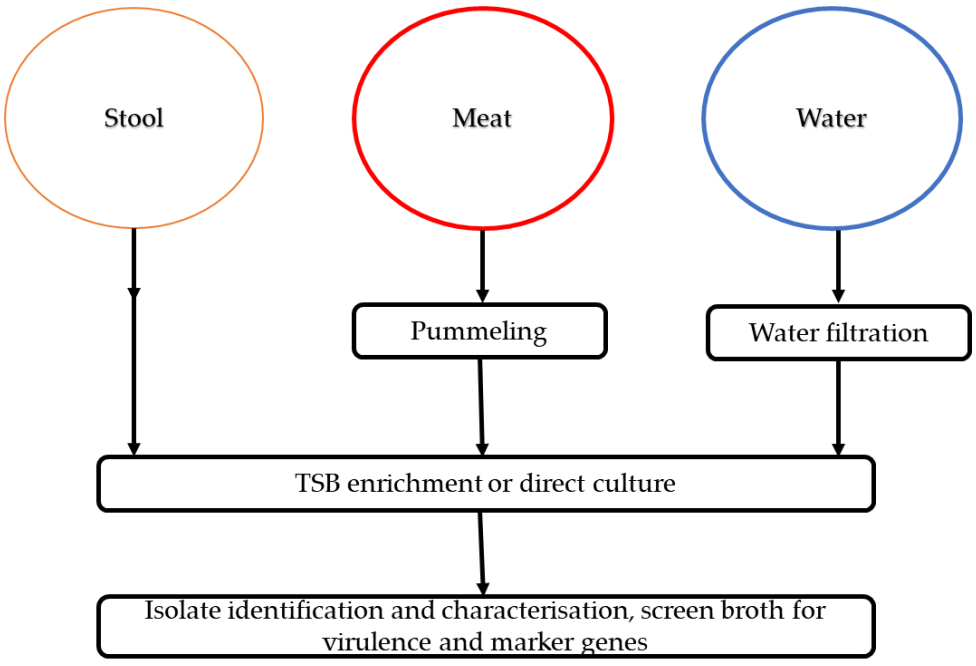

Figure 1:3 Summary of the laboratory work flow for meat, stool and surface water samples from Nyanga.

1.6 Outline of the thesis

Chapter Two is a narrative of literature focusing mainly on STEC surveillance including serotypes, virulence factors, phylogenetic analyses, laboratory diagnosis, antimicrobial susceptibility testing, source attribution, and national surveillance strategy. **Chapter Three** involves the validation of an in-house developed real-time PCR, which is then used as a gold standard to assess the performance of CHROMagar™STEC for screening stool, surface water, and meat from the informal abattoirs in Nyanga. **Chapter Four** covers the characteristics of the undiagnosed STEC and other diarrheic *E. coli* at the Groote Schuur Hospital. **Chapter Five** involves proteomic comparison of the proteomes of three diarrheic *E. coli* isolated on CHROMagar™STEC. **Chapter Six** focuses on Nyanga. It considers the prevalence and characteristics of STEC (tellurite resistant strains), and other foodborne pathogens in meat, surface water, and stool. **Chapter Seven** is about the source tracing of STEC and other diarrheic *E. coli* in Nyanga. Here we determined the MLVA genotypes of diarrheic *E. coli* from the Lotus River surface water, meat from the informal abattoir, and stool from children less than 12 years of age attending the NCHC. **Chapter Eight** is a general discussion of the main findings in this study, and the general conclusions.

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Chapter Two

2 One Health- an integrated approach to surveillance of foodborne bacterial pathogens and Foodborne antimicrobial resistance: A Review of the Literature

2.1 The Global Foodborne Disease Burden

According to the World Health Organisation (WHO), foodborne diseases are defined as illnesses resulting from the ingestion of foodstuffs contaminated in the process from food production to consumption with microorganisms or chemicals. Often, foodborne diseases present clinically as gastro-intestinal symptoms like diarrhoea.[1] In the Global Enteric Multi-center Study that was conducted in sub-Saharan Africa and Asia, most attributable cases of moderate to severe diarrhoea (considering all the study sites in sub-Saharan Africa and Asia) were caused by four pathogens namely; rotavirus, *Shigella*, enterotoxigenic *E. coli* that produces the heat stable toxin (ETEC-ST), and *Cryptosporidium*.[2] Other pathogens like *Aeromonas* (important in Bangladesh and Pakistan), and *Campylobacter* (important in India, Bangladesh, and Pakistan) were associated with moderate-to-severe diarrhoea in some sites but not the others. Of the four main pathogens reported in the GEMS (rotavirus, *Shigella*, enterotoxigenic *E. coli*, and *Cryptosporidium*), rotavirus is mainly transmitted from human to human, *Shigella* is mainly transmitted via water, while both *Cryptosporidium* and ETEC-ST are mainly transmitted via food. Not all bacterial pathogens causing foodborne disease are transmitted solely by food; other transmission routes such as animals, humans, and the inanimate environment exist. Foodborne bacterial pathogens such as Shiga toxin producing *Escherichia coli* (STEC), other diarrheic *E. coli,* and *Salmonella enterica* are mainly transmitted via food at several points during production and processing while others like *Listeria monocytogenes* are almost 100% foodborne.[3] For this reason, estimation of the global burden of foodborne disease is complicated. In order to have reliable estimates of the burden of foodborne disease, it was necessary to determine the relative contributions of food (as a transmission route) to the burden of foodborne disease. The most recent estimates of the burden of foodborne disease by the World Health Organisation (WHO) are reliant on a structured expert elicitation study whose main purpose was to estimate the relative contribution of food to the global burden of diseases transmitted via food.[3] Consequently, it was established that the global burden of foodborne disease in 2010 caused by 31 hazards (bacterial and non-bacterial) was 33 million disability adjusted life years (DALYs) with 40% of the burden bore by children under the age of five years.[4]

The impact or effect of the different pathogens can be weighted in terms of DALYs per case or per 100,000 population.[5] Per the Foodborne Epidemiology Research Group of the WHO, enteropathogenic *E. coli* (EPEC) had the highest global DALYs per 100,000 for children under the age of five years as compared to other foodborne bacterial and viral pathogens (**Figure 2:1**). *Listeria monocytogenes* had the highest DALYs per case as compared to other foodborne bacterial and viral pathogens.

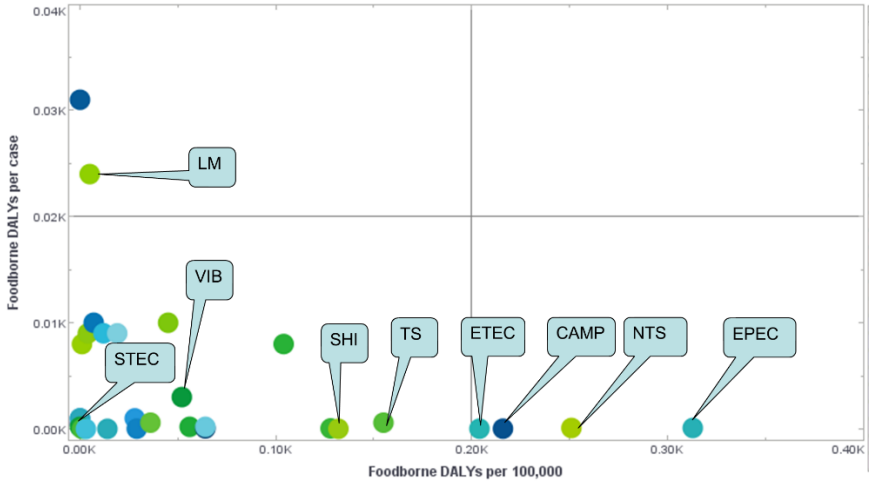

Figure 2:1 Foodborne Disability-adjusted life years per 100,00 and per case attributable to the different biological and non-biological hazards

LM= *Listeria monocytogenes*, VIB= *Vibrio cholerae*, SHI= *Shigella*, TS= Typhoidal *Salmonella*, ETEC= enterotoxigenic *E. coli*, CAMP= *Campylobacter*, NTS= non - typhoidal *Salmonella*, EPEC = *enteropathogenic E. coli*. The unlabeled coloured points represent non-bacterial foodborne hazards. This map was adopted (and modified) from the WHO FERG website.[5]

2.2 Surveillance for foodborne disease

2.2.1 The One- Health approach

The concept of One Health is hinged on the integration of human, animal and environmental health in addition to scrutiny of socio-economic drivers of infectious disease.[6,7] Due to pathogen transmission between humans, animals and the inanimate environment, it is important to focus surveillance and intervention across the three facets.[8] The interconnectedness of individual, regional, global public health and planetary environments, will require a One Health approach to food safety. One Health has been defined as a multidisciplinary approach to attain optimal health for people, animals, and the inanimate environment.[9] Also, social and economic drivers

influence transmission of infectious disease while policy and political issues become key when carrying out cross-institutional interventions.[10,11] The concept of "One Health, One City" assumes a dynamic equilibrium at the human-animal-inanimate environment interface with pathogen transmission across the three facets without limitation of geographical, administrative or political boundaries; this is also referred to as "global interconnectedness".[7] One example of global interconnectedness is the global food chain. The global food chain refers to the complex networks through which food is transported from one region to another, and across nations and continents such that the distance from the "farm" to the "fork" is increased. For example, a meal consumed in South Africa, comprising of onions produced in Brazil, tomatoes grown in Zimbabwe, and mushrooms grown in Botswana. In effect emergence of foodborne disease in any of the countries involved in the food chain means that all the other countries in the food network may become affected. Three main factors have been shown to play a key role in the emergence of foodborne disease globally; (1) intensification of agricultural production[12] (2) increased interaction between human, domestic animals, wild animals[13] (3) environmental "commons" such as water.[14] The intensification of agricultural production is such that huge numbers of animals are reared on the same farm with increased contact between the animals. Such a farm would be producing large quantities of meat and meat products for sale and with a wide consumer base. In the case of a foodborne disease on such a farm, it would spread widely affecting many people.

2.3 Foodborne Antimicrobial Resistance

2.3.1 A general overview

On the whole, the consumption of antibiotics in the clinical setting is rising globally due to improved standards of living, access to health insurance and the high burden of infectious disease. However, in resource-limited settings as is the case in many parts of Africa, over a million children die annually from infectious disease due to a lack of access to antibiotics.[15,16] Despite the limited access to antibiotics in a clinical setting, there is often resistance to first-line antibiotics which would be used to treat common infectious diseases. Additionally, there is a lack of effective public health facilities in resource-limited settings, lack of access to clean water, poor hygiene, and inadequate waste disposal systems encourage the spread of infectious disease usually caused

by drug-resistant pathogens. Consequently, resistant pathogens (in humans) are propagated from person to person and into the environment.[17] Concomitantly, antibiotics are used for the treatment of animal diseases among the food animals. Given the poor veterinary infrastructure in many resource-limited countries, these antibiotics are misused, and antibiotic residues end up in the environment (surface waters) and in foods of animal origin such as milk, eggs, and meat.[18] Also, antibiotic resistant bacteria from the gut of the slaughtered food animals might get disseminated in the food of animal origin.[19-22] Consequently, consumption of these foods of animal origin leads to exposure to sub-therapeutic levels of different antibiotics, and to antibiotic resistant bacteria with zoonotic potential.

2.3.2 Informal abattoirs and storm water as reservoirs of antibiotic resistant pathogens

2.3.2.1 Stormwater

Stormwater is runoff that is tunnelled in streams after rainfall and is a significant component of the urban aquatic systems. Stormwater washes different environmental niches such as homesteads, hospitals, farms, and industries. It affects the water quality in the urban aquatic systems because it feeds into the general sewage treatment system. Sewage has to be treated for recycling - very contaminated sewage is not easily treated for recycling. Stormwater has been shown to be contaminated with bacterial pathogens (which could be drug resistant) washed from soil[23] and with toxic compounds such as Lead and Copper.[24] Visceral content, salts used in curing, as well as feathers and other animal waste products from informal abattoirs are also washed into the storm water channels.[25] According to the Department of water affairs and forestry, Cape Town, rainwater runoff leads to the introduction of untreated waste into rivers thus affecting river health and posing a health risk to the communities that use these waters.[26] One such risk is the propagation of drug-resistant organisms.[27]

2.3.2.2 Informal abattoirs

Informal abattoirs are unlicensed animal slaughter facilities whose operations are unsupervised by veterinary and other public health professionals. Informal abattoirs infrastructure is often poor and unsuitable for the purpose. Usually, such slaughter places are sited wrongly- in the

immediate neighbourhood of residential areas, public toilet facilities, waste disposal areas, shelter sites and busy access roads.[28]

Informal slaughter could be drivers for antimicrobial resistance in the communities through two main routes; (1) the slaughter personnel who might carry drug-resistant bacteria from animals to the general population, (2) through the disposal of animal viscera into the storm water drains and thus contaminating the environment with drug-resistant bacteria.[27,29]

2.3.3 The South African situation analysis, strategy, and challenges

The Global Antibiotic Resistance Partnership–South Africa situation analysis report highlights the emerging threat of antimicrobial resistance.[30] The Antimicrobial Resistance National Strategy Framework 2014-2024 aims to optimise surveillance and early detection of antimicrobial resistance through strengthening surveillance of local and national resistance patterns and antimicrobial use. Additionally, the framework seeks to prevent infection through high coverage vaccination programs (vaccination reduces the need for antibiotic use) and adequate infection control to combat antimicrobial resistance.[31] This strategy, however, is faced with significant challenges. For example, in an informal settlement setting, with unacceptable slaughter practices, high diarrhoeal disease burden, and inadequate waste disposal, infection control is difficult to implement. In such settings, the drivers of antimicrobial resistance could include environmental discharge of resistant pathogens (Environmental contamination with enteric bacteria can be a result of inappropriate disposal of faecal waste or animal slaughter waste) into water streams.[32] Consequently, through storm water, drug-resistant enteric pathogens are disseminated in the inanimate environment. Wastewater and sewerage treatment methods could kill the pathogens but not the resistance genes which could effectively be spread through the distribution of chlorinated water which is often labelled as “safe” for drinking.

Unlike South Africa, developed countries face fewer challenges in as far as environmental contamination with enteric bacteria. The Center for Disease Control (CDC), through the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS)- a division of the National Center for Emerging and Zoonotic Infectious Disease (NCEZID), antimicrobial resistance experts track antimicrobial resistance patterns of bacteria commonly transmitted

through food- bearing in mind that antibiotic resistance in other parts of the world could affect treatment options in the United States.[33]

Broadly, the CDC recommends four essential avenues to control the spread of foodborne antimicrobial resistance. These include prevention of the spread of disease (thus eliminating the need to use antibiotics), tracking of resistance patterns and the development of new antibiotics and diagnostic tests primarily targeting pathogens affecting large portions of the population.[33] Diarrhoea, especially among children under five years, is one of the leading causes of mortality and morbidity and therefore diagnostic and prevention strategies aimed at reducing diarrhoea would reduce the spread of resistance (fewer antibiotics would be used to manage invasive diarrhoeal disease).[33,34] It is, therefore, important to track antimicrobial resistance patterns of foodborne pathogens and then conduct surveillance of its environmental drivers to establish appropriate control measures.[17]

2.4 Source Attribution for STEC and other foodborne bacterial pathogens

As per the Public Health Agency of Canada, source attribution is the association of gastrointestinal pathogens causing disease in the human population with different animal reservoirs and vehicles. It is important because it helps to consider a variety of factors that play a role in the causation of disease and for each, to assess the number of human cases, hospitalisations, deaths, and the related costs.[35] Even though several methods exist for determining the source and burden of human illness, their contributions are complementary and as such should be used in combination. The various methods include; microbial subtyping comparison, comparative exposure assessment, outbreak data analysis, case-control study, intervention study, and expert elicitation. Outbreaks of STEC are identified by determining the similarity between patients, for example, exposure to the same potential environmental reservoir, microbial properties among geographically related cases that happened at around the same time, or if the number of cases in an area is higher than the background incidence.

At the Gastrointestinal Bacteria Reference Unit in England, the national reference laboratory for gastrointestinal pathogens in England, confirmed foodborne pathogens such as STEC O157:H7 are analysed using Whole Genome Sequencing (WGS). Also, enhanced surveillance

questionnaires are administered to all suspect *E. coli* O157:H7 cases to determine similarities in exposure, location and time of onset of illness.[36] At this unit, cases can be categorised as domestic, travel, household, cluster or sporadic while the groups are classified as family, known or wgMLST identified.[36] PulseNet International is a global laboratory network dedicated to laboratory-based surveillance for bacterial food-borne diseases which comprises of national and regional laboratory networks of Africa, Asia Pacific, Canada, Europe, Latin America, the Middle East, and the United States. Recently, this network shared its vision to standardize the use of whole genome sequencing (WGS) to identify and subtype food-borne bacterial pathogens worldwide.[37] Presently, this network relies on the use of Pulse Field Gel Electrophoresis (PFGE) coupled with Multi-Locus Variable Number of Tandem repeats Analysis (MLVA) to identify and investigate foodborne disease outbreaks. However, besides outbreak identification and investigation, little information about the outbreak strains can be obtained using these methods. Comparatively, WGS data can be used to determine relatedness between outbreak strains as well as information about strain characteristics such as serotype, antibiogram, and virulence attributes. Additionally, further in-silico analysis can be done on the WGS data retrospectively. Challenges to implementation of global use of WGS by the PulseNet International laboratories include (1) cost of sequencing, (2) bioinformatics skills need to handle WGS data, (3) Storage of short read sequence and contig sequence data generated, and generation of standardized protocols to be implemented by all the laboratories in the network.

2.5 Diarrheagenic *Escherichia coli*

The Genus *Escherichia* was named after Theodor Escherich who first isolated *Escherichia coli.* It belongs to the order proteobacteria, phylum gamma proteobacteria, order enterobacteriales, and family enteroacteriaceae.[38] *Escherichia* are straight gram negative rods [(1.1 – 1.5) μm * (2.0 – 6.0) μm] that are either non-motile or motile by means of peritrichous flagella and exist singly or in pairs (**Figure 2:2**). They metabolise by means of respiration or fermentation and rarely produce hydrogen sulphide. The species in this genus include *Escherichia coli* (commensals in the intestinal tract of warm-blooded animals*), Escherichia hermanii* (intestinal and extra-intestinal sites of warm-blooded animals), *Escherichia fergusonii* (intestinal and extraintestinal sites of warm-blooded animals), *Escherichia vulneris* (intestinal and extra-intestinal sites of warm-blooded animals), and *Escherichia blattae* (commensals in the hindgut of cockroaches).[38] The type species of genus *Escherichia* is *Escherichia coli* (*E. coli*).

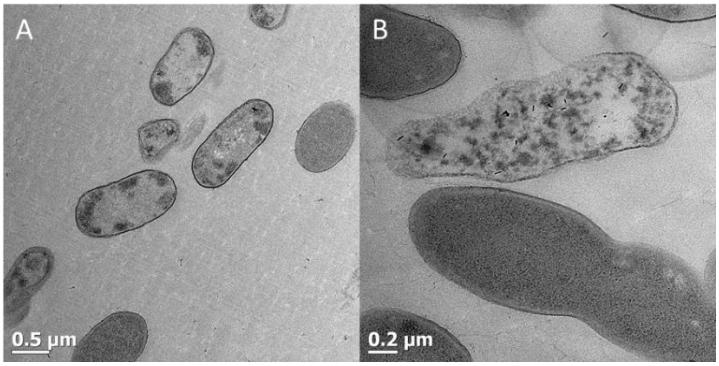

Figure 2:2 Transmission Electron Micrograph of E. coli showing rod-shaped cells in A and B at a magnification of X10,000

This micrograph of *E. coli* was taken by the PhD candidate and has not yet been published. It was taken at the Electron Microscope Unit, Center for Imaging and Analysis, University of Cape Town.

Some *E. coli* are pathogenic while others are not. The non-pathogenic strains can acquire virulence genes through horizontal transfer and thus become pathogenic. The pathogenic *E. coli* possess virulence factors that enable them to cause disease. The weakening of the host immune system, production of toxins and other virulence factors such as invasins may lead to diarrhoea and or extra-intestinal disease in humans and or animals.[39,40]

Pathogenic *E. coli* can be categorised into two groups based on the ability to invade beyond the intestinal tract. The pathogenic intestinal *E. coli* cause diarrhoea using a myriad of virulence factors while the extraintestinal pathogenic *E. coli* have possess extra virulence properties that enable them to invade extra-intestinal tissues and thus causing infections such as urinary tract infections (caused by uropathogenic *E. coli*-UPEC) and neonatal meningitis (neonatal meningitis causing *E. coli*-NMEC).[41] The Avian Pathogenic *E. coli* (APEC) are also categorised as extra-intestinal and cause generalised systemic infections in poultry, leading to the death of chicken embryos,[42] but could also have zoonotic potential.[43] A virulence marker protein for extraintestinal pathogenic *E. coli* pathotypes NMEC, UPEC, and APEC is esterase B, which is encoded by the *aes* gene.[44]

The extraintestinal pathogenic *E. coli* have been shown to possess less antimicrobial resistance attributes, especially fluoroquinolone resistance, as compared to the intestinal pathogenic *E. coli.* [45,46] The phenomenon of reduced virulence traits among the more antibiotic resistant intestinal pathogenic *E. coli* is referred to as the fitness cost of antimicrobial resistance. However, this theorem (on the fitness cost of antimicrobial resistance) has not always held true, especially with the pandemic spread of antibiotic resistant strains such as the ESBL-producing strain of *E. coli*[47-49] that carried the CTX-M-15 allele located on conjugative IncFI plasmids.[50] In this case, the potential to spread so widely was a measure of fitness.

The intestinal pathogenic *E. coli* include six main pathotypes: Shiga-toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAggEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and diffusely adherent *E. coli* (DAEC) whose marker genes are *stx*$_1$ and *stx*$_2$, *aggr*, *LT*/*ST*, *ipa*, *eaeA* and *daaC* genes respectively. All of these may cause diarrhoea, especially in children and the immunocompromised.[51] In general, diarrheic *E. coli* could also be carried by normal children without diarrhoea. [52] The more recently described pathotypes of diarrheic *E. coli* include the cell-detaching *E. coli* and the cytolethal distending toxin–producing *E. coli,* which by epidemiologic associations, have been shown to cause diarrhoea.[51]

2.5.1 Enteropathogenic *E. coli*

In developing countries, EPEC cause infantile infectious diarrhoea accompanied by fever, vomiting, and dehydration.[39,40] In the Global Enteric Multicenter Study (GEMS), typical EPEC (EPEC with the bundle-forming pili gene) was significantly associated with moderate to severe diarrhoea among infants in Kenya.[53] This pathotype forms Attachment and Effacement (A/E) lesions on the brush border of the small intestine. There are mainly two variants of this pathotype; the typical EPEC which carries the *E. coli* adherence factor plasmid (pEAF) and the atypical EPEC which does not have this plasmid.[54] The typical EPEC mainly possess virulence factors encoded by the *E. coli* adherence factor plasmid and the LEE (Locus of Enterocyte Effacement) (except for a few that carry the cytolethal distending toxin-CDT and the enteroaggregative heat stable toxin-EAST1). The LEE contains the *eaeA* gene which encodes intimin. The atypical EPEC are more closely related to the LEE-positive STEC in terms of serotypes, genetic characteristics, virulence properties, and reservoirs.[54] The atypical EPEC are divided into two main categories; (1) those with only the LEE-encoded virulence factors, and (2) those with both the LEE and the non-LEE-encoded virulence factors (for example the CDT and EAST1).[55]

In developing countries, typical EPEC are commonly reported in the causation of infantile diarrhoea in children while in the developed countries atypical EPEC is a commoner cause of diarrhoea.[55]

Adherence of these bacteria to the host's intestinal epithelial cells is initially mediated via the bundle-forming pili which are a type IV protein that forms cell to cell connections leading to micro-colonies of EPEC along the intestinal surface.[56] Adherence is followed by signal transduction and intimate attachment. Intimate attachment is a result of interaction between the translocated intimin receptor and intimin both of which are encoded by the LEE.[39] The secreted proteins that play a role in the formation of attaching-effacing (A/E) lesions include the translocated intimin receptor protein (Tir)[57], mitochondrion-associated protein (Map)[58], serine protease F (EspF), serine protease Z (EspZ), serine protease G (EspG)[59], serine protease H (EspH)[60], and serine protease B (EspB).[61] The formation of A/E histopathology leads to the effacement of the brush border microvilli at the site of attachment.[62]

2.5.2 Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) is a facultatively intracellular pathogen that causes bacillary dysentery mainly by means of alteration of the secretory activity of the intestinal epithelial cells by producing the EAST1 (heat stable enterotoxin of enteroaggregative *E. coli*) toxin and other virulence factors encoded by the *pINV* plasmid. It has very similar virulence and genetic properties to *Shigella*.[63,64] The chromosomally located invasion plasmid antigen H gene (*ipaH*) is responsible for the invasion of epithelial cells.[64]

2.5.3 Enteroaggregative *E. coli* and the enteroaggregative haemorrhagic *E. coli*

Enteroaggregative *E. coli* (EAggEC) possesses the *aggr* gene as the virulence marker and can be classified broadly into two groups namely, the atypical (without *aggr*) and the typical (with *aggr*).[65] It causes persistent diarrhoea and traveler's diarrhoea[66] in poorly developed countries.[67] EAggEC have also previously been reported to cause community acquired urinary tract infection outbreaks in Denmark.[68] The enteroaggregative-haemorrhagic *E. coli* (EAHEC) or enteroaggregative Shiga-toxin producing *E. coli* strain O104:H4 was shown to be well adapted to adverse environmental conditions associated with food processing by the production of a bacteriocin, giving it a competitive advantage over other foodborne bacteria.[69] The EAHEC lacks the LEE and so does not attach to the intestinal cells by means of intimin. Instead, it attaches by means of the aggregative adherence fimbrial pili whose gene is carried on the enteroaggregative *E. coli* plasmid.[70]

2.5.4 Adherent-Invasive *E. coli* (AIEC)

An increase in the relative proportions of AIEC (pathobiont expansion) in the gut due to inflammation has been associated with the onset of inflammatory bowel disease (IBD).[71] IBD is a result of a dysregulation of gut bacteria resulting in increased permeability of the epithelial barrier in two conditions of the intestinal tract namely; Crohn's disease (CD) and ulcerative colitis (UC).[72,73]

Even though most of the AIEC that have been associated with IBD belong to phylotype B2, some strains belong to the other phylotypes. AIEC are distinct from other diarrheic *E. coli* pathotypes because they are difficult to identify due to the lack of a specific genetic marker. Particularly, they

lack the type III secretion system which is possessed by all the other pathotypes. AIEC Pathogenesis involves three main stages namely; (1) Interaction with the intestinal epithelial cells, (2) adherence, and (3) invasion. Attachment of AIEC to the intestinal epithelial cells is facilitated by the upregulation of carcinoembryonic antigen – related cell – adhesion molecule 6 (CEACAM 6) on the apical surface of epithelial cells in the large intestine.[74] CAECAM 6 attaches to the type 1 pili on the surface of AIEC prior to cellular invasion. Invasion is by means of pinocytosis and vacuolization.[75] Following invasion of the intestinal epithelial cells and macrophages, they survive and replicate leading to barrier dysfunction by affecting the intercellular junctions between the intestinal epithelial cells.[71]

2.5.5 Diffusely Adherent *E. coli*

Diffusely Adherent *E. coli* (DAEC) causes watery diarrhoea which is most severe in children aged between 18 months and 5 years.[76] It causes disease by secretion of adhesins that attach to molecules on polarised epithelial cells leading to alteration of the epithelial cell cytoskeleton. This then leads to destruction or rearrangement of the microvilli which are responsible for nutrient absorption in the intestinal tract.[76]

2.5.6 Cell Detaching *E. coli*

These cause diarrhoea by way of attachment by means of the P-pili, production of alpha haemolysin, and the release of the Cytotoxic Necrotizing Factor.[77] The cell-bound haemolysin (hlyA) and not the cell-free hlyA, secreted in vitro during the exponential and stationary phase of growth, is responsible for the cell detaching activity of cell detaching *E. coli* (CDEC).[78] By epidemiologic associations, it has been linked to diarrhoea in children.[77]

2.5.7 Cytolethal -distending toxin-producing *E. coli*

This pathotype of *E. coli* has been reported to cause severe diarrhoea in children in Mexico[79], Japan[80], and Bangladesh[81] and was isolated from meat in Northern Ireland.[82] It causes disease by the production of a potent toxin called the cytolethal distending toxin (CDT). This toxin is coded for by three adjacent overlapping genes called the *cdtA*, *cdtB*, and *cdtC*. The toxin leads to arrest of the eukaryotic cell cycle at the G1 or G2 phase and thus leading to cell distension and eventual

cell death. A variant of the CDT toxin called the CDT-V has been shown to be produced by STEC especially the non-O157 STEC that lack the intimin coding locus of enterocyte effacement (LEE).[83]

2.5.8 Shiga-toxin-producing *E. coli*

Shiga toxin producing *E. coli* / Verocytotoxigenic *E. coli* (STEC/VTEC) possess *stx* genes and have previously been associated with global foodborne pandemics resulting in food trade sanctions and massive economic losses in the food industry.[84] Enterohaemorrhagic *E. coli* (EHEC) are STEC/VTEC that have frequently been associated with severe human disease marked by bloody diarrhoea, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). EHEC strains (for example STEC O157, O26, O103, O111, and O145) possess a bacteriophage carrying the *stx* genes which code for the Shiga toxin and a Locus of Enterocyte Effacement (LEE) which encodes the intimin protein used in intimate attachment to the intestinal epithelial cells. The locus of enterocyte effacement (LEE) is a 35.5kb pathogenicity island which carries genes that code for the intimin and type III secretion system proteins (EspA, EspB, and EspD), Outer Membrane Protein (OMP) and the translocated intimin receptor (Tir).[85] However, LEE-negative STEC use alternative virulence factors such as a subtilase (*subAB*), mucinase (*epeA*), and an adhesin (*saa*).[86] Subsequently, they invade the host epithelial cells using a process mediated by bacterial proteins and host cell microfilaments. LEE-negative STEC can also achieve intimate attachment using the aggregative adherence fimbrial pili.[70]

Even though Levin coined the term enterohaemorrhagic *E. coli* (EHEC) to refer to a special category of STEC that caused severe disease such as *E. coli* O157,[87] the nomenclature term EHEC is being replaced with the term verocytotoxin producing E. *coli* (VTEC) or Shiga-toxin producing *E. coli* (STEC) because of the emergence of outbreaks (marked by severe disease) due to strains that don't fit the definitive criteria of EHEC.

The number of STEC serotypes involved in outbreaks is increasing steadily.[88] Shiga-like toxins are encoded by genes in the genomes of functional or defective lambdoid bacteriophages termed *stx*-phages.[89] The *stx*-phages are defined by the occurrence of the Shiga toxin operon and can integrate into specific sites in the host bacterial chromosome. Bacterial host cells can carry multiple *stx* phages, allowing them to produce several Shiga toxin variants, with recombination

of various phage sequences creating potentially novel phages.[90] This is the mechanism by which new variants of the toxins arise. During the lytic cycle of the *stx* phages, the bacterial host cell is lysed releasing the toxin. The use of certain antibiotics (such as the DNA targeting quinolones) induces the SOS DNA repair response which in these bacteria is often synchronised with the activation of the phage lytic cycle and release of the Shiga-like toxins.[91]

The most important virulence determinants for STEC are the two Shiga toxin families (*stx*$_1$ and *stx*$_2$).[92] Shiga toxin 2 is 1000 times more cytotoxic to human nephrons as compared to Shiga toxin 1 and therefore STEC isolates that carry the *stx*$_2$ genes are more commonly associated with severe disease.[93] Different variants of the *stx*$_2$ genes are related to differing disease severity. The main *stx*$_2$ variants that have been described based on sequence homology and immunological cross-reactivity are *stx*$_{2a}$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_e$, *stx*$_{2f}$, *and stx*$_{2g}$.[94,95]

Stools should be tested for Shiga toxin as early as possible during illness as it becomes impossible to detect them after one week of sickness.[84] Loss of the *stx* phages has been reported in humans-initially, *stx*-positive STEC O26:H11/NM and sorbitol-fermenting O157:NM strains were isolated from patients; however, the isolates were *stx*-negative when isolated several days later from the same patient.[96,97] Since *stx*-negative STEC fit the definition of atypical EPEC, Bielaszewska, and colleagues looked at these isolates recovered from the bloody stool and suggested that they were not atypical EPEC and so they coined the term EHEC-LST (EHEC that had lost the *stx*) or STEC-LST (STEC that had lost the *stx*). Thus the *stx* status of STEC was proven to fluctuate.[98]

Besides the use of the *stx* and *eae* genes as virulence markers for STEC, recent research has suggested the use of enterohemolysin A gene (*ehxA*) and *ureC* (one of the components of the urease gene operon) as additional pathogenicity markers in addition to *stx*$_{2a}$ and *eae*.[99,100] Possession of the *ureC* gene was commonly associated with co-carriage of the *eae* gene, one of the pathogenicity indicators for STEC.[100]

2.5.8.1 Shiga toxin

In 1978, Konowalchuk discovered the presence of a verocytotoxin secreted by some *E. coli* strains.[101] Later, O'Brien showed that this toxin could be neutralised by antibodies against the

toxin produced by *Shigella dysenteriae* type 1. Thus this *E. coli* toxin was referred to as Shiga-like toxin.[89] The rationalised nomenclature for the toxins in the Shiga toxin family was developed by Calderwood.[102] The genes that code for the Shiga toxins are carried on Shiga toxin converting lambdoid phages of different types depending on the toxin subtype.[89] These lambdoid phages are diverse and cause lysogenic or lytic infection of a diverse array of O157 and non-O157 *E. coli.* Research has proved that commensal *E. coli* bacteria that are susceptible to infection by the stx converting lambdoid phages can amplify Shiga toxin leading to more severe disease.[103] All the AB halo-toxins (each toxin molecule has one A unit and one B unit that has five subunits) in this family cause disease by direct damage to the endothelial cells and thus destabilising the homeostatic balance of the affected cells.[104-106] The Shiga toxins and the shiga-like toxins belong to a large group of toxins secreted by plants and bacteria that cause cellular damage by initial binding and then induction of cellular damage by inhibition of protein synthesis and induction of apoptosis. Shiga-like toxins are not only released by STEC but also by *Citrobacter freundi*, *Aeromonas hydrophilia,* and *Enterobacter cloacae.*[84] These toxins affect a wide range of host cells including the macrophages and the proximal tubular cells of the human kidney.[106] The A unit of the toxin is enzymatically active while the B unit has five subunits and is necessary for the attachment to the host cell glycolipid receptors. Strictly, these toxins attach to the Gb3 glycolipid receptors on the surface of cells and or Gb4 in the case of the *stx2e* toxins released by some strains. Upon attachment, they induce uptake into the Claritin pits and are endocytosed to the Golgi apparatus and the endoplasmic reticulum. The protease named furin then cleaves the A unit into two fragments, A1 and A2, by cleaving a region flanked by two arginine amino acids. The affected cells are induced to secrete either interleukin 1 (IL-1), interleukin 6 (IL-6) or Tumor Necrosis Factor (TNF). The secreted cytokines then induce an overexpression of Gb3 receptors on the surface of adjacent cells and thus provide attachment sites for more toxic moieties. The A1 subunit of the A fragment removes adenine from the 28S RNA of the 60S ribosomal subunit and thus prevents attachment of the aminoacyl transfer RNA and in so doing effectively inhibiting protein synthesis.[84] The A1 fragment also induces programmed cell death (apoptosis) by inducing nuclear blebbing, DNA degeneration and release of cellular contents.[107]

2.5.8.2 Other known and Putative virulence factors of STEC

Since STEC is transmitted through food, including meat and leafy vegetables,[108] virulence factors possessed facilitate not only the ability to survive and cause disease in the human host but also to withstand the stresses it is subjected to during food processing and in the non-human ruminant host. Such could include resistance to harsh temperatures, osmotic pressures and heavy metals like Zinc and Copper. [109,110]

The infectious dose for STEC is 1-100 CFU which is low compared to other diarrhoea causing *E. coli*.[111] This is due to the ability of STEC to survive the acidic conditions in the stomach of the host. Acid resistance is mediated by the *rpoS* gene which encodes a stationary phase sigma factor enabling the organism to survive at acidity levels below pH=2.5.[112,113]

2.5.8.3 STEC Serotypes

Globally, over 470 STEC serotypes have been reported. Some of these have commonly been associated with severe disease in humans but are not prevalent in wild and domestic ruminants, while others are prevalent in ruminants but also cause severe disease in humans; an example of such a strain is *E. coli* serotype O157:H7.[114] The serotypes that commonly cause human disease have most frequently been reported in domestic and wild ruminants as opposed to the non-ruminants.[108,115]

The severity and frequency of clinical disease are the basis of the seropathotype classification scheme that was designed by Karmali *et* al. This scheme categorises STEC serotypes into five seropathotypes (A-E) on the basis of relative incidence, the frequency of involvement in outbreaks, and association with severe disease.[116] In this scheme, the *E. coli* serotype O104:H11, which caused the recent spinach-contamination related outbreak in Germany, belonged to seropathotype C as it was rarely (until after the outbreak)[117] involved in outbreaks and with a relatively low incidence. (**Table** *2.1*). Seropathotype classification could be useful for predictive hazard identification of STEC serotypes.[118]

Table 2.1 STEC seropathotype classification scheme

Seropathotype	Relative Incidence	Frequency of Involvement in outbreaks.	Association with severe disease.	Serotypes
A	High	Common	Yes	O157:H7, O157: NM
B	Moderate	Uncommon	Yes	O26:H11, O103:H2, O111: NM, O121:H19, O145: NM
C	Low	Rare	Yes	O91:H21, O104:H21, O113:H21; others
D	Low	Rare	No	Multiple
E	Non-human only	NA	No	Multiple

This table is an extract from Karmali *et* al.,2003. NA-Not applicable.

2.5.8.4 Phylogenetic analyses of STEC

Phylogenetic analysis serves to determine the evolutionary origins and relations of STEC. Phylogenetic grouping is using three PCRs targeting the *chuA*, TSPE4.C2 DNA fragment, and the *yjaA*. The presence or absence of the three fragments determines the group of a pathogenic *E. coli* strain. This analysis is important because it can help predict the potential health risk of an isolate from the environment.[119] For example, STEC belonging to phylogenetic group A are less likely to cause human disease.[120]

Of the four main *E. coli* phylogenetic groups (A, B1, B2 and D), STEC has been shown to belong to phylogenetic groups A, B1, and D.[121] Extra-intestinal *E. coli* belongs mainly to phylogenetic groups B2 and D while most commensal and other diarrheagenic *E. coli* belong to phylogenetic groups A and B1.[120]

2.5.8.5 Laboratory diagnosis of STEC

Laboratory practice guidelines call for routine investigations for STEC in all diarrhoeal stool samples in European countries.[122] According to the Centre for Disease Control (CDC), all acute community-acquired diarrhoea cases should be screened for STEC in a timely and accurate manner since early treatment helps to avoid severe clinical outcome such as renal failure.[123] Commonly, culture for STEC is done on media selective for *E. coli* O157: H7 and Shiga toxin proteins detected from stool by immunochromatography methods following overnight enrichment. Currently, in South Africa, at the Groote Schuur Hospital, screening is done for only sorbitol-negative *E. coli* O157:H7 (after 24 hr incubation at 37°C) on sorbitol MacConkey agar at

the explicit request of a physician whose basis to request for a laboratory test for *E. coli* O157:H7 is usually presence of blood in stool or clinical presentation of HUS.[124]

Selective testing of bloody stools, targeting only sorbitol-negative (O157) *E. coli*, may lead to underestimation of the incidence of STEC disease in South Africa, and hinder the laboratory detection of STEC outbreak strains. Foodborne disease outbreaks, for example, those caused by STEC, in South Africa are a common occurrence but are rarely reported.[125] Recently, (February 2017), there has been an outbreak of STEC at the Red Cross War Memorial Hospital, Cape Town leading to HUS in four children (laboratory testing done by this PhD candidate, not yet published). In this suspected outbreak, which was caused by *eaeA* and *hlyA* positive STEC O26, routine screening for *E. coli* O157:H7 and further screening for the other sorbitol-fermenting *E. coli* strains was required to identify the causative bacteria. However, most NHLS (state) laboratories in South Africa focus only on detection of the non-sorbitol fermenting *E. coli* O157:H7. This is not limited to only South Africa but is common in many developing countries, where there is difficulty detecting non-O157 STEC as reported by Brooks *et* al.[126]

2.5.8.5.1 Chromogenic media for isolation of STEC

Several chromogenic media have been developed to screen for the presence of STEC. Some have been specifically designed to detect specific serotypes of STEC (CHROMagar™STEC O104 for isolation of *E. coli* O104: H4) while others broadly identify several STEC serotypes for example, CHROMagar™STEC.[127]

2.5.8.5.1.1 CHROMagar™STEC

Only tellurite resistant STEC are able to grow on this medium. Tellurite resistance is a result of possession of the tellurite resistance gene complex Ter-ZABCDEF.[128] Only *TerB*, *TerC*, *TerD* and *TerE* genes are necessary for tellurite resistance.[129] Pathogenic STEC have been shown to be widely diverse regarding their tellurite resistance properties, with some being resistant and others being susceptible. However, the possession of tellurite resistance genes has been shown to be associated with an increased resilience to oxidative stress and survival inside the macrophage for tellurite resistant strains compared to the susceptible ones.[130,131]

2.5.8.5.1.1.1 Performance on stool

A recent study has shown that CHROMagar™STEC has a high negative predictive value and has the potential to detect the non-sorbitol fermenting *E. coli* (STEC O157:H7 and O157:NM), as well as the conventional sorbitol fermenting strains producing Shiga toxin.[132] Of the top six non-O157 *E. coli* O-serogroups (seropathotype B) known to cause severe illnesses in humans, CHROMagar™STEC efficiently detects O26, O111, O121, and O145 but its performance has been shown to be weaker in as far as detection of ter D negative O103 (only growing if a higher inoculum is streaked).[133] Using archived isolates and human stool samples, this medium was shown to be sensitive for detection of most common STEC serotypes as well as the detection of other diarrheagenic *E. coli* in Finland. The adequately detected serotypes included: O2: [H29], O5, O26, O104, O111, O121, O130, O145, O157: [H7], and OX182. However, the medium showed inadequate detection for tellurite susceptible sorbitol-fermenting O157: NM and a common clinical serotype O103:H2.[132] Gouali and Wylie evaluated the same agar for use on stool and found that it had high sensitivity, specificity, and negative predictive value.[134,135] The main limitation, therefore, with the use of CHROMagar™STEC for detection of STEC in stool , is its failure to detect tellurite susceptible STEC (**Figure 2:3**).

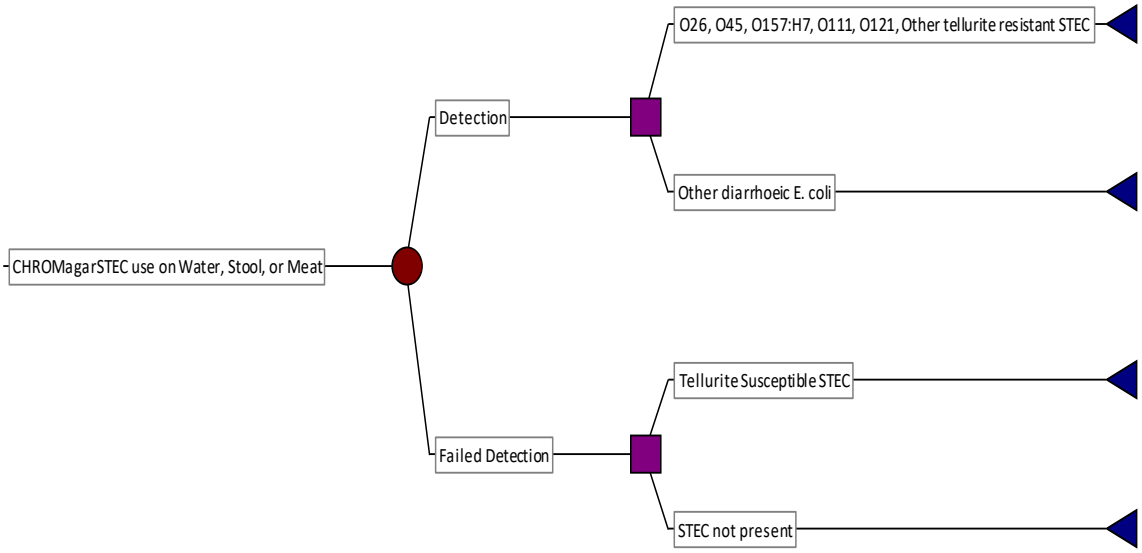

Figure 2:3 Event tree on use of CHROMagar™STEC to screen for STEC in stool, meat, and water

2.5.8.5.1.1.2 Performance on food samples

Per the manufacturer of this medium (CHROMagar, Paris, France), the common serotypes of STEC form mauve coloured colonies, while the other enterobacteriaceae form blue colonies or are inhibited. Tzschoppe *et* al, 2012 evaluated the performance of CHROMagar™STEC on vegetables and reported low detection rates for STEC O103:H2 but efficient detection of STEC serotypes O26, O103, O111, O118, O121, O145, and O157.[136] A comparative study, which compared the performance of different chromogenic media for isolation of STEC in food, reported that of six chromogenic media compared (including CHROMagar™STEC, Rapid *E. coli* O157:H7, Tryptone Bile X-glucuronide agar, Rainbow® Agar O157, Modified MacConkey Agar, and chromID™EHEC), only CHROMagar™STEC and Rapid *E. coli* O157:H7 were selective enough to inhibit the growth of non-STEC strains totally.[137]

2.5.8.5.1.2 Other chromogenic media used for isolation of STEC

2.5.8.5.1.2.1 Performance on stool

The STEC strain O157: H7 is commonly associated with severe disease and is identified in the clinical laboratory because it does not ferment sorbitol and shows marked resistance to tellurite

and cefixime.[138] The media used for this purpose are sorbitol MacConkey (to test for sorbitol fermentation) and Cefixime-Tellurite Sorbitol MacConkey (to test for sorbitol fermentation as well as resistance to cefixime and tellurite).[139] Other selective and non-selective media have been used to screen for both O157 and non-O157 STEC with varying sensitivity and specificity for the different serotypes. Highly STEC selective media such as R&F *E. coli* O157: H7 agar, Rainbow agar and modified Rainbow agar, actually inhibit the growth of the non-STEC organisms but also hinder the growth of the non-tellurite resistant strains which grow weakly except on addition of washed blood cells.[140] Less selective media, such as the STEC Heart Infusion Broth agar with Mitomycin C (SHIBAM), although lacking in the inhibitory components possessed by the selective agars, rely on the characteristic hemolytic pattern shown by STEC and enable growth of STEC that would otherwise be inhibited.[141]

In summary, only CHROMagar™STEC and Rapid *E. coli* O157:H7 are selective enough for inhibition of non-target strains totally in both clinical and non-clinical samples – an attribute of the concentration of inhibitory chemicals present in them (**Table 2.2**).

However, Rapid *E. coli* O157:H7 does not favor the growth of non-O157 STEC. Less selective media such as SHIBAM facilitate the growth of tellurite sensitive STEC but are less specific in a sense that they permit growth of other microflora in stool or food sample. Therefore, a paired use of a more selective and less selective medium may be a better option for isolation of O157 and or non- O157 STEC.[142]

Table 2.2 A comparison of the performance of different chromogenic media for STEC detection

Media	Level of selectivity	O157 STEC	Non-O157 STEC
CHROMagar™STEC	High	+	+
SHIBAM	Low	+	+
Rapid *E. coli* O157:H7	High	+	-
CT-Sorbitol MacConkey	High	+	-
R&F *E. coli* O157:H7	High	+	-
Rainbow® Agar O157	Low	+	-
chromID™EHEC	Low	+	-
Tryptone Bile X-glucuronide agar	Low	+	+

2.5.8.5.2 Microbiological testing of foods

2.5.8.5.2.1 Testing meat for STEC

To aid identification of food safety risks and prevent microbial hazards associated with the animal slaughter process, the Hazard Analysis Critical Control Points System (HACCP) is increasingly becoming necessary for all meat slaughterhouses to ensure safety and quality of meat. All operations during slaughter must be evaluated to ensure that the final product complies with stringent food safety objectives (maximum level of microbiological contamination acceptable for human consumption).[143] In Canada for example, all meat must have zero levels of STEC or risk being recalled.[144] However, successful implementation of an HACCP system lies hugely on the successful identification of the critical control points.[145] Based on these, microbiological testing of meat is done following both the local and international standards with priority given to pathogens of public health importance. International standards on food testing include; Bacteriologic Analytical Methods (BAM of US FDA), International Commission for the Microbiological Specification of Foods (ICMSF), American Public Health Association (APHA), and Health Protection Agency (HPA of UK). In South Africa, the International Standards Organisation (ISO) methodology was adopted by the South African Bureau of Standards and referred to as South African National Standards (SANS) methodology or the methodology prescribed by the Codex Alimentarius. These methods are prescribed by law in the regulations of the Foodstuffs, Cosmetics & Disinfectant Act (Act 54 of 1972) and are generally applied for non-automated analytical procedures. Generally, for all these methods, coliform counts per 25g of meat are an indicator of how contaminated the meat is with microbes from the gastro-intestinal tract of the slaughtered animal. Research has shown that while swabbing with gauze, a tenfold increase in the surface area swabbed doubles the number of samples from which coliforms were recovered.[146]

2.5.8.5.2.1.1 Enrichment for STEC in foods

The International Organization for Standardization (ISO) recommends that following enrichment of a food sample in suitable broth medium, isolation of STEC from all PCR (for *stx*) positive broths should be attempted. Enrichment for STEC in processed foods is necessary because various processing methods frequently damage the cells. The use of modified tryptic soy broth

supplemented with novobiocin (10mg/liter) is highly selective, but could lead to false negative results.[147] Despite the fact that various enrichment media and conditions have been compared, there is no standardized method for the enrichment of O157 and non-O157 STEC.[148] No single STEC enrichment protocol has conclusively been recommended for STEC enrichment.[148] Enrichment broths are often supplemented with selectively inhibitory chemicals such as antibiotics. But it is now recommended to use non-selective enrichment such as Buffered Peptone Water (BPW) because of the potential damage to the already stressed cells by the inhibitor chemicals.[147] Tryptone Soya Broth (TSB) and *E. coli* broth (EC) are the commonly used enrichment broth for O157 and non-O157 STEC, but the Food Safety Inspection Services of the U.S. has recently recommended the modified Tryptone Soya Broth (mTSB) to favor combined enrichment for both *Salmonella enterica* and STEC.[149] Incubation for O157 and non-O157 STEC is usually done at 35-37°C for 16-24h, but if the food or food product has a lot of background competing bacteria, sometimes incubation is done at 42°C.[127] The elevated incubation temperature help to suppress the antagonistic activity of the background microflora even though such an approach may interfere with growth of injured STEC cells.

2.5.8.5.3 Molecular assays

Varyingly rapid, sensitive and accurate PCR-based assays have been used to target the primary virulence and marker genes carried by STEC. The most notable virulence genes for STEC are *stx*$_1$ and *stx*$_2$ which may rarely be carried by other organisms such as *Citrobacter freundi*. [150] Other virulence genes include *eae*, *bfp* and *hlyA*.[85] Hybrid strains of STEC carry additional virulence genes such as the *est*, *elt*, *ipa*, *aat* and *daaC*.[151,152] The presence of these, in addition to the *stx* genes, has been associated with increased likelihood of HUS.[153,154] Serotype-specific genes for *E. coli* O and H antigens are often included to aid detection of serotypes commonly associated with severe clinical disease. Such genes include *rfbE* and *wbdl* for STEC serotypes O157 and O111 respectively.[155,156] Sero-groups O26 and O113 are detected by PCR amplification of the serotype-specific genes *wzx* and *wzy* respectively.[126] However, WGS eliminates the need for additional detection of serotype-specific genes.[157]

Subtyping of *stx* is necessary for risk profiling of STEC strains because some subtypes have frequently been linked to clinical disease, while others have rarely associated with clinical disease. The World Health Organization (WHO) Collaborating Center for Reference and Research on *E. coli* and *Klebsiella* uses conventional PCR amplification to detect the three stx_1 and seven stx_2 subtypes.[158] Other approaches have relied on the use of electrospray ionization mass spectrometry to identify and differentiate the STEC strains.[159] Since there are different amino-acid subtypes for stx_1 and stx_2, the proteomic fingerprint of each of these can be used to quickly delineate the different subtypes even though there is a high level of similarity between stx_{2a}, stx_{2c}, and stx_{2d} at nucleotide sequence level.[160] In this approach, the more abundant B subunit of the AB_5 toxin (Shiga toxin is an AB_5 toxin composed of the A and B subunits) and the A2 fragment of the A subunit are identified.[89] Thus, mass spectrometry, in addition to being rapid and cost efficient, would remedy the challenge of subtyping closely related subtypes such as stx_{2a}, stx_{2c}, and stx_{2d}.[161] More recently, Whole Genome Sequencing (WGS) technologies have been used for public health surveillance of STEC and have shown to be comparable to the PCR-based methods in terms of sensitivity and specificity.[161-164]

2.5.8.5.4 Antibody-based methods

Murine monoclonal and polyclonal antibodies against Shiga-toxin have been developed.[165] These antibodies can be used to differentiate between stx_1 and stx_2 because these two types of toxins are antigenically different.[39] They have been employed in different Enzyme-Linked Immunosorbent Assay (ELISA) kits to detect and differentiate Shiga-toxin. ELISA assay kits that have been developed to detect the different subtypes of Shiga-toxin include: ProSpecT Shiga toxin *E. coli* (STEC) Microplate Assay (Remel Inc., Lenexa, KS), ImmunoCard STAT! EHEC (Meridian Bioscience, Cincinnati, OH) , Ridascreen (r-Biopharm AG, Darmstadt, Germany), Premier EHEC test (Meridian Bioscience), Verotoxin Enzyme Immunoassay (r-Biopharm AG), Shiga Toxin Chek (TECHLAB, Inc., Blacksburg, VA), and VTEC-PRLA (Denka Seiken, Japan).[166] These kits are however limited in the scope of Shiga-toxin subtypes that they are able to detect.[167]

Shiga toxin can also be identified by use of Vero-cell lines or HeLa cell lines, which are rich in globotriaosylceramides (Gb3 and Gb4). In these cell lines, the toxins cause cell death due to

protein synthesis inhibition. The fluorescent d2EGFP protein has been developed to assess effects of the Shiga toxin on these cell lines.[168]

The other antibody based option is immunomagnetic separation. During immunomagnetic separation, affinity purified monoclonal and polyclonal antibodies against O and H antigens of O157 and non-O157 STEC (especially the top six non-O157 STEC serotypes) are coated on the immuno-magnetic-separation beads to assist target specific serotypes on culture.[169] The beads are placed in the sample before it is cultured and are expected to bind to the O and H antigens (on the bacteria of interest) in the sample that matches the antibody bound to the bead surface. The super paramagnetic beads (bacteria-bead complexes) are then placed on a suitable medium and incubated to grow the bacteria attached to them. Immunomagnetic beads have particularly been used to detect O26 and O111 in ground beef. [117]

2.5.8.6 Antimicrobial resistance in STEC

There is a steady increase in antimicrobial resistance to commonly used antimicrobials globally. The WHO, through its global action plan, has recently urged governments to implement policies that control the misuse of antibiotics in both human and non-human users. *E. coli* is one of the bacteria associated with several non-human sources and has the potential to cause severe human disease. For this reason, the antimicrobials used to treat STEC (if indicated) infections are categorized as critically important antibiotics (CIA) by the WHO.[170]

As described above, the use of DNA-targeting antibiotics like fluoroquinolones to treat STEC infection triggers the SOS DNA repair response and the expression of Shiga toxin genes.[91] Antimicrobials that rupture the bacterial cell wall also lead to the release of Shiga toxins and thus accelerate development of HUS.[171] STEC infections are sometimes treated with antimicrobials to reduce the risk of progression to HUS and yet there have already been reports of multidrug resistance.[172-174] The antibiotic resistance profile of STEC can be influenced by the use of antibiotics in ruminants since they are the main reservoirs of STEC.[175,176] Recent reports have shown multiple drug resistance in both O157 and the non-O157 STEC with the most common antimicrobial resistance profile being sulfisoxazole-streptomycin-tetracycline.[177]

2.5.8.7 Epidemiology of STEC

The WHO Foodborne Disease Epidemiology Reference Group (FERG) estimated the global impact of foodborne disease to be more than six hundred million cases, 420,000 deaths, and 33 million Disability-Adjusted Life Years (DALYs) in 2010.[178] The estimated global burden of STEC was: 2,481,511 illnesses (95%Confidence Interval= 1,594,572 – 5,376,503), 269 deaths (95%CI=111 - 814), and 26,827 DALYs (95% CI=12,089 – 72,204). Of all the STEC infections, the proportion that was foodborne was 0.48 (95%CI=0.33 – 0.60).[179] However, this estimate which considered data from only 21 countries- may not accurately represent the true global burden given that there was no data from many WHO sub-regions and countries.

2.5.8.7.1 Geographical differences in STEC disease

Previous research has attempted to explain the differences in the STEC disease burden per region and particularly, to explain the low prevalence (or few reports) of STEC disease in Africa. For instance, compared to the Europe and America, fewer outbreaks of bloody diarrhoea due to STEC have been reported in sub-Saharan countries.[180-182]

It has been postulated that the different domestic ruminant feeding diets (grain-based vs. non-grain based diets) are responsible for these differences.[183] However, an alternative explanation is the differences in laboratory diagnostics between Africa, and more developed countries. However, even in developed countries, there are differences in the serotypes of STEC causing disease. Many studies, mainly in Europe and the United States, have reported the occurrence of *E. coli* O157:H7 in several outbreaks.[184] There are however geographical differences in the non-O157 STEC serotypes reported in Europe, Asia, and the United States. Even though serotype O26 is common in many countries in Europe, Asia, and the United States, it was not reported in France and Sweden. Detection depends on a high index of suspicion for these serotypes in the countries where they were frequently detected and on the availability of suitable laboratory methods to detect them.[185]

2.5.8.7.2 Transmission modes of STEC in the human and non-human environment

On explicit request by the Codex Committee on Food Hygiene (CCFH), the joint FAO (Food and Agricultural Organisation)/WHO (World Health Organisation) Core Expert Group Meeting on

STEC was held in Geneva, Switzerland in July 2016 to discuss three aspects of STEC namely; (1) global burden of STEC (2) hazard identification and characterisation of STEC (3) current monitoring and assurance programs for STEC. During this meeting, it was noted that while there was considerable knowledge of specific STEC serotypes such as STEC O157:H7, there was limited knowledge on foodborne STEC caused by the other STEC serotypes. The experts believed that the linkage between the illness causing strain and the STEC in foods was often influenced by the region's environment and food handling context. It was also recommended that the food categorization scheme by the United States' Interagency Food Safety Analytics Collaboration (IFSAC) be used to categorise foods with slight modifications to suit the different countries or regions. In this scheme, foods are broadly categorised into three groups namely: (1) aquatic animal foods, (2) land animal foods, and (3) plant foods. The aquatic foods are divided into three commodities namely; fish, crustaceans, and molluscs. The land animal foods are divided into six commodities namely: dairy, eggs, beef, game, pork, and poultry. The plant foods are divided into six commodities namely: grains, oils, fruit-nuts, fungi, leafy, root, sprout, and vine-stalk vegetables.[186]

Over a ten-year period, between 1998 and 2008, the percentage of annual foodborne illness in the USA caused by STEC was attributable to eight commodities of food subtypes namely: dairy, beef, game, pork, poultry, grains, fruit-nuts, leafy, and sprout[186] (**Table 2.3**).

Table 2.3 Percentage annual foodborne illness due to O157 and non-O157 STEC in the USA (1998-2008) [186]

	Proportion of foodborne STEC associated with food subtypes annually	
Food subtypes	O157 STEC	Non-O157 STEC
Dairy	6.7%-9.8%	-
Beef	33% - 41.3%	29.7%
Game	0.5% - 0.8%	-
Pork	0.7% - 4%	-
Poultry	0.8% - 2.5%	-
Grains	0.1% - 10.4%	-
Fruit-nuts	18% - 22.5%	62.2%
Leafy	19.3% - 31.5%	8.1%
Sprout	1.1% - 1.7%	

This means that in the USA, over the ten-year period, non-O157 STEC was mainly transmitted from fruit-nuts, while the O157 STEC was mainly transmitted from beef.

Besides the USA picture, globaly, various other transmission vehicles are involved in the transmission of STEC to humans and are of different levels of importance for both non-O157 STEC and O157 STEC (see **Table 2.4**). Non-O157 STEC are five times more likely to be present in the environment than the *E. coli* O157.[187] Due to their ubiquitous distribution, the non-O157 STEC have frequently been reported as contaminants of agricultural produce. Increased cross-border trade in agricultural produce, therefore, provides additional risk to the importation of STEC outbreak strains as exemplified by the recent O104 STEC outbreak which originated in Germany and affected 16 countries causing 4,000 cases of disease, including 908 cases of HUS.[117] Indeed, most of the recent foodborne STEC pandemics could easily be traced among the trade partner countries.[117] There is probably limited trade linkage (in food products) between Africa and the countries where STEC disease is commonly reported (America and Europe).

Table 2.4 Comparison of the relative importance of transmission vehicles associated with outbreaks of O157 and non-O157 STEC.

Vehicle	***E. coli* O157:H7**	***E. coli* Non-O157:H7**
Animal Contact	6.2%	9.7%
Water	10%	25.6%
Person to person	28.8%	6.8%
Dairy	10%	12.5%
Meat	11.2%	24.6%
Produce	6.2%	9.2%
Other food	8.8%	5.8%
Unknown	18.8%	5.8%

This table has been adopted from Charles Kasper, Doyle, *et al.*, 2009[188].

Globally, most of the described STEC infections are associated with vehicles relating to beef and livestock related products.[188] Food products of ruminant origin such as meat and milk are contaminated with STEC during harvesting, handling and processing.[138] During the animal slaughter process, bacteria from the heavily contaminated hide and the gastrointestinal tract may be spread on the carcas to the extent that even the post-slaughter washing may not eliminate them. The level of carcas contamination with STEC varies depending on conditions such as the

physiological state of the animal at slaughter, animal handling prior to slaughter, and management.[189] For instance, animals that shed more than 10^4 CFU of STEC per gramme of faeces prior to slaughter are considered heavy shedders and are more likely to be linked to carcas contamination. Research by Gyles *et* al. estimated high levels of carriage of STEC in healthy cattle using the occurrence of the *stx* genes in the stool.[190] However, this would probably not be an accurate estimate of the health risk posed because *stx* genes can be carried by non-pathogenic organisms or no organism at all (as freely occurring bacteriophages in the gut/faeces). Since the infectious dose of STEC is low, it is prudent to track the shedding levels of ruminants prior to slaughter. [191,192]

2.5.8.7.2.1 The African picture

The non-human environment that has been studied in Africa includes animal faeces, water, carcases; value added meat products especially the value-added beef products, fish, and dairy products (

Table 2.5).[193,194]

Table 2.5 Reported environmental reservoirs of STEC in Africa.

Environmental reservoir	Genes detected	STEC serotypes identified	References
Beef, pork, water, human, animals.	*rfbE* O157 and *fliC*H7, *hlyA* and *eaeA*.	O157	[195]
Raw meat and beef intestines at local markets.	*stx*1 and *stx*2.	No isolation	[196]
Zebu Cattle, fish, and water	None	O157: H7, and O157: NM	[197]
Shellfish and coastal environments	*stx*1 mainly, *stx*2	O157	[193]
Meat and dairy products, human	*stx*1, *stx*2, *ehxA* and *eaeA*.	O157	[194,198]
Beef, pork, chicken, deer, boar, bison, and retail rabbit meats. Springbok	*stx*1	O45	[199,200]
Water and cattle	*stx*1 and *stx*2		[201,202]
Cattle	*stx*1 and *stx*2	STEC	[180]
Calves, camels, humans,	STEC		[203]
Beef and beef value-added products	*stx*1 and *eaeA*.	Non-O157 STEC. O111,055, O26, O128	[204]

Raw milk cheese	*stx*$_1$, *stx*$_2$	O22: H8, O26: H11, O86: H21, O103: H2, O113: H21 and O146: H21	[205]
Home cooked food samples		O157	[206]
Meat and faeces of food animals	*stx*$_1$, *stx*$_2$, and *eaeA*	O157, O26, O91, O103, O111, O128, O145	[207]
Hides and faeces		O157: H7	[208]
Meat and dairy products	*stx*$_1$, *stx*$_2$, *eaeA*		[194]

2.5.8.7.3 STEC infections in South Africa

Research as early as 1978 by Seedat *et* al., suggested bacterial infections as the predominant cause of acute renal failure (ARF) in South Africa.[209,210] HUS was reported as the most common cause of ARF in children between 1986 and 2002 in South Africa.[211] Of note, hemolytic uremic syndrome, which is overwhelmingly caused by STEC, is the leading contributor to ARF in paediatric patients at the Pretoria Academic Hospital.[211,212]

Since the first report of severe STEC infection in South Africa in 1990, which was seven years after the release of the first report on HUS and haemorrhagic colitis-associated *E. coli* by Karmali et al., there have been scattered reports of both O157 and non-O157 STEC albeit with the earlier research efforts mainly based on serology and only in cases that were hospital-based.[213] Later, in 1993, the second STEC report of a waterborne outbreak was noted in Swaziland and South Africa. This outbreak which killed over 2000 people was found to originate in Swaziland on a sugar plantation which was contaminated by STEC via contaminated water flowing from an area with sick cattle.[214] Since then, with the initiation of the National Institute of Communicable Diseases (NICD) in 2004, Tau *et* al and Ateba *et* al reported the occurrence of STEC O104 (no *stx* genes were detected, but the strains were human diarrhoea associated) and STEC O157 in archived hospital isolates and the non-human environment respectively.[195,215] Investigators at the NICD screened 2378 suspect diarrheic *E. coli* isolated from stool of patients with diarrhoea received from collaborating clinical microbiology laboratories across the country between 2006 and 2009 for *stx* genes. Results showed that only 14 of them were STEC. Of these 14 STEC, only one was *E. coli* O157:H7.[216]

In 2011, at the Mafikeng provincial hospital, up to 56.5% (11/19) of stool samples from HIV-infected patients with diarrhoea in the North West province, tested positive for the presence of STEC O157:H7.[195] Immunocompromised individuals are particularly at risk of poor clinical outcome from HUS as up to 9% of immunocompromised adults and children die within four years after developing HUS.[51]

Recent environmental survey data describe a relatively high prevalence of *E. coli* O157: H7 identified by PCR from meat (27.7 – 67.7%), water (2.3 – 25.5%), and vegetable (21.7%) samples from the Eastern Cape and North West provinces of South Africa.[217] A separate study on piglet oedema by Mohlatlole *et* al. 2013, reports the occurrence of *stx* genes in diarrhoeagenic *E. coli* in piglets, though the serotypes were not determined.[218]

Most recently, in February 2017, there has been an outbreak of STEC O26 (associated with HUS) at the Red Cross War Memorial Hospital in Cape Town affecting four children (unpublished data).

In most of the instances where STEC was reported in a patient, there was no success in terms of, or effort made in as far as identification of the non-human sources of the outbreak strain.

2.5.8.8 Regional surveillance and monitoring strategies for STEC

Regional surveillance strategies differ, depending on the presence or absence of an outbreak in a country. In the absence of an outbreak, efforts may be focused on food safety (particularly for foods for export) or public health and may differ from country to country.

Regions should have a STEC surveillance strategy because of the public health impact as well as the economic impact of STEC infection.

Surveillance of *stx* related disease should, in addition to a hospital laboratory-based identification of cases, include attempts to identify the non-human sources of the infection through the administration of food history surveys in addition to testing of the food samples themselves.[219] Such a strategy should consider the possibility of more than one STEC strain causing disease and also the fact that an implicated food source could have travelled more than the geography of the patient.[115,220] The National STEC surveillance strategy by the Centers for Disease Control (CDC)

emphasises the passive model where laboratory-confirmed STEC (from food and water samples) and *stx* positive broths are sent to central public health laboratories where they are further characterised. However, this is in addition to a unified nationwide clinical diagnostic strategy for STEC where all stool samples presented for bacterial culture are tested for STEC and for presence of the Shiga toxin using a non-culture based method.[221]

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Chapter Three

3 Development of a real-time PCR test and comparison to culture on CHROMagar™STEC for detection of Shiga-toxin-producing *E. coli* in Stool, Water, and Food samples.

3.1 Summary

Introduction: Shiga toxin producing *Escherichia coli* (STEC) is an emerging pathogen with the potential for severe clinical manifestations including Haemolytic Uremic Syndrome, and is associated with economic losses in the food industry. CHROMagar™STEC is a selective and differential screening medium for STEC whose performance is serotype dependent. Molecular detection of STEC is increasingly used in diagnostic laboratories. Even though previous studies have compared the performance of CHROMagar™STEC to real-time PCR in Europe, no studies have been done to assess its performance in Africa.

Objectives: This project was undertaken to optimise a real-time PCR-based assay for simultaneous detection of *stx*$_1$ and *stx*$_2$. We aimed to achieve the following objectives:

i. To optimise and determine the limit of detection of the in - house developed real-time PCR assay.
ii. Assessment of the performance of CHROMagar™STEC in comparison to the in – house developed real-time PCR assay for detection of *stx* in stool, water and meat.
iii. To determine the prevalence of *stx* in the stool at a tertiary academic hospital laboratory in Cape Town, surface water from the Lotus River, and meat from the informal slaughter setting in Nyanga, Cape Town, South Africa.
iv. To characterise the *E. coli* strains that formed mauve colonies on CHROMagar™STEC.

Methods: Between September 2014 and May 2015, residual stool (submitted for bacterial pathogen culture) after routine testing were collected from 226 consecutive stool specimens (irrespective of age or related clinical data) from the National Health Laboratory Services located at the Groote Schuur Hospital in Cape Town, a tertiary care academic teaching laboratory affiliated with the University of Cape Town. This tertiary academic hospital serves the greater Cape Town area.

Between September 2015 and May 2016, forty-nine (49) raw and 36 ready – to - eat meat samples were collected from 53 randomly selected local stalls along main access roads in the Nyanga Township. Sixty-four (64) water samples were collected over a period of ten months (between 7

a.m and 12 noon) from July 2015 to March 2016 by using the depth integrated grab sampling method with samples being collected at a depth of approximately 30cm below the surface of the river. A multiplex real-time PCR for detection and differentiation of *stx*$_1$ and *stx*$_2$ was optimised, and performance characterised using stool spiked with plasmids containing cloned stx genes. We enriched for STEC in Tryptic Soy Broth (TSB). Real-time PCR was used to screen TSB, and mauve isolates on CHROMagar™STEC for *stx*, while agarose gel detection PCR was used to detect other diarrheic *E. coli* (DEC) virulence genes possessed by the isolates. The ImmunoCard STAT! EHEC® rapid test was used to test the isolates for Shiga toxin production.

Results: The real-time PCR limit of detection was 5.3 target copies/µl of broth. The mean melting temperature on melt-curve analysis for detection of *stx*$_1$ was 58.2 ℃ and for *stx*$_2$ was 65.3 ℃; detection of both targets could therefore occur in the same run. Of 226 stool specimens screened, real-time PCR detected *stx* in 14 specimens (6.2%, 95% Confidence Interval=3.43%-10.18%), comprising eight *stx*$_1$, five *stx*$_2$ and one with both *stx*$_1$ and *stx*$_2$. Using the in-house real-time PCR as reference, for clinical specimens, the sensitivity, specificity, negative predictive value and positive predictive value of the CHROMagar™STEC were 33.3%, 77.4%, 95.3% and 11.3% respectively.

The limit of detection of the in-house developed assay for *stx* targets in meat and water was 116 ±4.03 CFU per 25g of meat and 144 ±6.4 CFU/100ml of Lotus River surface water for both targets. Of the 64 water specimens screened, real-time PCR detected *stx* genes in 19 samples (29.7%, 95% Confidence Interval= 18.91%-42.42%), while the sensitivity, specificity, negative predictive value and positive predictive value of the CHROMagar™STEC were 5.26%, 66.7%, 62.5% and 6.25%, respectively. Of the 85 meat specimens, real-time PCR detected *stx* genes in 18 samples (21.2%, 95% Confidence Interval= 13.06%-31.39%), while the specificity and negative predictive value of the CHROMagar™STEC were 52.94% and 66.7%, respectively.

Conclusions: CHROMagar™STEC cannot be relied on solely to screen stool for STEC in this setting. To improve patient management, and to ensure the safety of food and water for public use, a robust nucleic acid amplification-based assay is needed in limited resource settings.

3.2 Background

Globally, food and water-borne outbreaks of both O157 and non-O157 STEC (Shiga-toxin-producing *Escherichia coli*) have been successfully detected due to the availability of good baseline data and efficient active laboratory-based surveillance systems.[1-4] Early detection of outbreaks is important to minimise morbidity, mortality, and associated economic losses[5]. There is a lack of good baseline data on STEC in Africa, which can be attributed to a lack of laboratory resources and the surveillance strategy employed. STEC has been reported in outbreaks of bloody diarrhoea in sub-Saharan countries.[6-8] However, these have been difficult to manage due to laboratory weakness.[9,10] Furthermore, typical Haemolytic Uremic Syndrome (tHUS), which is overwhelmingly caused by STEC, was reported as the leading contributor to acute renal failure (ARF) in paediatric patients at a South African academic hospital.[11]

Laboratory methods to isolate STEC involve the use of sorbitol MacConkey containing cefixime and tellurite to culture the non-sorbitol fermenting O157: H7 STEC[12], coupled with the use of immunochromatographic methods to detect Shiga toxin in stool.[13] However, non-O157 STEC have recently emerged as causes of serious STEC disease.[14] For this reason, several chromogenic media like CHROMagar™STEC have been developed to target both O157 and non-O157 STEC in clinical and environmental samples.[15] Even though several studies have evaluated the performance of CHROMagar™STEC by comparison to molecular and antigen detection methods in developed countries[16,17], no study has so far evaluated its performance in Africa. This may be important, given that there are geographical differences in characteristics of STEC prevalent per region. Detection of the prevalent STEC serotypes in an area is dependent on the index of suspicion for the different STEC serotypes and on the availability of suitable laboratory methods to detect them.[14]

In many South African (and African) laboratories, stool specimens are not routinely tested for STEC, although physicians may request testing specific for *E. coli* serotype O157:H7 if clinically suspected. In the National Health Laboratory System (NHLS, South Africa) laboratories, testing is often based on the non-sorbitol fermenting property of some STEC, using sorbitol MacConkey, and only on request by a physician. This practice is likely to underestimate the real magnitude of

STEC since not all serotype O157 strains are non-sorbitol fermenting (O157: NM), and > 470 non-O157 serotypes have been associated with clinical disease.[18]

Laboratory capacity for molecular detection is increasingly available in African countries, and may in some cases be simpler than culture-based detection. Therefore, we validated the use of a duplex hybridization probe-based real-time PCR assay and compared findings to those obtained using a chromogenic screening culture medium. We used the PCR assay to assess the prevalence of STEC in specimens submitted to the National Health Laboratory Services at Groote Schuur Hospital, a tertiary academic hospital laboratory in Cape Town, South Africa. We further characterised the *E. coli* isolates that formed mauve colonies on CHROMagar™STEC from the same specimens by serotyping, detection of Shiga toxin production, as well as detection of the fimbrial adhesion gene (*daaC)* for diffusely adherent *E. coli* (DAEC), the anti-aggregation protein transporter gene for Enteroaggregative *E. coli* (EAggEC) *(aat)*, heat-stable (*ST*) and heat-labile (*LT*) enterotoxin genes of enterotoxigenic *E. coli* (ETEC), the intimin coding gene *eae* for enteropathogenic *E. coli* (EPEC), and the bundle forming pili gene (*bfp*) for typical EPEC.

3.2.1 Aims and objectives

i. To optimise and determine the limit of detection of the in - house developed real-time PCR assay.

ii. Assessment of the performance of CHROMagar™STEC in comparison to the in – house developed real-time PCR assay for detection of *stx* in stool, water and meat.

iii. To determine the prevalence of *stx* in the stool at a tertiary academic hospital laboratory in Cape Town, surface water from the Lotus River, and meat from the informal slaughter setting in Nyanga, Cape Town, South Africa.

iv. To characterise the *E. coli* strains that formed mauve colonies on CHROMagar™STEC.

3.3 Methods

3.3.1 Target plasmid preparation.

The real-time PCR previously described by Grys *et al*. [19] was used to amplify *stx*$_1$ and *stx*$_2$ gene targets from STEC O157:H7 NCTC control strain (C4193-1) with both stx$_1$ (subtype 1a) and stx$_2$ (subtype 2a). PCR amplicon size was confirmed visually by agarose gel detection (~208bp for stx$_1$ and ~204bp for stx$_2$) before confirmation by sequencing using the Big Dye® Terminator v3.1 Cycle Sequencing Kit. We used primers 1a and 2a for unidirectional Sanger sequencing of the amplicons (**Table 3.1**).

Table 3.1 Primers and probes used for real-time PCR

Primers/probes	5′	Sequence	3′	Reference
stx1a-primer		CAAGAGCGATGTTACGGT		[19]
stx1b-primer		AATTCTTCCTACACGAACAGA		[19]
stx1f-probe		CTGGGGAAGGTTGAGTAGCG	Fluorescein	[19]
stx1r-probe	CAL Fluor 610	CCTGCCTGACTATCATGGACA	3' phosphor	[19]
stx2a-primer		GGGACCACATCGGTGT		[19]
stx2b-primer		CGGGCACTGATATATGTGTAA		[19]
stx2f-probe		CTGTGGATATACGAGGGCTTGATGTC	Fluorescein	[19]
stx2r-probe	CAL Fluor 610	ATCAGGCGCGTTTTGACCATCT	3' phosphor	[19]

Resultant sequences were then trimmed and submitted for BLAST analysis against the NCBI database and confirming *stx*$_1$ or *stx*$_2$ target sequences in comparison to O157:H7 EDL933 (NCBI Reference: NC_002655.2)[20]. Purified amplicons (Mini Elute Gel extraction kit, Qiagen, Madrid Spain) were cloned using CloneJet PCR cloning kit (Thermofisher Scientific, Austin Texas, USA) into a pJet 1.2 / blunt vector using the sticky end cloning protocol and transfected into the JM109 competent cells by calcium chloride transformation. Plasmids containing *stx*$_1$ and *stx*$_2$ were separately extracted using a Genopure plasmid Maxi kit (Roche Life Sciences, Industriestrasse, Switzerland) and quantified by spectrophotometry. To verify successful preparation purified

plasmids were subjected to PCR amplification using primers 1a and 1b for *stx*$_1$ and 2a and 2b for *stx*$_2$ with amplicon size visually confirmed by agarose gel detection and subsequent sequence analysis. Plasmid quantification was performed spectrophotometrically employing the BioDrop-µlite (Isogen Life Science, B.V, Veldzigt, Netherlands). The A_{260} was used to calculate the plasmid concentration expressed as the number of molecules/µl. Three measurements were made and the average determined. Briefly, the plasmid concentration was calculated as the product of the A_{260}, the dilution factor, and the concentration with one A_{260} being the equivalent of 50µg/ml and 1 pico mole being equivalent to 660pico grammes.

3.3.2 PCR Assay Validation

To assess the potential for PCR cross-reactivity and assess the analytical specificity of the hybridization probe-based real-time PCR described by Grys et al[19] the primer and probe sequences were subjected to BLAST analysis on the NCBI database. The PCR reaction was optimized for use on the LightCycler®480 Instrument II (Roche Life Sciences, Industriestrasse, Switzerland) employing the LightCycler® 480 Probes Master mastermix (Industriestrasse, Switzerland) with modification to the thermal cycling conditions for amplification consisting of denaturation at 95℃ for 10 minutes followed by 45 cycles of 95℃ for 5s, 56℃ for 5s, and 72℃ for 15s. A positive amplification signal was defined as an increase in fluorescence signal that crossed the threshold before 35 cycles. Amplicon identity was determined using the melt-curve analysis program of 95℃ for 30s, 40℃ for 60s, and 85℃ 5s with continuous fluorescence acquisition. The Multi-color HybProbe detection format was used for analysis, combining the Red 610, Red 640 and FAM filter pairs (LightCycler®480 Instrument II Manual, Roche Life Sciences). The resulting amplicon was visualized using agarose gel electrophoresis and subjected to DNA sequencing and BLAST alignment to reference *stx1a* and *stx2a* sequences (NC_002655.2).

Stool: To mimic the sample matrix for sensitivity determination, TSB was inoculated with a pea-size amount of stool (from a single donor shown to be *stx*-negative by PCR). To this inoculated broth, 1 ml of plasmid stock ($5.3*10^6$ copies/µl or $5.3*10^9$ copies/ml) containing both *stx*$_1$ and *stx*$_2$ was added and serially diluted eight times in 9 ml of TSB, to the lowest dilution of $1:10^8$ (53 plasmid copies/ml).

Meat samples: Firstly, we prepared a 0.5 McFarland concentration of C4193-1 O157: H7 (using 1% PBS). Twenty-five grammes of meat were rinsed by pummelling in 225ml of TSB (TSB; Oxoid, Basingstoke, UK). Tenfold serial dilutions of the bacterial suspension were made in the TSB meat rinsate to a final dilution of 10^0 CFU/ml. From the third dilution to the highest dilution (10^5 to 10^0), 1ml of TSB with C4193-1 O157: H7 bacteria was spread out on a 2% Blood agar plates using a plastic spreader and incubated for 24hrs at 35℃. For each dilution, the culture was carried out on five 2% Blood agar plates, and the mean of the counts from the five plates represented the number of colony forming units at a dilution.

Water samples: A 0.5 McFarland concentration of C4193-1 O157: H7 (using 1% PBS) was prepared. Ten millilitres of the 0.5 McFarland solution was resuspended in 90ml of Lotus River surface water. Then, this solution (100ml) was filtered through a 0.45µM membrane filter on a calibrated filtration unit attached to a vacuum pump. This membrane was placed in 100ml of sterile TSB (Oxoid, Basingstoke, UK) and agitated with vortexing for at least 15 minutes to release bacteria from the membrane into the TSB (Oxoid, Basingstoke, UK) to a final concentration equivalent of 10^7CFU/ml. Seven tenfold serial dilutions of the TSB-bacterial suspension were made in the TSB rinsate to a final dilution of 10^0 CFU/ml (this procedure was used to mimic the routine water testing process). From the third dilution to the highest dilution (10^5 to 10^0), 1ml of TSB (Oxoid, Basingstoke, UK) with C4193-1 O157: H7 bacteria was spread out on a 2% Blood agar plates using a plastic spreader and incubated for 24hrs at 35℃. For each dilution, the culture was carried out on five 2% Blood agar plates, and the mean of the counts from the five plates represented of the number of colony forming units at a particular dilution.

Nucleic acid extraction was performed on 200µl TSB broth (without enrichment/incubation for TSB rinsates of meat, Lotus River surface water and stool) employing the MagNApure LC instrument (Roche Diagnostics, Industriestrasse, Switzerland) to yield 100µl of extract.

Initially, real-time PCR was performed in triplicate using a template from each of the 8 dilutions to estimate a limit of detection (LOD). Subsequently, real-time PCR was performed in eight replicates on the dilution (replicated only the real – time PCR and not the extractions) with the

estimated LOD, as well as one dilution above and one dilution below the estimate. The LOD was defined as the lowest plasmid concentration spiked into TSB, before nucleic acid extraction, yielding a positive signal, as described above in all eight replicates. Nucleic acid extractions from STEC subtypes 1d (Reference strain MH1813, GenBank accession No. AY170851), 2b (Reference strain EH250, GenBank accession No. AF043627), 2c (Reference strain 031, GenBank Accession No. L11079), 2d (Reference strain C165-02, GenBank accession No. DQ059012), 2e (Reference strain S1191, GenBank accession No. M21534), 2f (Reference strain T4/97, GenBank accession No. AJ010730) and 2g (Reference strain 7V, GenBank accession No. AY286000) were also subjected to PCR amplification to assess impact of strain variation on detection. The reproducibility of melting temperature assessment for *stx*$_1$ and *stx*$_2$ differentiation was determined by testing 24 replicates of TSB (Oxoid, Basingstoke, UK) spiked with *stx*$_1$ and *stx*$_2$ plasmids. To further assess the reproducibility of melting temperature, the three stx$_1$ subtypes and seven stx$_2$ subtypes were tested similarly.

3.3.3 Clinical specimen testing

Between September 2014 and May 2015, we collected residual stool (submitted for bacterial pathogen culture) after routine testing from 226 consecutive stool specimens (irrespective of age or related clinical data) from the National Health Laboratory Services located at the Groote Schuur Hospital in Cape Town, a tertiary care academic teaching laboratory affiliated with the University of Cape Town. This tertiary academic hospital serves the greater Cape Town area.

A pea sized stool sample was inoculated in 90ml of TSB and vortexed before incubation at 37°C for 18h. Two hundred microliters of broth were subsequently extracted employing the MagNA Pure LC Total Nucleic Acid isolation kit (Roche Diagnostics, Industriestrasse, Switzerland) using the total variable elution volume protocol and following the manufacturer's manual (version 14) to yield 100µl of nucleic acid extract.

In addition, CHROMagar™STEC (CHROMagar Microbiology, Paris, France) was inoculated with a loop full of overnight incubated broth and incubated at 37 ℃ for 18h. Bright mauve colonies were sub-cultured onto MacConkey agar with crystal violet, sorbitol MacConkey agar, and 2%

Blood agar (Green point Media, NHLS, Albertynshof). *E. coli* was presumptively identified as lactose-positive, oxidase-negative, spot indole-positive, and pyrrolidonyl arylamidase (PYR)-negative with confirmatory identification using VITEK® 2 automated system (bioMerieux, USA).

3.3.4 Environmental sample testing

We collected 64 surface water samples over a period of ten months (between 7 a.m and 12 noon) from July 2015 to March 2016 by using the depth integrated grab sampling method. These were collected 30cm below the Lotus River surface. Between October 2015 and May 2016, we collected Forty-nine (49) raw and 36 ready – to - eat meat samples from 53 randomly selected local stalls along main access roads in the Nyanga Township.

Meat: Twenty-five grammes of meat tissue was thoroughly pummeled using a sterile mortar and pestle, and inoculated into 225ml of Tryptic Soy Broth (TSB; Oxoid, Basingstoke, UK) and incubated at 35°C for 24 hours as previously described but with slight modifications[21].

Water: One hundred millilitres of surface water was filtered through a 0.45µM nitrocellulose membrane (Pall Corporation, Port Washington, USA) on a filter funnel mannifold (Pall Corporation, Port Washington, USA). The post – filtration membrane was placed in 90ml of Tryptic Soy Broth (Oxoid, Basingstoke, UK) using sterile forceps, vortexed and then incubated at 35℃ for 18-24h. This was in accordance with the International Standards Organisation guidelines on microbiology of water (ISO 9308-3: 1998)[22]. Two hundred microliters (200µl) of broth was then loaded into the sample cartridge and processed using the MagNA Pure LC (Roche) instrument using the total variable elution volume protocol and following the manufacturer's manual (version14) to yield 100µl of nucleic acid extract.

CHROMagar™STEC (CHROMagar Microbiology, Paris, France) was inoculated with a loopful of overnight incubated broth and incubated at 37℃ for 18h. Bright mauve colonies were processed as described in **3.3.3**.

3.3.5 Isolate characterization.

Isolates yielding mauve colonies on CHROMagar™STEC and presumptively identified as *E. coli* were subjected to *stx* characterization employing the real-time PCR assay described here as well as a conventional PCR performed as previously described by Paton and Paton (1998).[23] The presence of virulence genes including the fimbrial adhesion gene for diffusely adherent *E. coli* (*daa*C), the anti-aggregation protein transporter gene for Enteroaggregative *E. coli* (*aat*), heat-stable (*ST*) and heat-labile (*LT*) enterotoxin genes of enterotoxigenic *E. coli* (ETEC), the invasive plasmid antigen gene for enteroinvasive *E. coli* (*ipa*) and the intimin coding gene (*eaeA*) for EPEC were determined using standard gel-based PCR as previously described[23] using primers as shown below (**Table 3.2**).

Table 3.2: Primers used for detection of diarrheic E. coli virulence genes

Reaction	Target genes	Primers used	Primer sequence	Product size
A	*eaeA*	eae-F	TCAATGCAGTTCCGTTATCAGTT	482bp
		eae-R	GTAAAGTCCGTTACCCCAACCTG	
	Bfp	bfp-F	GGAAGTCAAATTCATGGGGGTAT	298bp
		bfp-R	GGAATCAGACGCAGACTGGTAGT	
	stx$_1$	stx$_1$-F	CAGTTAATGTGGTGGCGAAGG	348bp
		stx$_1$-R	CACCAGACAATGTAACCGCTG	
	stx$_2$	stx$_2$-F	ATCCTATTCCCGGGAGTTTACG	584bp
		stx$_2$-R	GCGTCATCGTATACACAGGAGC	
B	*Est*	ST-F	ATTTTTCTTTCTGTATTGTCTT	190bp
		ST-R	CACCCGGTACAAGCAGGATT	
	Elt	LT-F	GGCGACAGATTATACCGTGC	440bp
		LT-R	CGGTCTCTATATTCCCTGTT	
C	*Ipa*	ipaH-F	CTCGGCACGTTTTAATAGTCTGG	933bp
		ipaH-R	GTGGAGAGCTGAAGTTTCTCTGC	
	Aat	pCVD432-F	CTGGCGAAAGACTGTATCAT	630bp
		pCVD432-R	CAATGTATAGAAATCCGCTGTT	
	daaC	daaC-F	CAGGTCATCCGGTCAGTCGG	212bp
		daaC-R	CAATGCCACGTACAACCGGC	

Screening of E. coli colonies for Shiga toxin production: The Immunocard STAT! ® EHEC was used to detect Shiga toxin 1 and Shiga toxin 2 by the isolates employing immunochromatography with toxin-directed monoclonal antibodies labelled with red-coloured gold particles (Meridian Biosciences, Inc., USA).

Serotyping: All mauve isolates found to carry virulence genes were serotyped at the Centre for Enteric Diseases, National Institute of Communicable Disease (NICD), Johannesburg employing

(Statens Serum Institut, Copenhagen, Denmark) antisera and the detection of somatic O- antigens as previously described.[24,25] H-antigen serotyping was not undertaken.

3.3.6 Data Analysis

All data were entered into an Excel database (Microsoft Corporation, USA). Two-by-two tables were created. The performance of CHROMagar™STEC was compared with that of the in-house qPCR (reference standard) in order to determine its sensitivity, specificity, negative and positive predictive values for the different sample matrices.

3.4 Results

3.4.1 Real-time PCR validation

The BLAST analysis of the primers and probe sequence specificity yielded no significant homology to non-*stx* targets (data not shown). Real-time PCR amplicons generated were confirmed as 208bp and 204bp for *stx*$_1$ and *stx*$_2$, respectively (**Figure 3:1**).

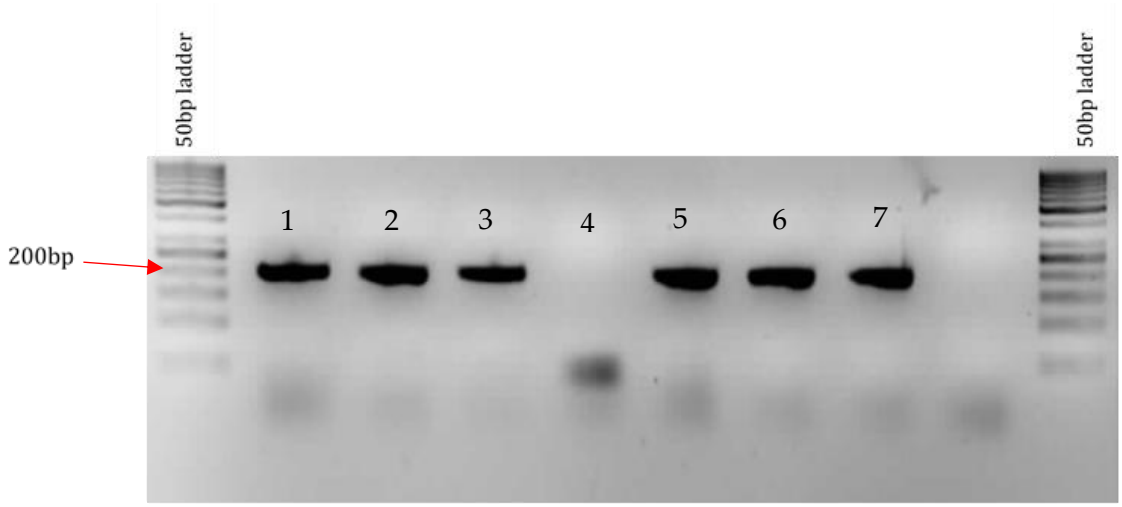

Figure 3:1 PCR amplicons of stx$_1$ *and stx*$_2$

Lanes 1,2 and 3 show *stx*$_1$ amplicon bands. Lane 4 shows the negative control. Lanes 5,6, and 7 show the *stx*$_2$ amplicon bands.

Sequencing and BLAST analysis confirmed the identity of both stx$_1$ and stx$_2$ amplicons. The serially diluted plasmid-stool-TSB was successfully amplified in 8/8 replicates in the fifth dilution. The sixth and seventh dilutions yielded amplification signal in 8/8 and 3/8 replicates respectively, yielding a LOD of 5.3 target copies / µl of broth. All other *stx* subtypes investigated (stx1a, stx1b, stx2a, stx2b, stx2c, stx2d, stx2e, stx2f, and stx2g) were successfully amplified by this assay (data not shown). *Stx*$_1$ and *stx*$_2$ were successfully distinguished by a melting temperature of 58.2 ℃ (SD=0.033) and 65.3 ℃ (SD=0.037), respectively (**Figure 3:2**).

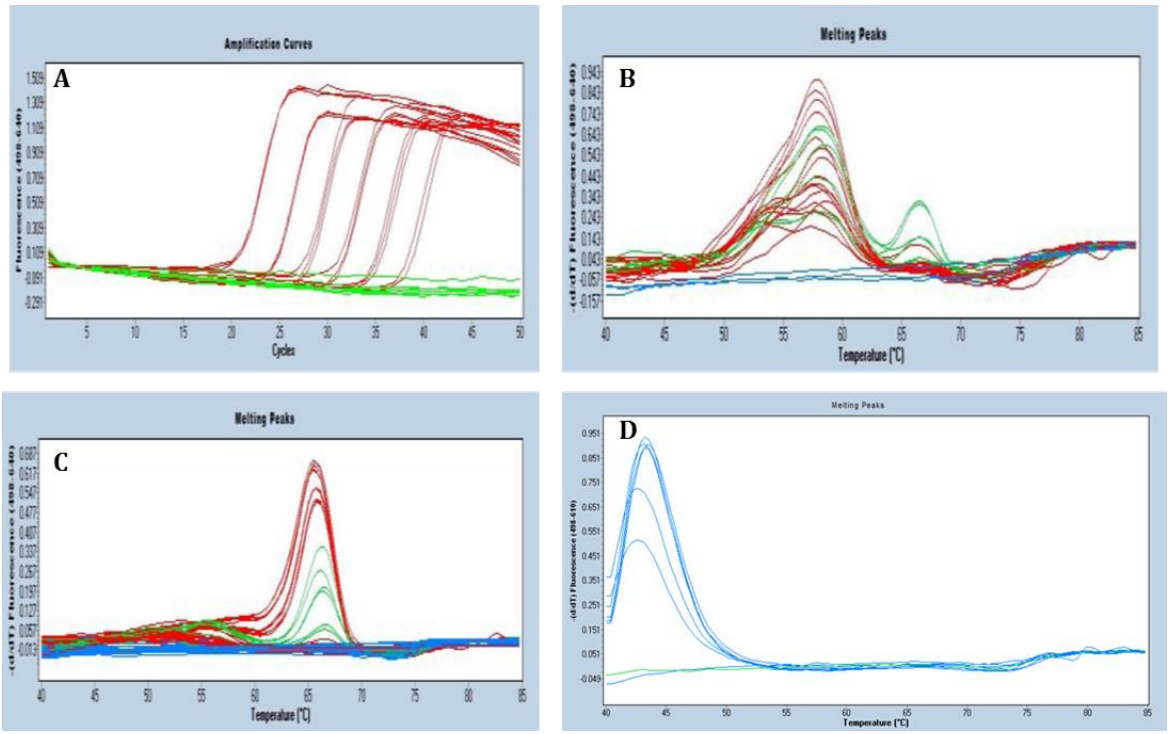

Figure 3:2: Performance of the optimised real-time PCR

A- Amplification curves on amplification of serially diluted template DNA (eight dilutions from 10^7 to 10^0), B-Melting curves for the duplex real – time PCR (stx1a peak at 58.2℃ and stx2a peak at 65.3℃) on the serially diluted DNA (eight dilutions from 10^7 to 10^0), C-Melting curves for stx$_2$ on serially diluted *stx*$_2$ plasmids (eight dilutions from 10^7 to 10^0), D-Melting curves for stx1d on serially diluted genomic DNA (eight dilutions from 10^7 to 10^0).

The T_m for subtypes *stx*$_2$ subtypes 2a, 2b, 2c, 2d, 2e, 2f and 2g were the same at 65.3℃ (SD=0.037, 0.041, 0.035, 0.039, 0.034, 0.033 and 0.032 respectively) while that of 1d was 45.7℃ (SD=0.042). The efficiency of the assay was 1.99 as calculated from the amplification curves generated using the Light Cycler® 480 software. The duplex assay detected both targets in the same run, and these could be differentiated by the melt curve with two distinct peaks at 58.2℃ for stx$_1$ and 65.3℃ for stx$_2$ (**Figure 3:*3***).

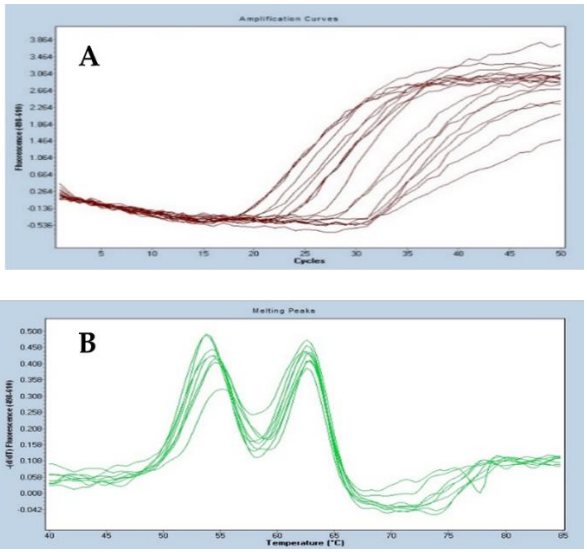

Figure 3:3: Amplification curves and Melting Peaks for the in-house optimised real-time PCR assay.

A- Amplification curves for the duplex real – time PCR targeting both *stx*$_1$ and *stx*$_2$ simultaneously on serially diluted DNA extracts from TSB rinsates of meat.

B- Melting peaks for the duplex real-time PCR assay targeting both *stx*$_1$ and *stx*$_2$ simultaneously on serially diluted DNA extracts from TSB rinsates of meat.

Determination of the LOD of the assay for *stx*$_1$ and *stx*$_2$ in water and meat

The mean count of colony forming units growing on 2% Blood agar for increasing dilutions for TSB rinsate of 25g of meat were 116 ±4.03, 29 ±2.61 and 2 ±1.9 for the sixth, seventh and eighth dilutions, respectively (the extrapolated original CFU/ml values were 10^2, 10^1, and 10^0 for the sixth, seventh and eighth dilutions respectively). The mean count of colony forming units on 2% Blood agar for increasing dilutions for Lotus River water in TSB were 212 ±6, 32 ±5 and 5 ±0.8 (The extrapolated original CFU/ml were 10^2, 10^1, and 10^0 for the sixth, seventh and eighth dilutions respectively) (**Table 3.3**). We observed amplification curves for DNA template from the serial dilutions in TSB up to the sixth dilutions for template from 25g of meat and Lotus River surface water. The mean crossing points were 18.87, 21.7, 23.5, 27.6, 31.02 and 34.02 for the first, second, third, fourth, fifth and sixth dilutions, respectively, for template from TSB rinsate of 25g of meat. The mean crossing points were 19.53, 22.53, 25.43, 28.20, 31.21 and 34.23 for the first, second, third, fourth, fifth and sixth dilutions, respectively, for template from TSB dilutions of Lotus River

surface water (**Table 3.3)**. The limit of detection of the in-house developed assay for *stx* targets in meat and water was 116 ±4.03 CFU per 25g of meat and 144 ±6.4 CFU/100ml of Lotus River surface water for both targets.

Table 3.3 Mean cell counts/ml of TSB rinsate (river water and meat samples) with crossing points, and melting temperatures for stx_1 and stx_2 targets resulting from a duplex qPCR run in triplicate on broth DNA extracts from TSB dilutions.

TSB rinsate of 25g of meat following pummelling					**Lotus River water**				
(Estimated CFU/ml)	Mean Cp	Mean count ±SD	Tm1	Tm2	(Estimated CFU/ml)	Mean Cp	Mean count ±SD	Tm1	Tm2
10^7		ND	55.12	61.90	10^7		ND	55.23	62.80
10^7	18.87	ND	54.97	61.91	10^7	19.53	ND	55	60.81
10^7		ND	55.12	61.90	10^7		ND	55.3	62.78
10^6		ND	54.46	61.76	10^6		ND	54.6	61.66
10^6	21.7	ND	55.28	61.96	10^6	22.53	ND	56	61.96
10^6		ND	54.88	61.84	10^6		ND	56.4	61.54
10^5		ND	55.48	62.20	10^5		ND	55.3	62.0
10^5	23.5	ND	54.92	61.93	10^5	25.43	ND	54.8	62.83
10^5		ND	54.73	61.94	10^5		ND	55.4	62.44
10^4		ND	55.11	61.99	10^4		ND	55.87	62.14
10^4	27.6	ND	55.37	62.38	10^4	28.20	ND	55.95	62.10
10^4		ND	53.67	61.72	10^4		ND	54.5	61.52
10^3		TNTC	53.73	61.89	10^3		TNTC	53.73	62.8
10^3	31.02	TNTC	56.25	62.26	10^3	31.2	TNTC	55.8	62.0
10^3		TNTC	55.71	62.26	10^3		TNTC	55.71	62.07
10^2			53.79	61.88	10^2			54.3	62.5
10^2	34.20	116 ±4.03	55.35	62.24	10^2	34.23	144 ±4.0	55.2	62.6
10^2			55.44	62.40	10^2			55.65	62.44
10^1			56.61	62.55	10^1			56.78	62.64
10^1		29 ±2.61	62.39		10^1		32 ±2.4	62.45	
10^1			56.54	62.64	10^1			56.89	62.48
10^0			-	-	10^0			-	-
10^0		2 ±1.9	-	-	10^0		2 ±1.3	-	-
10^0			-	-	10^0			-	-
Mean Tm			55.10 ±0.82	62.09 ±0.27	Mean Tm			55.6 ±1.72	62.2 ±0.53

SD: standard deviation, ND: not detectable, TNTC: too numerous to count, Tm: melting temperature.

The mean melting temperatures (Tm) for *stx*$_1$ (Tm1) and *stx*$_2$ (Tm2) detection in TSB rinsate of 25g of meat over 24 DNA replicates using the duplex qPCR were 55.10 ℃±0.82 and 62.09 ℃ ±0.27, respectively. The mean melting temperatures for *stx*$_1$ (Tm1) and *stx*$_2$ (Tm2) detection in TSB dilutions of Lotus River surface water over 24 DNA replicates using the duplex qPCR were 55.10 ℃ ±0.82 and 62.09 ℃ ±0.27, respectively.

3.4.1 Clinical and Environmental specimen testing

Clinical specimen testing: Of the 226 specimens screened, real-time PCR detected Shiga toxin genes in 14 samples (6.2%), comprising eight *stx*$_1$, five *stx*$_2$ and one specimen containing both *stx*$_1$ and *stx*$_2$. CHROMagar™STEC yielded mauve colonies from 23.45% (53/226) of the stool broth cultures.

Environmental specimen testing: Of the sixty-four water sample broths, 19 (29.7%, 95% Confidence Interval= 18.9%-42.4%) tested positive for the stx$_1$ using the in-house developed qPCR. None of the samples tested positive for *stx*$_2$. Eighteen of the 86 meat sample broths (21.2%, 95% Confidence Interval= 13.1%-31.4%) from the informal slaughter setting tested positive *stx* genes with the qPCR, 15 had the *stx*$_1$ genes; one sample had both the stx$_1$ and stx$_2$ genes while four samples possessed the *stx*$_2$ genes.

3.4.2 The performance of CHROMagar™STEC for clinical and Environmental specimen testing

Clinical specimen: Of the 53 mauve isolates, 48 were negative for *stx* genes using the real-time PCR assay. Of the 14 broths that were positive on real – time PCR, nine did not yield any mauve colonies on CHROMagar™STEC culture (diagnostic odds=1.898).

Lotus River surface water: Of the 64 water samples screened, 16 yielded mauve colonies on CHROMagar™STEC following overnight incubation (one mauve colony was selected from each sample). Of the 19 Lotus River surface water enrichment broths that tested positive for *stx*$_1$ using the in-house optimised real-time PCR, only five formed mauve colonies on CHROMagar™STEC. Of these five, one isolate tested positive for *stx*$_1$. The other 11 mauve colonies (to form a total of 16 mauve colonies from all the 64 water samples) were isolated from *stx* negative broths.

Meat samples: Of the 85 meat samples processed, 32 formed mauve colonies on CHROMagar™STEC. All the 18 meat sample broths that tested positive for stx genes using the in-house developed real-time PCR formed mauve colonies on CHROMagar™STEC.

3.4.3 Isolate characteristics for isolates from clinical and environmental testing

Two hundred twenty-six stool isolates, 85 meat samples and 64 water samples were screened. Fifty-three, 32, and 16 mauve isolates were detected in stool, meat, and water respectively. Of these, five, zero, and one tested positive for *stx* using the real – time PCR (**Figure 3:4**).

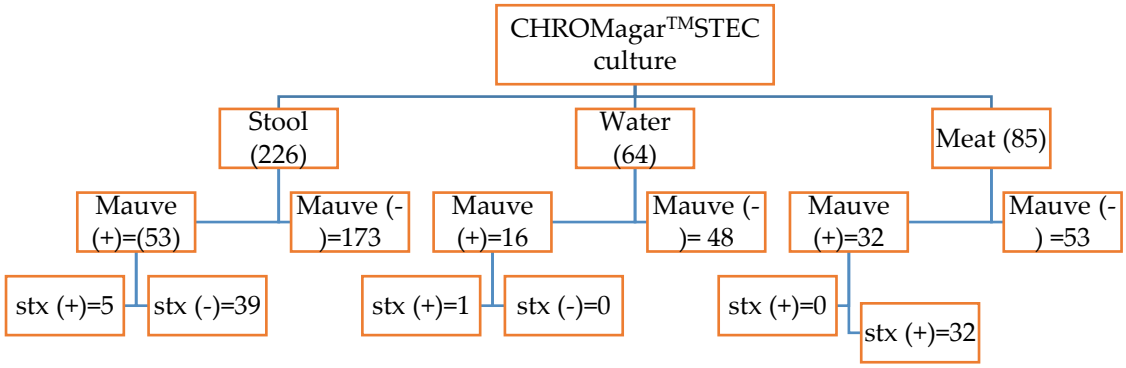

Figure 3:4: Summary of results showing growth properties on CHROMagar™STEC and the detection of stx genes from the mauve isolates.

Clinical testing: Forty-four (83%) of the 53 mauve colonies fermented lactose on MacConkey agar with crystal violet. Eleven (25%) of the 44 lactose fermenters were non-sorbitol-fermenting. Real-time PCR on the 44 *E. coli* confirmed the presence of *stx* genes in five (11%) while 39 were negative for the *stx* gene.

Real-time PCR was not done on the nine non-lactose fermenting isolates as these were found not to be *E. coli* on biochemical testing. Four of the five *stx* positive *E. coli* colonies were from corresponding real-time PCR positive broths. Of the 39 *stx*-negative *E. coli*, only four (12.5%) carried *eae* genes, while four possessed *aat* genes. Of the four eae positive isolates, two also had the *bfp* genes and were typical EPEC. The other two *eae* positive isolates did not possess the *bfp*

genes and were classified as atypical EPEC. The four enteroaggregative *stx*-negative isolates all belonged to *E. coli* serotype O104. All the typical EPEC belonged to serotype O55 while one of the two atypical EPEC belonged to serotype O101. The atypical EPEC serotype O101 was from stx_2 positive broth. The other atypical EPEC isolate was untypeable. None of the 53 *E. coli* isolates that were screened by immunochromatography was positive for Shiga toxins. The sensitivity, specificity, negative predictive value and positive predictive value of the CHROMagar™STEC were 35.71%, 77.4%, 95.3% and 11.3% respectively (**Table 3.4**). The diagnostic accuracy of the test for stool screening was 74.78%.

		qPCR assay		
CHROMagar™STEC		+	_	**Total**
	+	5	48	53
	_	9	164	173
Total		14	212	226

Table 3.4 Two-by-two table showing the performance of CHROMagar™STEC in comparison to the qPCR on clinical stool specimens.

Sensitivity =5/14*100=35.71% (95% Confidence Interval=16.34 - 61.24)

Specificity=164/212*100=77.4% (95% Confidence Interval=71.27 – 82.48)

Positive Predictive Value= 5/53*100=9.4% (95% Confidence Interval=4.097 – 20.25)

Negative Predictive Value =164/173*100=95% (95% Confidence Interval=90.41 – 97.24)

Isolates from water: From the 64 water samples, mauve colonies were detected in 16 water samples (16 mauve colonies). All the 16 mauve colonies were lactose fermenters, and we confirmed them to be *E. coli*. However, of these, only four carried virulence genes. Two of these isolates carried *daaC* genes while one had the *eae* gene and one carried the *ipa* gene. One mauve isolate tested positive for Shiga toxin 1 on immunochromatography. The sensitivity, specificity, negative predictive value and positive predictive value of the CHROMagar™STEC were 5.3%, 66.7%, 62.5% and 6.3%, respectively (**Table 3.5).** The diagnostic accuracy of this media was 48.44%.

Table 3.5 Two-by-two table showing the performance of CHROMagar™STEC in comparison to the qPCR to detect STEC in Lotus River water samples.

		qPCR assay		
CHROMagar™STEC		+	_	**Total**
	+	1	15	16
	_	18	30	48
Total		19	45	64

Sensitivity= 1/19*100= 5.3% (95% Confidence Interval= 0.9352 – 24.64)

Specificity= 30/45*100= 66.7% (95% Confidence Interval= 52.07 – 78.64)

Negative predictive value= 30/48*100= 62.5% (95% Confidence Interval=48.36 – 74.78)

Positive predictive value= 1/16*100= 6.3% (95% Confidence Interval= 1.112 – 28.33)

Isolates from the informal slaughter: Of the 85 meat samples, only 32 formed mauve colonies on CHROMagar™STEC. The 32 mauve colonies were confirmed to be *E. coli* using the VITEK®2 automated system. Only three of the 32 mauve colonies on CHROMagar™STEC carried virulence genes. Of these three, two possessed the *daaC* genes while only one carried the *eae* gene. None of the mauve colonies had *stx* genes. To detect *stx* genes from 25g of meat samples, the sensitivity, specificity, positive predictive value and negative predictive value of the CHROMagar™STEC were 0%, 52.9%, 0%, and 66.7%, respectively (**Table 3.6).** The diagnostic accuracy of this media was 41.86% for screening meat.

Table 3.6 Two-by-two table showing the performance of CHROMagar™STEC in comparison to the qPCR on meat samples.

		In-house developed assay		
CHROMagar™STEC		+	_	Total
	+	0	32	32
	_	18	36	54
Total		18	68	86

Sensitivity=0.0% (95% Confidence Interval=0.0 – 17.59)

Specificity= 36/68*100= 52.9% (95% Confidence Interval=0.0 – 17.59)

Positive Predictive Value=0.0% (95% Confidence Interval=41.24 – 64.33)

Negative Predictive Value= 36/54*100= 66.7% (95% Confidence Interval=53.36 – 77.76)

3.5 Discussion

Validation and determination of the limit of detection of the in - house developed real-time PCR assay.

We validated the use of and modified a previously described duplex real-time PCR assay to detect and differentiate *stx*$_1$ (melting temperature=58.2℃) and *stx*$_2$ (melting temperature=65.3℃) from overnight broth enrichment cultures with a LOD of 5.3 target copies/ul broth, and compared findings to those of culture-based CHROMagar™STEC. The limit of detection of the in-house developed assay for *stx* targets in meat and water were 116 ±4.03 CFU per 25g of meat and 144 ±6.4 CFU/100ml of Lotus River surface water for both targets. The in-house developed hybridisation probe based assay is more sensitive than CHROMagar™STEC (sensitivity and specificity of 35.7% and 77.4, 5.3% and 66.7%, 0% and 52.9% for screening stool, Lotus River surface water and meat from the informal slaughter setting respectively).This is in agreement with previously published findings.[26]

The performance of CHROMagar™STEC in comparison to the in – house developed real-time PCR assay for detection of stx genes in stool, water and meat.

Compared to the validated duplex real-time PCR, CHROMagar™STEC showed a sensitivity, specificity, negative predictive value and positive predictive value of 35.7%, 77.4%, 9.4% and 95% respectively for detection of STEC in stool following TSB enrichment. Specifically, of the 53 mauve isolates, 48 were negative for *stx* genes on the real-time PCR while nine of the 14 PCR positive broths did not yield any mauve colonies when cultured on CHROMagar™STEC.

For screening of environmental samples, it showed sensitivity and specificity of 5.3% and 66.7%, 0% and 52.9% for screening Lotus River surface water and meat from the informal slaughter setting respectively.

Notably, this chromogenic media performed best at screening of stool (diagnostic accuracy=77.4%) and performed worst at screening of meat samples (diagnostic accuracy=41.86%). It showed a diagnostic accuracy of 48.4% for screening surface water for STEC. The differences in performance among the sample types could be due to the differences in the

prevalences of STEC and or tellurite resistant STEC among the different sample types. This is in agreement with previously published research.[27]

Comparison of CHROMagar™STEC performance in this setting to its performance in other contexts: This media contains chromogenic conjugates of a substrate-linked with a chromophore such that when the substrate is cleaved with an enzyme, insoluble chromophores are released giving a distinctive colour to the colonies. These media also contain selective inhibitors such as potassium tellurite (at a concentration of 2.5mg/L).[28,29] The exact chemistry of how CHROMagar™STEC works is proprietary information to the manufacturer.

For a chromogenic media to be considered for routine screening purposes, it must have high specificity so as not to waste resources on false positives.

CHROMagar™STEC has previously been used to screen for STEC from human and non-human sources. In this study, CHROMagar™STEC has sensitivity and specificity lower than has been reported in Europe, especially for clinical samples. Similar studies to evaluate these media were done in Canada, Finland, and Germany. In all these studies, higher sensitivities were shown for STEC serotypes O26, O111, O121, O145, O118, and O157 .[17,30,31] The specificity of 77.4% noted in this study was low compared to values reported in these studies that reported specificities between 95.8% and 98.9%.

For example, in comparison to a sensitivity of 89.1% and specificity of 83.7% as reported by Gouali et al.[16] Another related study performed in Canada showed a sensitivity and specificity of 85.7% and 95.8% respectively.[29]

Possible reasons for the poor performance of CHROMagar™STEC in this setting.

STEC numbers are sharply reduced in stool after one week of illness, and the Shiga toxin genes might be lost by the bacteria.[32] Therefore, it may be argued that due to delays in reporting of diarrhoea cases to a tertiary hospital, some of the mauve colonies may have lost *stx* genes or that there were no longer viable STEC in the stool. For example, one of the atypical EPEC belonged to serotype O101 which is one of the top five STEC serotypes globally.

These findings could be explained by the differences in the approaches used. For example, in this study, we did not strictly use stool from patients with bloody diarrhoea and or HUS as was the case in some of the studies conducted in Europe.[16] Instead, we screened all stools that were presented on the stool bench within the study period.

Some of the qPCR positive broths could have carried tellurite susceptible STEC strains and so were missed on CHROMagar™STEC culture. This is inferred from the fact that nine of the 14 PCR positive broths did not yield any mauve colonies.

Also, a negative *stx* PCR on a colony might indicate that the isolate carries a variant that may not be detectable using the available set of primers.[33] Whole Genome Sequencing would help to characterise further the tellurite susceptible *E. coli* that formed mauve in this study.

There is a question of how mauve a colony should be to be considered as suspect STEC. The high false positivity rate may be attributable to the various shades of mauve shown by colonies growing on this media. According to Claire Jenkins of the Public Health Laboratory in England (personal communication), the use of this media in their Laboratory was halted because of the different shades of mauve formed by *E. coli* on this media. The different shades could be attributable to the various levels of production of tellurium resistance proteins.

This media was designed for the screening of food, faeces and environmental samples for STEC. It was particularly meant to target the top six STEC serotypes globally including STEC serotypes O45, O157, O111, O121, O103 and O45. The detection of these serotypes is based on the production of tellurite resistance proteins, which varies among STEC from human and non - human sources, that enables them to grow on tellurite containing media such as CHROMagar™STEC.[27] However, not all STEC serotypes are able to produce tellurite resistance proteins. For this reason, not all STEC serotypes will form mauve colonies on this media or even grow on this media. There is need to determine the dominant STEC serotypes in this setting and then determine their ability to secrete tellurium resistance proteins.

When judging the performance of CHROMagar™STEC, it is important to remember that it was designed as a screening media to help to detect STEC in many samples. The findings of

CHROMagar™STEC screening would have to be confirmed using more sensitive and specific test such as real – time PCR.

The prevalence of stx in the clinical and environmental samples.

The prevalence of *stx* in stool samples was 6.2%. This is slightly lower than the 9% of STEC/EPEC reported in the same setting, but in the previous study, only the *eaeA* gene (as a proxy for EHEC/EPEC colonisation) was screened for in the stool DNA extracts.[34] Interestingly, an earlier study conducted at the Centre for Enteric Disease, National Institute of Communicable Disease (NICD), using STEC collected in the period 2006 – 2009, showed that of the 2378 DEC isolates screened, only 14 were EHEC (0.6%).[35] Only one of the 14 STEC belonged to serotype O157. According to STEC data collected over three years at the NICD, most of the isolates presented were from sporadic cases and not outbreak cases. Low levels of outbreak detection may be linked to the NHLS clinical microbiology diagnostic protocol according to which, DECs are not routinely screened for. The same protocol advises screening for only STEC O157:H7 and yet of the 14 STEC isolates collected over the three-year period, 2006 – 2009, only one was *E. coli* O157. Clearly,in the NICD study, non-O157 STEC serotypes O111 and O26 were dominant and may be dominant in the general population. And yet the NHLS diagnostic strategy prioritises *E. coli* O157, and this may partly explain low levels of STEC detection. The low levels of STEC infection in Africa have previously been attributed to the type of diet that is used to feed ruminants[36,37],and thus low levels of carcas contamination at slaughter and subsequent low levels of human infection. But this finding may not be relevant in the South African context because the cattle experiments only focused on *E. coli* O157 which is not dominant in South Africa.[35] So, diagnostic strategy, and the non- dominance of O157 in South Africa may play a role in the low STEC detection rates especially given the high number of typical HUS cases reported among children.[11]

Of the sixty-four water sample broths, 19 (29.7%, 95% Confidence Interval= 18.91%-42.42%) tested positive for the *stx*$_1$ using the in-house developed qPCR. This is higher than the 15% detected in the Berg River system (2015) in South Africa.[38] An earlier study that tested South Africa water sources (Eastern Cape) intended for direct and indirect consumption showed that they had no *E.*

coli O157:H7; but this study never tested for the non-O157 STEC.[39] A similar study conducted in the Eastern Cape showed a 25.5% prevalence of *E. coli* O157:H7 in the surface water.[40]

Eighteen of the 86 meat sample broths (21.2%, 95% Confidence Interval= 13.06%-31.39%) from the informal slaughter setting possessed *stx* genes on testing with the in-house developed qPCR. Previous reports conducted in South Africa, but mainly in the Eastern Cape, have reported a prevalence of 1.4%, 88.42%, 2.8%, and 35% for STEC in swine faecal samples (2016)[41], cattle faeces (2015)[42], meat/meat products (2009)[43], and raw meat (2008)[40] respectively. However, most of these have only targeted *E. coli* O157:H7 and did not attempt to isolate the non-O157 STEC.

Previous research done in some African countries, which targeted *E. coli* O157 resulted in no isolation of this pathogen. This was the case in Uganda[44], Burkina Faso[45], and the Central Africa Republic[46]. *E. coli* O157 was however successfully isolated from shellfish and meat in separate studies conducted in Morocco[47] (prevalence of 1.9% in meat samples) and Tunisia (prevalence of 1.2% in meat)[48] respectively. Another study conducted in Egypt[49] showed the presence of both O157 and non-O157 STEC in Egypt (prevalence of 13.8% in beef). In Namibia[50], another Southern Africa country, *E. coli* O157 was detected in meats of different livestock (prevalence of 1% – 20%).

Characteristics of the E. coli strains that formed mauve colonies on CHROMagar™STEC.

Among the 53 isolates that formed mauve colonies on CHROMagar™STEC, five were STEC (two serotype O26 and non-typeable), four were EAggEC (serotype O104), two were atypical EPEC (serotype O101 and non-typeable) while two were typical EPEC (serotype O55). This means that even though CHROMagar™STEC is intended for STEC culture, we detected other *E. coli* pathotypes are presenting as mauve colonies on this media. There is the possibility of hybrid strains losing *stx* genes. Notably, bacteriophages carrying the *stx* genes are very quickly lost both in vivo and in vitro[51]; and not all *stx* primers can detect all the *stx* gene variants.[33] Notably, serotypes O26 and O104 are among the top six STEC serotypes globally.[52,53] The high carriage rate of *eae* genes among isolates from TSB enrichment with *stx* genes could indicate increased pathogenic potential since possession of the genes by STEC has been associated with more severe disease[54]. None of the 53 *E. coli* isolates that were screened by immunochromatography was

positive for Shiga toxins. Firstly, not all of these tested positive for *stx* using the primers we utilised in this study. Secondly, even if they possessed the genes, not all the isolates may be expressing these genes as sometimes it may require exposure to antibiotics such as polymyxin B to induce the isolates to secrete toxin in-vitro. There is no evidence to suggest that these isolates were secreting toxin at the point they were isolated from the patients. We did not collect accompanying clinical data.

Limitations of this study

The evaluation of CHROMagar™STEC was carried out over a short period and on a limited number of samples. Some of the stx variants may have been missed due to a mismatch with the primers available.

3.6 Conclusions

CHROMagar™STEC can only be used as a supplementary to molecular tests such as PCR or detection of the toxin using enzyme immunoassays or immunochromatography. The integrated surveillance approach should occasionally be carried out to monitor the non-human sources of STEC.

3.7 References

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Chapter Four

4 Undiagnosed Shiga-toxin-producing *E. coli* and other diarrheic *E. coli* in stool processed at a tertiary referral hospital, Cape Town, South Africa.

4.1 Summary

Introduction: In the previous chapter, the performance of CHROMagar™STEC was assessed in comparison to an in - house validated real-time PCR assay. In this chapter, it was purposed to:

i. Determine the prevalence and pathotypes of tellurite resistant diarrheic *E. coli* isolated on CHROMagar™STEC from stool samples submitted to the National Health Laboratory Sciences, Groote Schuur Hospital.
ii. To characterize the tellurite resistant STEC and other diarrheic *E. coli* isolated from stool by determining their serotypes, antibiotic susceptibility patterns, and sorbitol fermentation ability.

This would inform diagnostic policy and provide baseline information to physicians on the prevalence of tellurite-resistant STEC and other diarrheic *E. coli* in stool samples submitted to the National Health Laboratory Services at Groote Schuur Hospital.

Methods: We tested all the stool samples presented to the stool culture bench at the National Health Laboratory Sciences, Groote Schuur Hospital between September 2014 and May 2015. We used CHROMagar™STEC with and without enrichment to isolate tellurite resistant *E. coli* strains, instead of the routinely used National Health Laboratory Services (NHLS) protocol involving the use Sorbitol MacConkey. Confirmed *E. coli* were tested for possession of the *aat*, *daaC*, *eaeA*, *ipa*, *bfp*, stx_1 and stx_2 using gel-based PCR and real – time PCR. All the *E. coli* strains that possessed virulence genes were then serotyped, tested for the ability to ferment sorbitol by culture on sorbitol MacConkey, and tested for Shiga toxin production by immunochromatography.

Results: The prevalence of tellurite resistant diarrheic *E. coli* in stool at the Groote Schuur Hospital was 5% (33/733, 95% Confidence Interval=3.2 – 6.2). Of the tellurite resistant diarrheic *E. coli* isolated, 15% (5/33, 95% CI=6.7 – 30.1) were STEC, 45% (15/33, 95%CI=29.84 – 62.01) were EAggEC, 18% (6/33, 95%CI=8.6 – 34.4) were atypical EPEC, 15% (5/33, 95% CI=6.7 – 30.1) were typical EPEC, and 3% (1/33, 95%CI=0.54 – 15.32) were DAEC.

Of the 33 diarrheic *E. coli* isolated, 45% (15/33, 95%CI=29.84 – 62.01) were non - sorbitol fermenting. The five STEC strains isolated belonged to serotype O186 and O101. Serotypes O104

(5/33, 15%) and 055 (6/33, 18%) were the dominant diarrheic *E. coli* serotypes in this setting. Of the 33 tellurite resistant diarrheic *E. coli* strains, 55% (95% Confidence Interval= 40.8 – 72.7) showed resistance to ampicillin.

***Conclusions*:** Culture on CHROMagar™STEC coupled with enrichment enabled detection of STEC plus other pathotypes that would have been missed by commonly used methods (sorbitol MacConkey). Further studies are needed to determine the proportion of STEC and other diarrheic *E. coli* in our setting which were tellurite-susceptible and might have been missed using CHROMagar™STEC.

4.2 Background

STEC are important causes of diarrhoea, haemorrhagic colitis, bloody diarrhoea, and haemolytic uremic syndrome (HUS).[1] HUS is characterised by acute renal failure, haemolytic anaemia, and neurological complications in about 10% of the cases especially the young and the elderly. The top STEC serotypes that are most commonly associated with severe disease globally include O26, O45, O103, O111, O121, O145 and O157.[2,3] According to a study conducted by the National Institute of Communicable Disease (NICD), over a three-year period from 2006 to 2009, the dominant STEC serotypes were the non – sorbitol fermenting STEC serotypes O26 and O111.[4] In the NICD study, only one of the 14 STEC isolated belonged to serotype O157:H7.[4] This means that STEC O157:H7 may not be the dominant non – sorbitol fermenting STEC in this setting.

STEC isolates that carry the *stx*$_2$ genes have been commonly associated with severe disease; and include the following variants; *stx*$_{2a}$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2e}$, *stx*$_{2f}$ and *stx*$_{2g}$ with *stx*$_{2a}$, *stx*$_{2c}$ and *stx*$_{2d}$ more frequently associated with severe clinical outcome.[5,6] In another related study that used Whole Genome Sequencing (WGS), strains that harboured *stx*$_{2a}$ were most commonly found to have multiple alleles (more than one copy of stx$_{2a}$ in the same genome) of the same subtype.[7] Genes *stx*$_{2d}$ and *stx*$_{2c}$ have often been associated with post infection HUS with *stx*$_{2c}$ being activated by the presence of intestinal mucus and urease.[8] As a result of interchange of genes among traditional diarrheic *E.coli*, new virulence genes have led to the creation of new STEC strains. For example, the hybrid EAHEC (enteroaggregative Haemolytic *E. coli*) outbreak strain O104: H11 in Germany that carried both *aat* and *stx* genes harboured a combination of virulence traits derived from two different ancestors; one enteroaggregative (EAggEC) and the other STEC. This hybrid carried the *AAF* genes for the Enteroaggregative *E. coli* (EAggEC) and the *stx* phage which is characteristic of STEC O157: H7 but it lacked the Locus of enterocyte effacement which contains the eae gene that STEC O157:H7 commonly carries.[9] Another emerging *E. coli* pathotype, EAHEC O104: H4, is endemic in Central Africa and has spread to Europe but its prevalence in South Africa is not known.[10]

Failed laboratory detection of STEC

Criteria for specimen selection for STEC testing may hinder STEC diagnosis. For example, many infections are missed due to testing only children, only those presenting with bloody diarrhoea (blood and or white blood cells in the stool) or only testing specimen submitted in the summer months.[11] This approach is questionable because STEC has been isolated from patients with non-bloody diarrhoea and some stools of STEC-infected patients do not contain white blood cells.[12,13]

As regards the selection of patients based on age, despite the fact that most of the cases have been reported in children, a significant proportion has been reported in cases older than 12 years.[13] Additionally, studies have reported the occurrence of infections throughout the year and not only during the summer months.[12]

Furthermore, bacteriophages carrying the *stx* genes are very quickly lost both in vivo, and in-vitro [14]; and not all *stx* primers can detect all the *stx* gene variants.[15] For these reasons, stools should be tested as early as possible in the course of illness, as it becomes less likely to detect them in stool after one week of the disease.[16]

Testing algorithms in well-resourced countries recommend an initial screening by use of selective media for *E. coli* O157: H7 as well use of immunoassays on overnight broth for Shiga toxin for all stool specimen submitted to the diagnostic laboratory.[11,17] The use of sorbitol MacConkey to screen for O157:H7, as is the case in many laboratories within the NHLS South Africa, has limitations due to the inability to detect emerging sorbitol-fermenting non-O157 STEC and the sorbitol-fermenting O157 strains. Indeed, new chromogenic media, for example, CHROMagar™STEC have been developed to detect both O157 and the non-O157 STEC with some targeting specific STEC serotypes.[18] CHROMagar™STEC is a screening media which selects for tellurite resistant *E. coli* strains.[18] Per the manufacturer, the common STEC serotypes form mauve colonies, other Enterobacteriaceae form blue or colourless colonies, while gram-positive bacteria are completely inhibited.[19] In order to isolate the tellurite resistant diarrheic *E. coli*, the mauve colonies are picked. Also, earlier research has shown that this media allows growth of

other diarrheic *E. coli* pathotypes other than STEC.[20] The chemistry behind the formation of the mauve coloured colonies is proprietary information.

Routine STEC screening at the National Health Laboratory Services, Groote Schuur Hospital

Groote Schuur is a referral (for primary or secondary health facilities) tertiary academic hospital that was opened in 1938 and provides tertiary and quaternary care for patients of the Western Cape and beyond.[21] In NHLS laboratories at Groote Schuur, stool specimens are not routinely screened for entero-haemorrhagic *E. coli*. Physicians are advised to contact the laboratory within 7 days if there is a clinical suspicion of EHEC infection or haemolytic-uraemic syndrome.[22] On specific request from a physician, screening is done using MacConkey –sorbitol and suspicious colonies forwarded to NICD for further identification and typing. Other diarrheic *E. coli* are not routinely screened for in the Groote Schuur laboratory (this practice is variable at different laboratories), unlike the other bacterial causes of infectious diarrhoea such as *Salmonella* and *Shigella*. The problem with this approach is that there are over 450 non-O157 STEC serotypes that have been associated with clinical disease globally[23] some of which are also non-sorbitol fermenting, for example, O26 and O104. Based on research done at the NICD considering STEC isolates collected between 2006 and 2009, the dominant STEC serotypes in S. Africa belonged to serotypes O26 and O111.

4.2.1 Aims and objectives

Given the many avenues through which we might fail to detect STEC and other diarrheic *E. coli* in the laboratory in this setting, we employed a multi-pronged approach to the diagnosis of STEC using culture (on CHROMagar™STEC), PCR (real-time and gel-based) and Shiga toxin detection by immunochromatography. CHROMagar™STEC was used instead of sorbitol MacConkey for primary isolation of STEC with and without enrichment in Tryptic Soy Broth (TSB). Since CHROMagar™STEC selects for only the tellurite resistant *E. coli*, and yet not all STEC are tellurite resistant, we don't report on the tellurite susceptible STEC in this study. Since the current STEC diagnostic strategy is based on non-sorbitol fermentation, we also sought to determine the prevalence of tellurite resistant non-sorbitol fermenting diarrheic *E. coli* in this setting. Even

though diarrhoea is not commonly treated using antibiotics, antibiotic profiles of diarrheic *E. coli* can be utilised for sentinel surveillance for antimicrobial resistance. This is because *E. coli* easily acquire and transfer antimicrobial resistance genes to transient pathogens in the gastro-intestinal tract. Even though diarrheic *E. coli* infections are usually self-limiting, persistent and invasive infections, especially in the immunocompromised persons, may necessitate the use of antibiotics.

Aim 1: Determine the prevalence and pathotypes of tellurite resistant diarrheic *E. coli* identified from CHROMagar™STEC culture of stool submitted to the NHLS, Groote Schuur Hospital, Cape Town.

Aim 2: Characterise the tellurite resistant STEC and other diarrheic *E. coli* isolated from stool by determining their serotypes, antibiotic susceptibility patterns, and sorbitol fermentation ability.

4.3 Methods

4.3.1 Sample collection and processing

In tandem with the routine stool processing in the clinical microbiology laboratory, Groote Schuur Hospital, we tested the residual stool sample. All stool samples from diarrhoea patients that were submitted to the NHLS for testing between September 2014 and May 2015 were included in this study irrespective of age. Some of the stool specimens (collected between September 2014 and November 2014) were directly streaked onto CHROMagar™STEC while others (collected between December 2014 and May 2015) were enriched in TSB (TSB; Oxoid, Basingstoke, U.K) before streaking on CHROMagar™STEC and were not directly plated.

Direct Streaking: Stool specimens received between September and November 2014 were directly streaked (A sterile loop was used to pick and streak a small piece of the stool sample) onto CHROMagar™STEC (CHROMagar, Paris) and incubated for 24h at 37°C. Mauve colonies were identified by visualisation under white light and considered suspect STEC. A maximum of five mauve colonies (depending on the number of mauve colonies formed) were selected from each of the culture plates which showed growth of a mauve colony. The mauve colonies were then streaked onto MacConkey agar with crystal violet (NHLS, Green point media) and sorbitol MacConkey (NHLS, Green point media) and incubated at 37°C for 18h.

Enrichment before streaking: From December 2014 to May 2015, stool samples that were presented to the stool culture bench at the clinical microbiology laboratory were processed. After the routine stool testing the residual sample was used in this study. .A pea-sized amount of stool was inoculated in 10ml of TSB and incubated for 24h at 37℃. A loopful of broth was then streaked on CHROMagar™STEC and incubated at 37℃ for 24h. Following incubation, plates were viewed under a light source and observed for mauve colonies. A single mauve colony was picked from 53 culture plates which showed mauve growth (**Figure 4:1**). The mauve colonies were then streaked onto MacConkey agar with crystal violet (NHLS, Green point media) and sorbitol MacConkey (NHLS, Green point media) and incubated at 37°C for 18h.

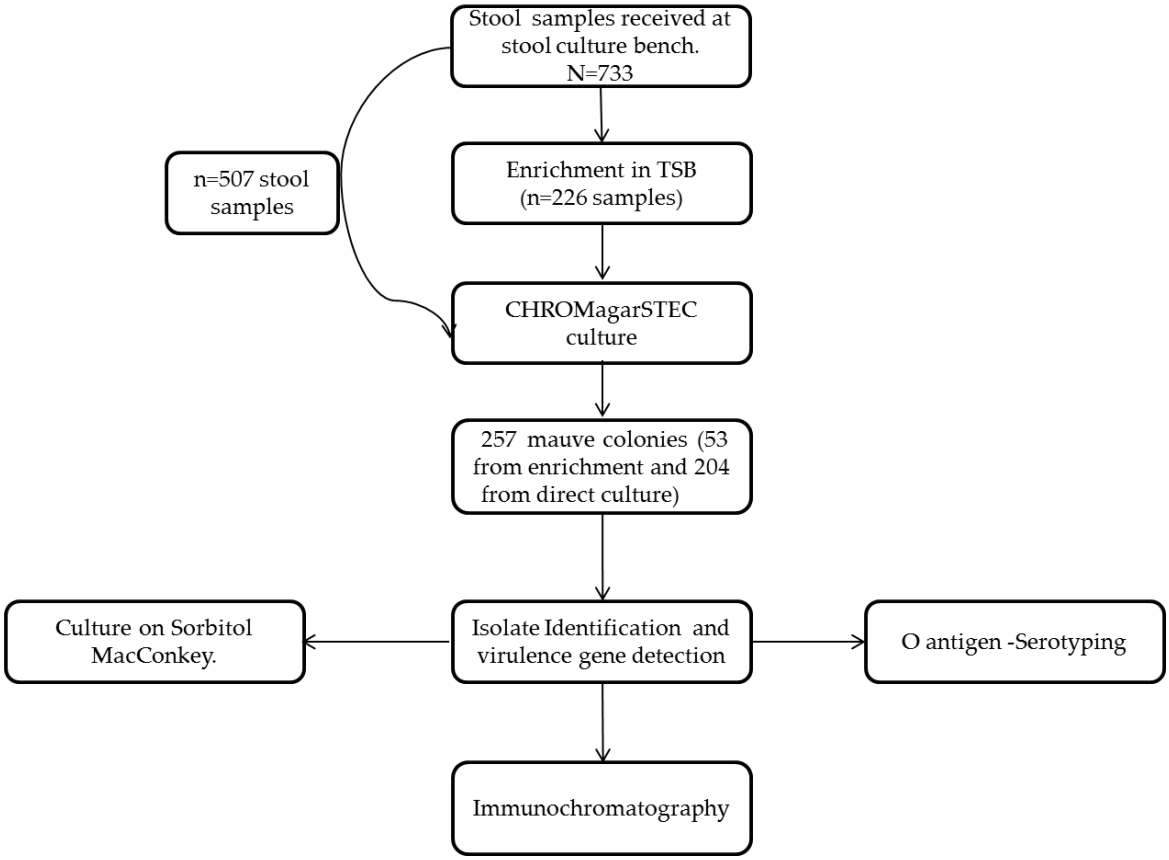

Figure 4:1 Summary of stool processing workflow indicating the samples directly streaked onto CHROMagar™STEC, and those streaked after enrichment in TSB.

4.3.2 Isolate Characterization

This was done as described in **3.3.5**. Briefly, the isolates identified as *E. coli* were subjected to *stx* characterization employing the in-house developed real-time PCR assay as well as a conventional PCR performed as previously described by Paton and Paton (1998).[24] The presence of virulence genes including the fimbrial adhesion gene for diffusely adherent *E. coli* (*daa*C), the anti-aggregation protein transporter gene for enteroaggregative *E. coli* (*aat*), heat-stable (*ST*) and heat-labile (*LT*) enterotoxin genes of enterotoxigenic *E. coli* (ETEC), the invasive plasmid antigen gene for enteroinvasive *E. coli* (*ipa*) and the intimin coding gene (*eae*) for EPEC were determined using standard gel-based PCR as previously described.[24]

The Immunocard STAT!® EHEC was used to detect Shiga-toxin 1 and Shiga-toxin 2 by the isolates employing immunochromatography with toxin-directed monoclonal antibodies labelled with

red-coloured gold particles (Meridian Biosciences, Inc., USA). We did not screen the broth for Shiga-toxin because the broth was not a suitable sample type for the Immunocard STAT! ® EHEC kit. We had already tested the broth for *stx* using the in-house validated real-time PCR. Serology using antisera (Statens Serum Institut, Copenhagen, Denmark) was done to detect *E. coli* somatic O- antigens.[25,26] H-antigen serotyping was not undertaken.

4.3.3 Antimicrobial Susceptibility Testing of the diarrheic *E. coli* using VITEK® automated system and broth microdilution.

Isolates were tested for susceptibility to 19 antibiotics following the Clinical Laboratory Standards Institute guidelines (CLSI, 2015). Since VITEK® automated system offers a limited MIC range, we further re-tested these isolates using the broth microdilution based GNX2F sensititre MIC plate (Thermo-scientific, USA) to confirm the MICs observed using VITEK®2 automated system. MIC values from broth microdilution were used as the final reference for classification as resistant, intermediate or susceptible. *E. coli* ATCC 25922 was used as the control strain. The antibiotics included in the VITEK®2 automated system panel were: ampicillin (AMP), amoxicillin – clavulanic acid (AMC), piperacillin – tazobactam (TZP), cefuroxime (CXM), cefuroxime Axetil (CXA), cefoxitin (FOX), cefotaxime (CTX), ceftazidime, (CAZ), cefepime (FEP), ertapenem (ETP), imipenem (IMP), meropenem (MEM), amikacin (AMK), gentamicin (CIN), ciprofloxacin (CIP), tigecycline (TIG), nitrofurantoin (NIT), colistin (COL), and trimethoprim – sulfamethoxazole (SXT).

The antibiotics included in the broth microdilution panel on the GNX2F sensititre plate were: amikacin, aztreonam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, colistin, doripenem, doxycycline, ertapenem, gentamicin, imipenem, levofloxacin, meropenem, minocycline, piperacillin – tazobactam, polymixin B, ticarcillin – clavulanic acid, tigecycline, tobramycin, and trimethoprim – sulfamethoxazole.

MIC data was entered in WHONET 5.6 software. Resistance to third generation cephalosporins and or carbapenems was confirmed using the ESB1F sensititre MIC plates (Thermo-scientific, USA).

4.3.4 Data analysis

Data on cultural characteristics, serotypes, and pathotypes was entered into Microsoft Office Excel 2016 (Microsoft Corporation, USA) software, coded and later exported to STATA version 12 for analysis. Further analysis was done using the R-statistical package.[27] Antimicrobial resistance data in the form of Minimum Inhibitory Concentrations (MICs) was entered into World Health Organization (WHO) antimicrobial resistance data management software called WHONET 5.6[28,29] and analysed as per the Clinical Laboratory Standards Institute (CLSI), 2016, USA guidelines and breakpoints for testing of *E. coli*.

4.4 Results

4.4.1 Isolate characteristics

Isolates from direct and indirect streaking:

Of the 733 specimens, 507 were directly inoculated on CHROMagar™STEC while 226 were first enriched in TSB before inoculation on CHROMagar™STEC. Of 733 specimens screened on tellurite-containing CHROMagar™STEC, 257 (35%) yielded mauve colonies. Of the 257 mauve colonies derived from direct and indirect streaking, 192 (75%) were lactose fermenting colonies on MacConkey agar with crystal violet. All the 192 lactose fermenters were confirmed to be *E. coli* using the VITEK® 2 automated system (bioMerieux, USA). Of these, 29% (56/192) were sorbitol fermenting.

Mauve colonies were obtained from 40% (204/507) of the directly inoculated specimens, and 24% (53/226) of stool samples that were enriched first in tryptic soy broth (p=0.001). Of the 204 mauve colonies obtained from direct culture, 12 were diarrheic *E. coli* while 21 of the 53 mauve colonies obtained following enrichment in TSB were diarrheic *E. coli*. Tellurite resistant diarrheic *E. coli* were therefore obtained from 5% (33/733) of the stool specimens.

4.4.1.1 Pathotypes and prevalence of tellurite resistant diarrheic E. coli

Real-time PCR on the 192 *E. coli* for *stx* yielded five positives. Of these five STEC isolates, three also carried the *eae* gene while two did not. Only one of the five STEC isolates carried both the stx_1 and stx_2 genes while four carried only the stx_1 gene. All the five STEC were obtained after enrichment in TSB. Of the 187 *stx*-negative *E. coli*, 11 (6%) carried *eae* genes, 15 (8%) carried *aat*, and only one (1%) possessed the *daaC* gene. Of the 11 *eae* positive isolates, five also had the *bfp* genes and were typical EPEC. The other six *eae* positive isolates did not possess the *bfp* genes and were classified as atypical EPEC. A total of 33 tellurite resistant diarrheic *E. coli* were therefore obtained.

Of the 33 tellurite resistant diarrheic *E. coli* , 64% (21/33) were obtained following enrichment in TSB while 36% (12/33) were obtained without enrichment (p=0.004). Of all the 33 diarrheic *E.coli* strains isolated on CHROMagar™STEC, 15% (5/33) were STEC; 45% (15/33) were EAggEC, 18%

(6/33) were atypical EPEC, 15.2% (5/33) were typical EPEC, and 3% (1/33) were DAEC. No Enteroinvasive *E. coli* (*E. coli*) were detected among the DEC pathotypes (**Figure 4:2**).

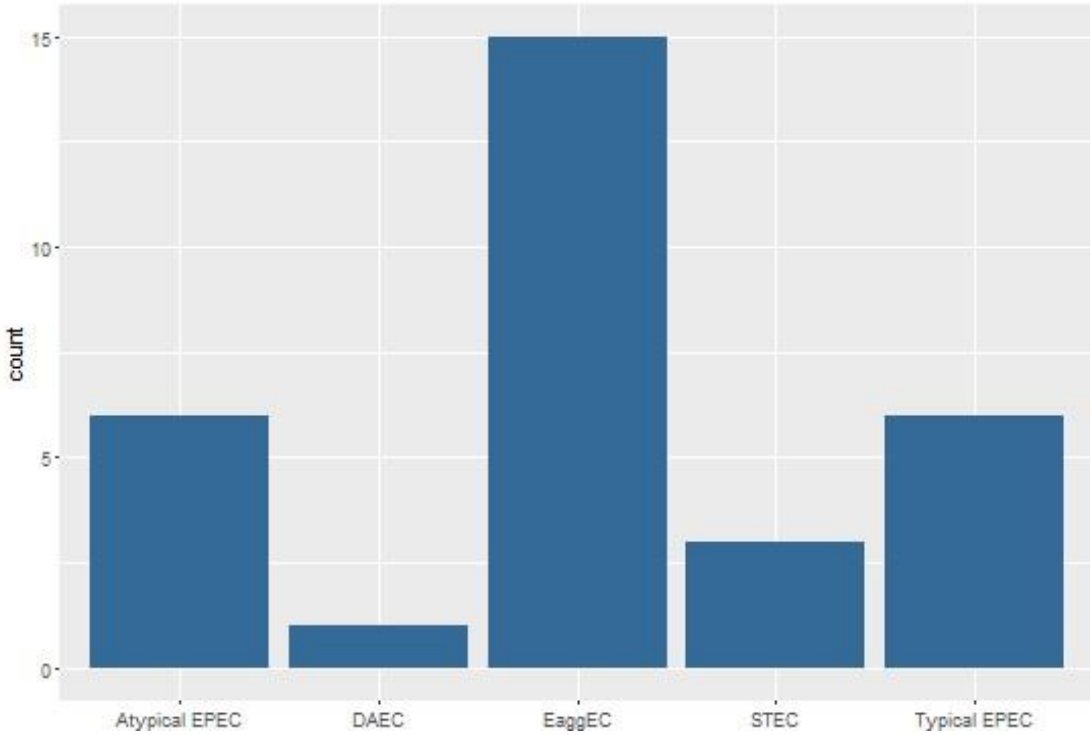

Figure 4:2 Bar graph showing the number of each of the diarrheic E. coli pathotypes isolated on CHROMagar™STEC from stool.

Shiga toxin production: Of the 33 diarrheic *E. coli* strains, two isolates that also tested positive for *stx*, tested positive for Shiga-toxin production on immunochromatography (the three STEC that tested positive for *stx* genes by PCR were negative for Shiga-toxin production on immunochromatography) with the formation of a band indicating the detection of Shiga toxin 1. All isolates showed the presence of the control band which was a marker for the validity of the test (**Figure 4:3**).

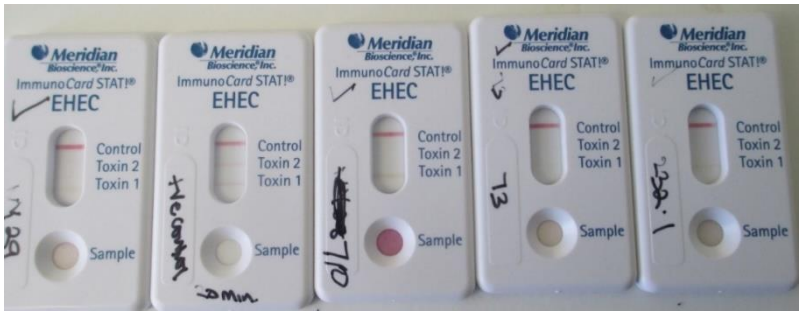

Figure 4:3 Images for the ImmunoCardSTAT® test panels showing the Shiga-toxin1 bands for the isolates that tested positive for Shiga-toxin 1 by immunochromatography

Isolates NY29, 710, 73, and 232.1 were positive for Shiga toxin 1 production. The upper red band in the test well is the positive control which should show in all valid tests. The positive control test (2nd from the left) showed both the Shiga toxin 1 and Shiga toxin 2 bands.

4.4.1.2 Serotypes

Of the five STEC serotypes, two belonged to serotype O186, while one belonged to serotype O101. The other two STEC were non-typeable (NT).

Of the six atypical EPEC isolates, two belonged to serotype O26, one of each belonged to serotypes O25, O182, O111, and one was not typeable. All the five typical EPEC belonged to serotype O55. Of the 15 EAggEC, five belonged to serotype O104; four belonged to serotype O175, while one of each belonged to serotypes O3, O8, O9, O16, O25, and O33.

Considering all the 33 diarrheic *E. coli* isolated on CHROMagar™STEC, **t**here were altogether 16 different serotypes. These included: O3, O104, O16, O25, O9, O8, O175, O101, O111, 055, 026, 0182, O186, O9, O33 and NT (Non - Typeable). **S**erotypes O104 (5/33, 15%) and O55 (6/33, 18%) were dominant serotypes among the tellurite resistant diarrheic *E. coli* isolated on CHROMagar™STEC (**Figure 4:4**). All the *E. coli* isolates that belonged to serotypes O104, and O55 were EAggEC and EPEC respectively.

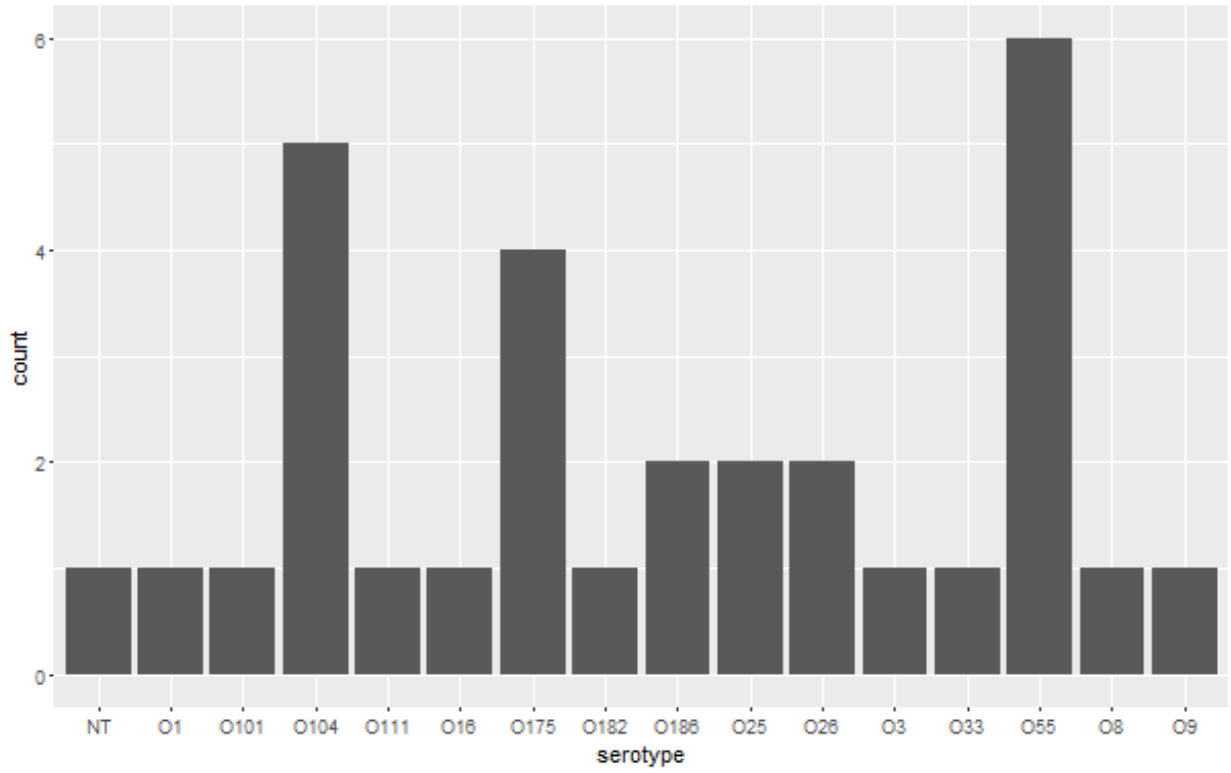

Figure 4:4 Bar graph showing the serotype distribution of diarrheic E. coli serotypes isolated from the stool with and without enrichment on CHROMagar™STEC.

'NT' means non-typeable, the isolate could not be serotyped using the available antisera as used at the Centre for Enteric Disease, National Institute of Communicable Disease, Johannesburg.

4.4.2 Sorbitol fermentation property of diarrheic *E. coli*

Of the 33 tellurite - resistant diarrheic *E. coli* isolated on CHROMagar™STEC, 45% (15/33) were non – sorbitol fermenting. Of the 15, seven were EPEC while seven were EAggEC (**Table 4.1**). Of the five STEC isolates, only one was non-sorbitol fermenting.

Table 4.1 Growth and Virulence properties of diarrheic E. coli that formed Mauve colonies on CHROMagar™STEC

Isolate No.	Bloody diarrhea	Serotype	Virulence genes.	Pathotype	Immunoassay	TSB enrichment	Sorbitol
271	-	O55	*eae, bfp*	Typical EPEC	-	-	-
722.2	-	O55	*eae, bfp*	Typical EPEC	-	+	-
722.1	-	O55	*eae ,bfp*	Typical EPEC	-	+	-
279	-	O55	*eae, bfp*	Typical EPEC	-	-	-
344	-	O55	*eae, bfp*	Typical EPEC	-	+	-
345.1	-	O55	*eae, bfp*	Typical EPEC	-	+	+
291	-	O182	*eae*	Atypical EPEC	-	-	+
286	-	O111	*eae*	Atypical EPEC	-	+	+
600	+	NT	*eae*	Atypical EPEC	-	+	+
15.1	+	O26	*eae*	Atypical EPEC	-	-	-
284	-	O25	*eae*	Atypical EPEC	-	-	+
15.2	+	O26	*eae*	Atypical EPEC	-	-	-
63.2	-	O1	*daaC*	DAEC	-	-	+
689	-	O175	*aat*	EAggEC	-	+	-
326	-	O16	*aat*	EAggEC	-	+	-
229.2	-	O104	*aat*	EAggEC	-	-	-
229.1	-	O104	*aat*	EAggEC	-	-	-
473	-	O33	*aat*	EAggEC	-	+	+
371.1	+	O25	*aat*	EAggEC	-	+	-
336	-	O175	*aat*	EAggEC	-	+	+
229	-	O104	*aat*	EAggEC	-	-	-
733	-	O104	*aat*	EAggEC	-	+	-
502	-	O9	*aat*	EAggEC	-	+	+
688	-	O3	*aat*	EAggEC	-	+	+
480	-	O8	*aat*	EAggEC	-	+	+
250	-	O175	*aat*	EAggEC	-	-	+
207	-	O104	*aat*	EAggEC	-	-	+
696	-	O175	*aat*	EAggEC	-	+	+
29.4	-	O186	*stx_1, eae*	STEC	-	+	+
29.5	-	O186	*eae, stx_1*	STEC	-	+	+
602	-	O101	*eae, stx_1, stx_2*	STEC	-	+	-
232.1*	-	NT	*stx_1*	STEC	Shiga toxin 1	+	+
73*	-	NT	*stx_1*	STEC	Shiga toxin 1	+	+

EAggEC-enteroaggregative *E. coli*, STEC-Shiga-toxin producing *E. coli*, EPEC-enteropathogenic *E. coli*, DAEC-diffusely adherent *E. coli*, NT-non typeable.

4.4.3 Antibiotic Susceptibility of STEC and other DECs from stool in GSH

There was a 100% agreement between the MIC data we obtained from using the VITEK® 2 automated system and the broth microdilution method.

Overall, of the thirty-three diarrheic *E. coli* we isolated from stool in GSH with and without enrichment, 55% (18/33) were resistant to ampicillin (AMP), 3% (1/33) to tazobactam – piperacillin (TZP), 6% (2/33) to cefuroxime (CXM), 3% (1/33) to cefoxitin (FOX), 21% (7/33) to nitrofurantoin (NIT), 9% (3/33) to cefuroxime-axetil (CXA), 3% (1/33) to tigecycline (TGC) and 3% (1/33) to ciprofloxacin (CIP) All the isolates in this study were resistant to trimethoprim – sulphamethoxazole (SXT) while none was resistant to a third-generation cephalosporin (**Figure 4:5**). The cluster of five atypical EPEC that belonged to serotype O55 had distinct antimicrobial resistance patterns despite being of the same serotype. The cluster of five serotype O104 EAggEC strains were noted within a collection period of 38 days, and had similar antimicrobial susceptibility patterns (Resistance to only SXT).

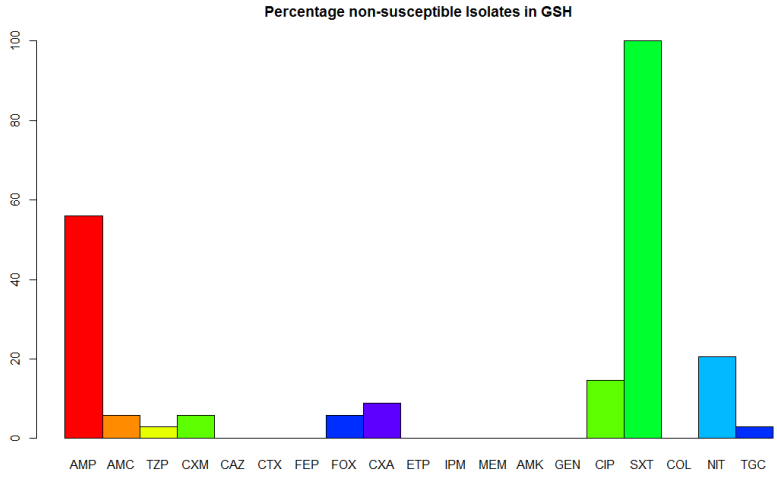

Figure 4:5 Bar graph showing the percentage of resistant isolates to the different antibiotics.

Only three isolates were Multidrug-resistant, one of which (EAggEC, serotype O25) was resistant to six antibiotics while the atypical (serotype O182) and typical EPEC (serotype O55) were resistant to three and four antibiotics respectively. The commonest resistance profile involved

resistance to only ampicillin (**Table 4.2**). All the STEC were susceptible to all the antibiotics included in this study except SXT.

Table 4.2 Resistance profiles of Diarrheic E. coli obtained with and without Tryptic Soy Broth (TSB) enrichment.

Isolate ID	Serotype	Resistance Profile	Pathotype
336	O175	AMP	EAggEC
279	O55	AMP	Typical EPEC
480	O8	AMP	EAggEC
696	O175	AMP	EAggEC
688	O3	AMP	EAggEC
326	O16	AMP	EAggEC
271	O55	AMP AMC CXA	Typical EPEC
291	0182	AMP CXM CXA FOX	atypical EPEC
371.1	O25	AMP AMC TZP CXM CXA FOX	EAggEC

4.5 Discussion

The main findings of this study were; (1) Five STEC, twelve EPEC, one DAEC, and fifteen EPEC strains were missed using the routine laboratory diagnostic strategy at the GSH, (2) All the diarrheic *E. coli* isolated in this study were resistant to trimethoprim-sulphamethoxazole while 55% (18/33) were resistant to ampicillin.

Prevalence of tellurite resistant diarrheic E. coli in stool

The inhibitory action of potassium tellurite on coliforms was first reported by Fleming in 1940.[30] Tellurite-containing media have been routinely used to screen for STEC[31], but not the other pathotypes. This is because the commonly reported STEC serotypes have a similar characteristic of being tellurite resistant. However, Hirvonen *et* al., 2012 reported the formation of mauve colonies by other tellurite resistant diarrheic *E. coli* on this medium.[20] Of the 733 stool samples processed in this study, 33 (5%) were tellurite-resistant diarrheic *E. coli*. However, tellurite media have limited ability to select for STEC. In a study conducted on STEC from human, animals and food in Austria, the prevalence of tellurite resistance amongst STEC was 26% - therefore 74% of the STEC would not be detected by the tellurite-containing medium.[32] Tellurite resistance is associated with high virulence and antimicrobial resistance among diarrheic *E. coli*.[32]

Recovery of *E. coli* from tellurite-containing solid media may select for strains carrying tellurite resistance determinants on plasmids (*IncHI1*,*IncHI2, IncP*) that are known to harbour multiple antibiotic resistance determinants.[33,34] Commercial selective media such as CHROMagar™STEC and Sorbitol MacConkey with cefixime and potassium - tellurite use this property to select STEC O157:H7 and other pathogenic STEC. This is the first study documenting the prevalence of tellurite resistant STEC in South Africa. There was a relatively low prevalence of tellurite-resistant diarrheic *E. coli* in stool samples submitted to NHLS, Groote Schuur Hospital. We were unable to determine the prevalence of non-tellurite-resistant STEC in this study.

Missed laboratory detection and characteristics of STEC

Five STEC were detected in this study. All the five were detected after enrichment in TSB, and none was obtained by directly streaking on CHROMagar™STEC. Overall, the number of

diarrheic *E. coli* obtained after enrichment in TSB was significantly higher than the number obtained without (p=0.004). This may suggest that enrichment in TSB may be a better method of recovering STEC (and other pathotypes) from a stool sample. The STEC diagnostic strategy employed by the NHLS clinical laboratory at the Groote Schuur Hospital targets only the O157 STEC. It is based on the non-sorbitol fermenting attribute of STEC O157:H7. However, not all the *E. coli* O157 are non-sorbitol fermenting. Therefore, this strategy would miss the non-O157 STEC and sorbitol-fermenting O157: NM (non-motile) (O157 STEC with no H-antigen). This would have public health implications in S. Africa because the 1990 STEC outbreak was caused by STEC O157 :NM.[35] Therefore, the five non- O157 STEC that we report in this study would have been missed. The low rate of STEC isolation could be due to a number of reasons: (1) loss of *stx* genes, since patients with diarrhoea may have had the condition for more than a week by the time they are referred from a primary or secondary health care centre to Groote Schuur hospital. (2) Criteria for specimen selection for STEC testing may hinder STEC diagnosis. For example, many infections are missed due to testing only children, only those presenting with bloody diarrhoea (blood and or white blood cells in the stool) or only testing specimen submitted in the summer months.[11] (3) Not all *stx* primers can detect all the *stx* gene variants.[15] (4) Low prevalence of STEC in stool samples processed especially if the stool specimens are not from patients presenting with bloody diarrhoea, or HUS. There are higher chances of recovering STEC from the stool of patients with bloody diarrhoea or HUS as opposed to those without.[36] In South Africa, like in many African countries, there may be a lower prevalence of STEC as compared to America and Europe. This has been thought to be related to the type of diet given to ruminants in America and Europe that favour the proliferation of STEC in cattle. This STEC later gets disseminated to the human population via the fecal-oral route.[37]

It is important to detect STEC in stool because the use of antibiotics such as the quinolones leads to bacterial lysis thus increasing the chances of HUS in affected patients.[38] Four of the five STEC detected in this study carried the stx_1 genes. Possession of stx_2 genes has been associated with the more severe form of illness.[5,6] Also, we detected *eae* in three of the five STEC, while two did not possess the *eae* gene. The *eae* gene which is located in the locus for enterocyte effacement (LEE) codes for the intimin protein which is necessary for the formation of attaching-effacing (A/E)

lesions in the intestinal tract.[39] STEC that carries *eae* (LEE-positive STEC) has been shown to cause more severe disease as compared to STEC without any attachment protein (LEE-negative STEC). In the two STEC where we did not detect the *eae* gene, it could be due to the two strains carrying a different variant of *eae* other than *eaeA* that was targeted in this study.

Serotypes of STEC

Per the manufacturer of CHROMagar™STEC, the commonly encountered STEC serotypes should form mauve coloured colonies on this medium. However, the serotypes categorised as "common" (O157, O26, O45, O145, O111, O121, and O103) were categorised as such, based on studies conducted in developed countries. This is partly because there have been fewer reports of STEC in Africa as opposed to America and Europe. In this study, STEC belonged to serotypes O101 and O186. These serotypes were not detected in an earlier study conducted at the NICD (2006 to 2009) which screened 2378 diarrheic *E. coli* isolates. The 14 STEC identified in that study belonged to serotypes O4, O5, O21, O26, O84, O111, and O157.[4]

Other related studies reported STEC serotypes O8 and O9 in pigs in South Africa[40]; while O104 was reported in humans albeit without the *stx* genes.[10] So, the serotypes of STEC reported in this study are rarely reported in South Africa.

We did not detect any *E. coli* O157:H7,possibly because some of the non-sorbitol fermenters were *E. coli* O157: H7 but without the *stx* genes; we only serotyped virulence gene positive non-sorbitol fermenting strains. The Centre for Disease Control (CDC) of the USA recommends that *E. coli* O157: H7 always be considered of public health importance even when the *stx* genes are not detected.[11] This is because the presence of this serotype could indicate a previously undetected STEC O157:H7 infection.[11] High sensitivity for the STEC serotype O157 has been previously reported for CHROMagar™STEC.[18]

Antimicrobial resistance of STEC

The STEC detected in this study were only resistant to SXT. This finding is similar to a 2011 study that was conducted in Kenya that reported a high level of resistance to SXT among STEC.[41] Contrary to other studies, for example in Mexico[42], the STEC in this study were susceptible to

ampicillin. The susceptibility of STEC to antibiotics is important because antibiotics are often used to decolonize long-term STEC carriers, and to treat invasive STEC disease[43]. However, the use of cell lysing antibiotics such as quinolones to treat invasive STEC infection has been linked with progression to HUS.[38]

Cultural characteristics of tellurite resistant STEC on sorbitol MacConkey

Sorbitol – negative STEC O157:H7 have been associated with more cases of HUS worldwide than other STEC serotypes.[44-48] However, STEC O157:H7 is not the only sorbitol – negative STEC or STEC that has been associated with HUS.[49] Others include O111, O145, O45, O26, and O104. Of the five STEC reported in this study, only one was non-sorbitol fermenting and belonged to serotype O101. Since failure to ferment sorbitol is the criteria used to screen for O157 STEC at the Groote Schuur Hospital, all the five STEC detected in this study would have been missed. This is because even the non-sorbitol fermenting non-O157 STEC would have tested negative for the O157 surface antigen by latex agglutination.

Shiga-toxin production by STEC

Using immunochromatography, we confirmed Shiga-toxin production by two strains which both carried the *stx*$_1$ genes and produced Shiga toxin 1. The other three *stx* gene-positive isolates tested negative for Shiga-toxin production. Despite possessing the stx genes, they were not expressed in the three strains. The production of Shiga toxin 2 by STEC has been associated with more severe illness[5,6] since Shiga-toxin 2 is 1000 times more potent than Shiga-toxin 1.[6]

Missed laboratory detection and characteristics of EAggEC, EPEC, and DAEC.

At the Groote Schuur Hospital, stool from diarrhoea patients is not routinely screened for diarrheic *E. coli*. The current protocol stipulates the use of sorbitol MacConkey to screen for only the non-sorbitol fermenting O157 STEC. The other pathotypes including EAggEC, EPEC and DAEC that were isolated in this study on CHROMagar™STEC would have been missed if sorbitol MacConkey had been used instead. CHROMagar™STEC favoured the growth of the other tellurite resistant diarrheic *E. coli* pathotypes. This medium does not favour the growth of the tellurite susceptible diarrheic *E. coli,* and so those are not reported in this study. Of all the 33

diarrheic *E.coli* strains isolated on CHROMagar™STEC, 45% (15/33) were EAggEC, 18% (6/33) were atypical EPEC, 15% (5/33) were typical EPEC, and 3 % (1/33) were DAEC. No enteroinvasive *E. coli* (*E. coli*) were detected. This finding is in agreement with an earlier study which showed that EAggEC were most likely to be cultured from stool since they cause a more persistent form of diarrhoea.[10]

Serotypes of EAggEC, EPEC, and DAEC

CHROMagar™STEC favours the growth of tellurite resistant *E. coli* (forming mauve colonies) irrespective of whether they carry the *stx* genes or not. Of the 16 diarrheic *E. coli* serotypes that were isolated using CHROMagar™STEC in this study, only serotypes O111, O104 and O26 were previously reported to be detectable on this medium by studies conducted in Europe.[18,20] In this study, we report the detection of the other tellurite resistant serotypes including O16, O175, O182, O186, O25, O3, O33, O175, O8, O9, O55, and O101 on CHROMagar™STEC. The dominant tellurite resistant diarrheic *E. coli* serotypes we identified in this study were O104 (15%), and O55 (18%). The cluster of six serotype O55 EPEC had clearly distinct antimicrobial resistance patterns and so was not an outbreak cluster. Since the cluster of five serotype O104 EAggEC strains were noted within a collection period of 38 days, and had the same antimicrobial susceptibility pattern, they are possibly an outbreak cluster of EAggEC. Further molecular epidemiology testing would be needed to confirm this.

Considering the recent German outbreak caused by an enteroaggregative- haemorrhagic *E. coli* strain, it is worth further investigating the epidemiology of enteroaggregative O104 at primary health care settings, especially among children. In the wake of the German outbreak, Tau *et*. al,2011 screened all *E. coli* O104 that had been associated with human diarrhoea and were received at the NICD between 2004 and 2011, for *stx* genes.[50] These isolates lacked the stx genes. Of the seven *E. coli* O104 isolates in that study, five were EAggEC, while two were EPEC. None of the *E. coli* isolates was STEC.

Antimicrobial resistance of EAggEC, EPEC, and DAEC

It's recommended that *E. coli* be tested for susceptibility to ampicillin, nalidixic acid, ciprofloxacin, tetracycline, chloramphenicol, gentamicin, ceftriaxone, and trimethoprim – sulfamethoxazole since these antimicrobials are clinically relevant for the management of community and hospital acquired *E. coli* infections.

EAggEC, EPEC, and DAEC in this study showed resistance to SXT (100%, 28/28) and Ampicillin (64%, 18/28). These findings are similar to reports from Kenya which showed a high prevalence of resistance to SXT among intestinal *E. coli*.[51] In the Kenya study, daily prophylactic use of SXT led to corresponding resistance to SXT by the enteric *E. coli*.

Of the 15 EAggEC isolates in this study, five were resistant to ampicillin while one was resistant to six antibiotics including ampicillin. There are increasing reports of resistance to multiple antibiotics among EAggEC.[52] Altogether, three strains were multidrug resistant with one (EAggEC serotype O25) being resistant to six antibiotics, while the atypical EPEC strain (serotype O182) was resistant to four antibiotics, and the typical EPEC strain (serotype O55) resistant to three antibiotics. Even though they are few, measures need to be put in place to avoid dissemination of such strains in the clinical setting.

Cultural characteristics of tellurite resistant EAggEC, EPEC, and DAEC on sorbitol MacConkey

Of the 33 diarrheic *E. coli* isolated on CHROMagar™STEC, 15 (45%) were non-sorbitol fermenting. Of the 15 non-sorbitol fermenting diarrheic *E. coli* isolated in this study, seven were EPEC (out of 12 EPEC in total), while seven (out of 15 EAggEC in total) were EAggEC. Only one of the non-sorbitol fermenting diarrheic *E. coli* was STEC. A similar study conducted in Tanzania reported a 14% prevalence of non-sorbitol fermenting *E. coli* in 1049 human stool and non-human samples.[53] This is higher than the 2% (15/733) prevalence of non-sorbitol fermenting *E. coli* reported in this study. The high number of non-sorbitol fermenting EAggEC can be explained by the fact that serotype O104 is one of the prevalent non-sorbitol fermenting serotypes.[18]

Limitations of this study

CHROMagar™STEC only permits the growth of tellurite resistant STEC and not the tellurite susceptible strains.

This study did not have a long sampling timeframe, and not all children that presented with diarrhoea may have had stool specimens taken. Only diarrheic *E. coli* that possessed virulence genes were characterised; therefore, we might have missed strains that lost the virulence genes.

4.6 Conclusions

We suggest that a new screening approach is required to detect STEC in this setting. The use of sorbitol MacConkey at the NHLS laboratory, Groote Schuur hospital to screen for possible STEC would lead to failed detection of the non-O157 STEC and of sorbitol-fermenting O157 STEC. None of the non-sorbitol fermenting *E. coli* strains reported in this study belonged to serotype O157:H7. The use of CHROMagar™STEC coupled with an enrichment step enables detection of both O157 and non-O157 STEC in addition to allowing the detection of the other diarrheic *E. coli* pathotypes. In this study, we could detect EAggEC, EPEC, and DAEC. Further work is required to determine what proportion of STEC (and other pathotypes) are tellurite susceptible and would be missed with this strategy.

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Chapter Five

5 Proteomic comparison of three clinical diarrhoeagenic drug-resistant *Escherichia coli* isolates grown on CHROMagar™STEC media.

5.1 Summary

Introduction: Shiga-toxin-producing *Escherichia coli* (STEC) and Enteropathogenic *Escherichia coli* (EPEC) are key diarrhoea-causing foodborne pathogens.

Objective: To investigate the use of high-throughput mass spectrometry-based proteomics and transmission electron microscopy of isolates grown on CHROMagar™STEC solid media culture, to compare the proteomes and morphotypes of three clinical EPEC/STEC strains isolated from patients with diarrhoea.

Methods: Here, we used mass spectrometry-based proteomics to characterise the virulence and antimicrobial resistance protein profiles of three clinical pathogenic *E. coli* isolates (two EPEC [one of which was resistant to ciprofloxacin] and one STEC) cultured on CHROMagar™STEC solid media after minimal laboratory passage.

Results: We identified a total of 4767 unique peptides from 1630 proteins groups across all three clinical *E. coli* strains. Use of a label-free proteomic approach also allowed the identification of virulence and drug resistance proteins unique to each of the clinical isolates. The B subunit of Shiga toxin, ToxB, was uniquely detected in the STEC strain only and additionally several other virulence factors including SheA, OmpF, OmpC and OmpX were significantly more abundant in the STEC strain. The ciprofloxacin resistant EPEC isolate possessed reduced levels of key virulence proteins compared to the ciprofloxacin susceptible EPEC and STEC strains.

Conclusions: Data presented here indicates that propagation of clinical isolates on a relevant solid medium followed by mass spectrometry analysis represents a convenient means to assess in a quantitative manner virulence factors and drug resistance determinants that might other wise be lost through extensive *in vitro* passage in enteropathogenic bacteria.

5.2 Background

Infections by pathogenic intestinal *Escherichia coli* constitute a major cause of worldwide morbidity and mortality. Diarrheagenic *E. coli* outbreaks are frequent in developing countries, resulting in several thousand deaths per year[1] and enormous economic losses due to trade sanctions.[2] Additionally, there is increasing concern regarding the occurrence of diarrhoeagenic *E. coli* outbreaks in developed countries.[3-5] A recent example is the 2011 international outbreak of a Shiga-toxin-producing *E. coli* (STEC) strain (STEC O104: H4) which originated in Germany and led to a total of 3816 cases including more than fifty deaths.[6]

Different types of virulence factors have been described in STEC and Enteropathogenic *E. coli* (EPEC) which have been classified mainly into adhesion group, toxin group, and the type III secretion group. These virulence factors are encoded by over 75 genes, including genes carried on the Locus of Enterocyte Effacement (LEE) and other Pathogenicity Islands.[7] Importantly, STEC and EPEC share particular virulence factors such as the LEE pathogenicity island.[8] Intimate attachment due to intimin encoded by the LEE genes and the production of Shiga toxin leads to heavy colonisation by STEC with levels reaching 90% of all aerobic faecal flora in the intestinal tract.[9,10] However, the emergence of human infections caused by LEE-negative STEC strains indicates the presence of other pathogenicity islands or plasmids that carry additional virulence factors.[11]

Recently, the co-occurrence of antibiotic resistance and virulence of pathogenic bacteria including *E. coli* has been a matter of great concern worldwide[12], particularly in developing countries.[1] The link between bacterial antibiotic resistance and virulence remains controversial.[13] For example, previous reports have suggested reduced virulence among multi-drug resistant *E. coli* isolates relative to sensitive strains.[14] However, other authors have emphasised the fact that acquisition of antimicrobial resistance does not necessarily compromise microbial fitness.[15] Consistent with this notion, recent epidemiological data indicate that antibiotic resistance and virulence factor carriage are linked in *E. coli* populations in some community settings.[16] A related study showed that the expression of virulence factors led to the formation of an antibiotic-tolerant subpopulation[17] and that antibiotic treatment indeed may select for virulence.[18] In addition to

drug resistance, treatment failure on the use of antibiotics in a clinical setting could be due to tolerance and or persistence to antibiotics.[19]

Mass spectrometry-based proteomics has been successfully employed to explore aspects of both bacterial virulence and antimicrobial resistance.[20-22] Mass spectrometry data has led to the better understanding of EPEC and STEC pathogenesis and identification of markers for laboratory diagnoses of these pathogens.[23] Recently, Pettersen *et al.*[24], employed label-free quantitative proteomics to characterise five extraintestinal pathogenic *E. coli* strains purified from clinical blood cultures associated with sepsis and urinary tract infections. Importantly, the report included a further comparison of the *E. coli* strains cultivated in blood culture on two different solid media, revealing differential responses in strains grown in the different culture environments.[24] Here, the use of high-throughput mass spectrometry-based proteomics and transmission electron microscopy of isolates grown on CHROMagar™STEC solid media culture was investigated. Consequently, the proteomes and morphotypes of three clinical EPEC/STEC strains isolated from patients with diarrhoea were compared.

5.2.1 Aims and objectives

To investigate the use of high-throughput mass spectrometry-based proteomics and transmission electron microscopy of isolates grown on CHROMagar™STEC solid media culture, to compare the proteomes and morphotypes of three clinical EPEC/STEC strains isolated from patients with diarrhoea.

5.3 Methods

5.3.1 *E. coli* isolation, identification, and antimicrobial susceptibility testing

Stool samples were collected between June 2014 and March 2015 from patients presenting with diarrhoea at Red Cross War Memorial Children's Hospital. Specimens were analysed at the clinical microbiology laboratory at Groote Schuur Hospital and the University of Cape Town, medical microbiology laboratory. Stool samples were inoculated onto CHROMagar™STEC plates (CHROMagar, Paris, France). Discrete, strongly mauve colonies were picked and streaked out on LB (Luria-Bertani) agar and MacConkey agar with crystal violet (Green point Media, NHLS, Albertynshof) and incubated for 18h. *E. coli* was identified as lactose-positive, oxidase-negative, spot indole-positive, and pyrrolidonyl arylamidase (PYR)-negative. Identification was confirmed using the VITEK® 2 automated systems (bioMérieux, USA).

The minimum inhibitory concentration (MIC) was determined with broth microdilution using the Trek sensititre GNX2F plates (Thermo Fischer Scientific, USA). The MIC values were interpreted following the Clinical Laboratory Standards Institute guidelines for antibiotic susceptibility testing using the WHONET software version 5.6.[25]

5.3.2 Polymerase chain reaction detection of *stx*$_1$, *stx*$_2$, and *eaeA*

We selected three suspect STEC isolates (from three different stool specimen) that formed mauve colonies on CHROMagar™STEC. To determine the presence of the *eaeA* and *stx* genes, discrete colonies were streaked onto 2% blood agar and incubated for 18h at 35 ℃ before nucleic acid extraction.[26] An endpoint PCR was done to detect *eaeA* on a 2720 Thermal Cycler detection platform (Applied Biosystems, Foster City, CA).

The following primers were used

Table 5.1).

Table 5.1 Primers used for conventional PCR to detect the eaeA gene

Target gene	PCR primer	Primer sequence	PCR product
eaeA	*eaeA*-F	TCAATGCAGTTCCGTTATCAGTT	482bp
	eaeA-R	GTAAAGTCCGTTACCCCAACCTG	

To detect *stx*$_1$ and *stx*$_2$, a real-time PCR reaction was set up on a LightCycler480 II (Roche Molecular Systems, Pleasanton, CA) instrument with *stx*$_1$ and *stx*$_2$ primers at a concentration of 0.45µM, probes (Inqaba Biotec Laboratory, South Africa) as previously described[27] The following primers and probes were used (**Table 5.2**).

Table 5.2: Primers and probes used in the Real-Time PCR assay to detect stx$_1$ *and stx*$_2$ *targets.*

Primers/probes	5′	Sequence	3′
stx1a-primer		CAAGAGCGATGTTACGGT	
stx1b-primer		AATTCTTCCTACACGAACAGA	
stx1f-probe		CTGGGGAAGGTTGAGTAGCG	Fluorescein
stx1r-probe	CAL Fluor 610	CCTGCCTGACTATCATGGACA	3' phosphor
stx2a-primer		GGGACCACATCGGTGT	
stx2b-primer		CGGGCACTGATATATGTGTAA	
stx2f-probe		CTGTGGATATACGAGGGCTTGATGTC	Fluorescein
stx2r-probe	CAL Fluor 610	ATCAGGCGCGTTTTGACCATCT	3' phosphor

5.3.3 Electron Microscopy

Sample preparation and initial fixation: Three sweeps of bacteria from CHROMagar™STEC were fixed in a 2.5% solution of glutaraldehyde in phosphate buffer (pH 7) with vortexing. The suspension was then pelleted by centrifugation at 4000 rpm for five minutes and then resuspended in buffered, 2.5% glutaraldehyde. The fixed bacteria were then washed twice with sterile 1x phosphate buffered saline (PBS) with the supernatant discarded after centrifugation at 4000 rpm after each wash. After the second wash, the pellet was re-suspended in an equal amount of 4% agarose (30µl) and allowed to solidify.

***High-Pressure Freezing*:** The glutaraldehyde fixed, agarose-embedded samples were thinly sliced (0.2mm) and loaded onto 6mm diameter freezing hats. Hexadecene was used as a filler, and the samples were high-pressure frozen using the Leica HPM100.

Freeze Substitution: The cryo-fixed specimens were immersed in a dry, pre-cooled (-85^{o}C) cocktail of 2% osmium tetroxide (OsO_4) in anhydrous acetone in a Leica AFS Automatic Freeze-Substitution unit. After 60h, the temperature was set to rise by 10 ℃ every hour to –60 ℃. The samples were left for 8h at this temperature. The temperature was gradually raised to room temperature, and the osmium tetroxide solution was replaced with acetone. The samples were washed with absolute acetone and prepared for embedding in Spurrs resin. The samples were infiltrated with increasing concentration of resin over a period of 3 days, and once in 100% resin. They were then positioned in molds and allowed to polymerize at 60 ℃.

Ultramicrotomy and transmission electron microscopy: Approximately 100-120nm sections were cut using a Leica EM UC7 and collected on copper grids. The samples were stained with uranyl acetate and lead citrate and viewed using an FEI Tecnai F20 transmission electron microscope operating at 200kV. Images were collected using a 4Kx4K Gatan CCD camera. We considered five sections of view at low magnification and five sections of view at 19,000X magnification. From these five sections, we observed each of the sections in detail at 100,000X magnifications. From each of these sections, we examined all the cells in the field of view.

5.3.4 Protein extraction

Single colonies of isolates (16.5, 29.4 and 424.2) described above were selected from CHROMagar™STEC plates and directly streaked onto a new Luria-Bertani agar plate and incubated for 48h at 37^{o}C. After incubation, a single loop of cells was scrapped from the solid media, suspended in 1% PBS (Sigma-Aldrich, St Louis, USA) and cells were broken using an ultrasonic cell disruptor (Virsonic; 6 x 30s on ice with a 3.2mm microprobe operated at 20% output power). Chloroform in methanol (1:1; v:v) was added, and the mixture was vortexed for 1 minute and centrifuged at 4000 rpm for 30 min. The upper phase was carefully removed, and 100% methanol was added. The whole cell lysate proteins were then pelleted by centrifugation at 4000

rpm. Air dry protein pellets were suspended in denaturation buffer (6M urea, 2M Thiourea, 10mM Tris buffer, pH 8.0). Protein was quantified using a modified Bradford assay.[28] For in-solution trypsin digest, 200 µg total protein was reduced with 1 mM 1,4-dithiothreitol (DTT) for 1 hour with agitation and alkylated with 5.5 mM 3-iodoacetamine (IAA) for one hour in the dark. Proteins were diluted with four-fold ammonium bicarbonate (ABC) (20 mM) before adding sequencing grade trypsin (NEB, New England Biolabs, UK, Ltd) supplemented with 20 mM CaCl2 (1:50; w/w) and digested overnight at 37 ℃ with agitation at 30rpm. Proteolysis was quenched by addition of trifluoroacetic acid (TFA) (Sigma-Aldrich). Before LC-MS/MS, peptides were dried and suspended in 0.1% formic acid (FA) and 2% acetonitrile (ACN) and cleaned up using stage tips.

5.3.5 Mass spectrometry analysis

Data-dependent acquisition of tandem mass spectrometry was performed on Orbitrap Q-Exactive mass spectrometer (Thermo Scientific) in a data-dependent manner, coupled to the Dionex Ultimate 3500 RS nano UHPLC (Thermo Scientific). Six hundred nanogram of peptide was loaded in technical triplicate onto an in-house packed pre-column (100 µm ID × 40 mm) packed with 5µm Luna C18 (Phenomenex 04A-4398) connected to an in-house packed analytical column (75 µm × 400 mm) packed with C18 3.5µm Aeris Peptide (Phenomenex 04A-4507) for liquid chromatography separation. The flow rate was set to 400nl/min with the gradient of 8% to 40% ACN for 118 minutes using Dionex curve 7, then increasing to 80% in 5 minutes. To wash the column, ACN was maintained at 80% for 10 minutes followed by a column equilibration at 2% ACN for 10 minutes. A "top ten" method with 30 s dynamic exclusion was used to acquire mass spectra, with automatic switching between MS and MS/MS scans.

5.3.6 Data processing

A total of nine MS raw data files were processed together in the MaxQuant (version 1. 5.0.30). Andromeda search engine integrated into the MaxQuant Suite[29] performed the spectra search against the *E. coli* O157: H7 proteome downloaded from Uniprot (10 December 2015). The search criteria were set as follows: tryptic digest with two missed cleavages; fixed modification

carbamidomethylation (C); variable modification of Acetyl (protein-N-term) and Oxidation (M). The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD005546.

5.3.7 Data analysis

We further analysed the Maxquant output data with the Perseus module. RAW files were analysed using the MaxQuant suite together with Perseus to determine significantly regulated proteins. Unique proteins (present in at least two strains, two or more replicates based on intensity values, two ≥ peptides per protein) in each isolate were excluded and analysed separately. Mean values of technical replicates were calculated, log transformed, and used in further analyses. Principal component analysis (PCA) was done by Perseus. One-way analysis of variance (ANOVA) with permutation-based FDR correction for multiple hypothesis testing (p-value cut-off 0.01) was used to compare protein LFQ intensities of all three strains in this study. Comparative analysis between strains was performed using a paired Student's t-test with p-values of 0.01. P-values were used to identify significantly up/down-regulated proteins and pathways. The identified proteins were functionally categorised based on universal Gene Ontology (GO) annotation terms using the Biological Networks Gene Ontology (BiNGO) program package.

5.4 Results

5.4.1 Characterization of the clinical *E. coli* isolates

5.4.1.1 Virulence profiles

Three suspected STEC strains which formed bright mauve colonies on CHROMagar™STEC were selected. The strains were numbered 29.4, 16.5, and 424.2, respectively. Virulence gene profiling using an endpoint PCR assay and a real-time PCR assay revealed that only strain 29.4 possessed both the *stx*$_1$ and *eaeA* genes while strains 424.2 and 16.5 possessed the *eaeA* gene only (**Figure 5:1 and Figure 5:2**).

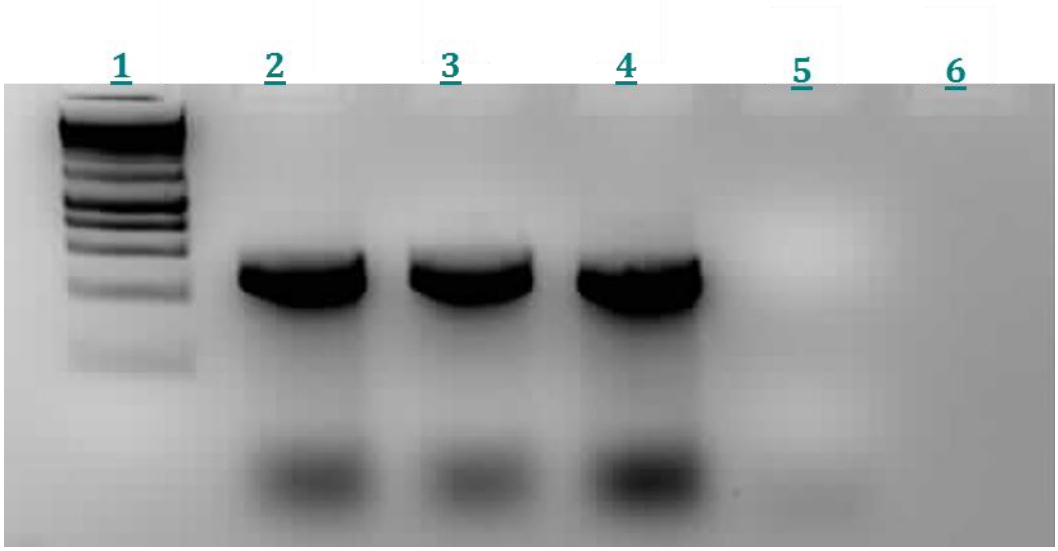

Figure 5:1: Gel image for PCR to detect eaeA.

Lane 1= 1kb Ladder, Lane 2= STEC strain ; Lane 3= ciprofloxacin resistant EPEC, Lane 4= ciprofloxacin susceptible EPEC; Lane 5= Negative control.

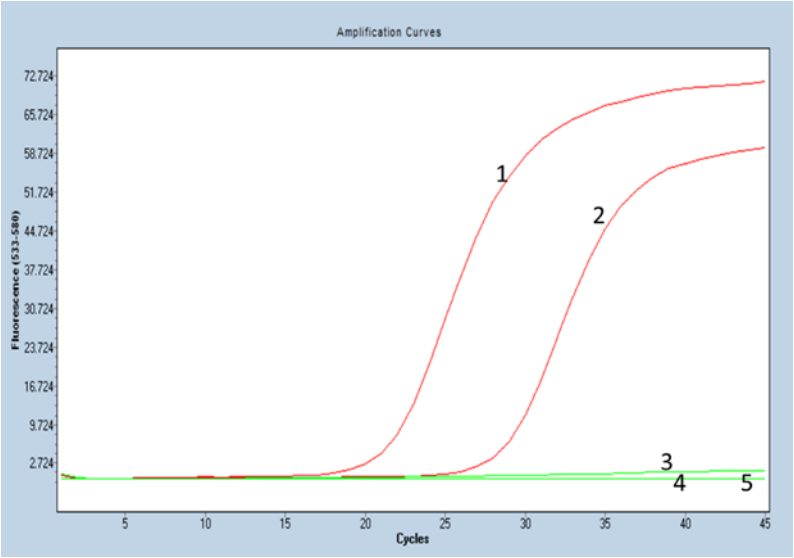

Figure 5:2 Showing the amplification curves for the stx positive mauve isolates on using the qPCR assay

1-positive control (*E. coli* O157:H7 strain C4193-1), 2-STEC strain, 3-ciprofloxacin susceptible EPEC strain, 4-ciprofloxacin resistant EPEC strain, 5-negative control.

None of the isolates carried *stx*$_2$. Additionally, hemolysis assays established that strain 29.4 was non-haemolytic on 2% sheep blood agar, while both 16.5 and 424.2 were β-haemolytic.

5.4.1.2 Drug resistance profiles

All three strains were resistant to potassium tellurite and thus formed mauve colonies on the CHROMagar™STEC substrate. To characterise their drug resistance profiles further, the isolates were tested against a total of 19 antibiotics (**Table 5.3**).

Table 5.3: Minimum Inhibitory Concentrations (μg/μl) to selected antibiotics of three E. coli clinical isolates.

Isolate/Antimicrobial	STEC	ciprofloxacin susceptible EPEC	ciprofloxacin resistant EPEC
Cotrimoxazole	R	R	R
	320	320	320
Ampicillin	S	S	R
	2	4	32
Cefuroxime	S	S	S
	4	4	4
Amoxicillin-clavulanic	S	S	S
acid	2	4	4
Ciprofloxacin	S	S	R
	0.25	0.25	4
Piperacillin/Tazobactam	S	S	S
	4	4	4
Cefotaxime	S	S	S
	1	1	1
Ceftazidime	S	S	S
	1	1	1
Cefepime	S	S	S
	1	4	1
Ertapenem	S	S	S
	0.5	0.5	0.5
Imipenem	S	S	S
	0.25	0.25	0.25
Meropenem	S	S	S
	0.25	0.25	0.25
Amikacin	S	S	S
	2	2	2
Gentamicin	S	S	S
	1	1	1
Tigecycline	S	S	S
	0.5	0.5	0.5
Nitrofurantoin	S	S	R
	32	16	256
Colistin	S	S	S
	0.5	0.5	0.5
Polymyxin B	S	S	S
	0.25	0.25	1

R=resistant, S=susceptible

Strain 16.5 was resistant to a wide spectrum of antibiotics, including trimethoprim-sulfamethoxazole, ciprofloxacin, ampicillin, and nitrofurantoin. Strain 29.4 and 424.2 were both resistant to trimethoprim-sulfamethoxazole. Even though all the strains were susceptible to cefepime, strain 424.2 showed a higher MIC (4 μg/μl) compared to strain 29.4 (1 μg/μl) and strain

16.5 (1 μg/μl). Based on these results, and for convenience in the text, strain 29.4 is hereafter referred to as the "STEC strain", and strain 16.5 as "ciprofloxacin resistant EPEC strain" while strain 424.2 is referred to as the "ciprofloxacin susceptible EPEC strain".

5.4.1.3 Electron Micrographs of the three Isolates

Proteomic data showed different levels of key cell envelope proteins including OmpC, OmpF and OmpA. Since low levels of OmpC coupled with higher levels of OmpF have been shown to influence bacterial response to antibiotic exposure[30], we conducted electron microscopy on the three isolates to examine the difference in their cell envelope thickness and see if it correlates with the observed differences in the cell envelop proteins we detected. The three strains exhibited distinct cell envelope features on the electron micrographs (**Figure 5:3**).

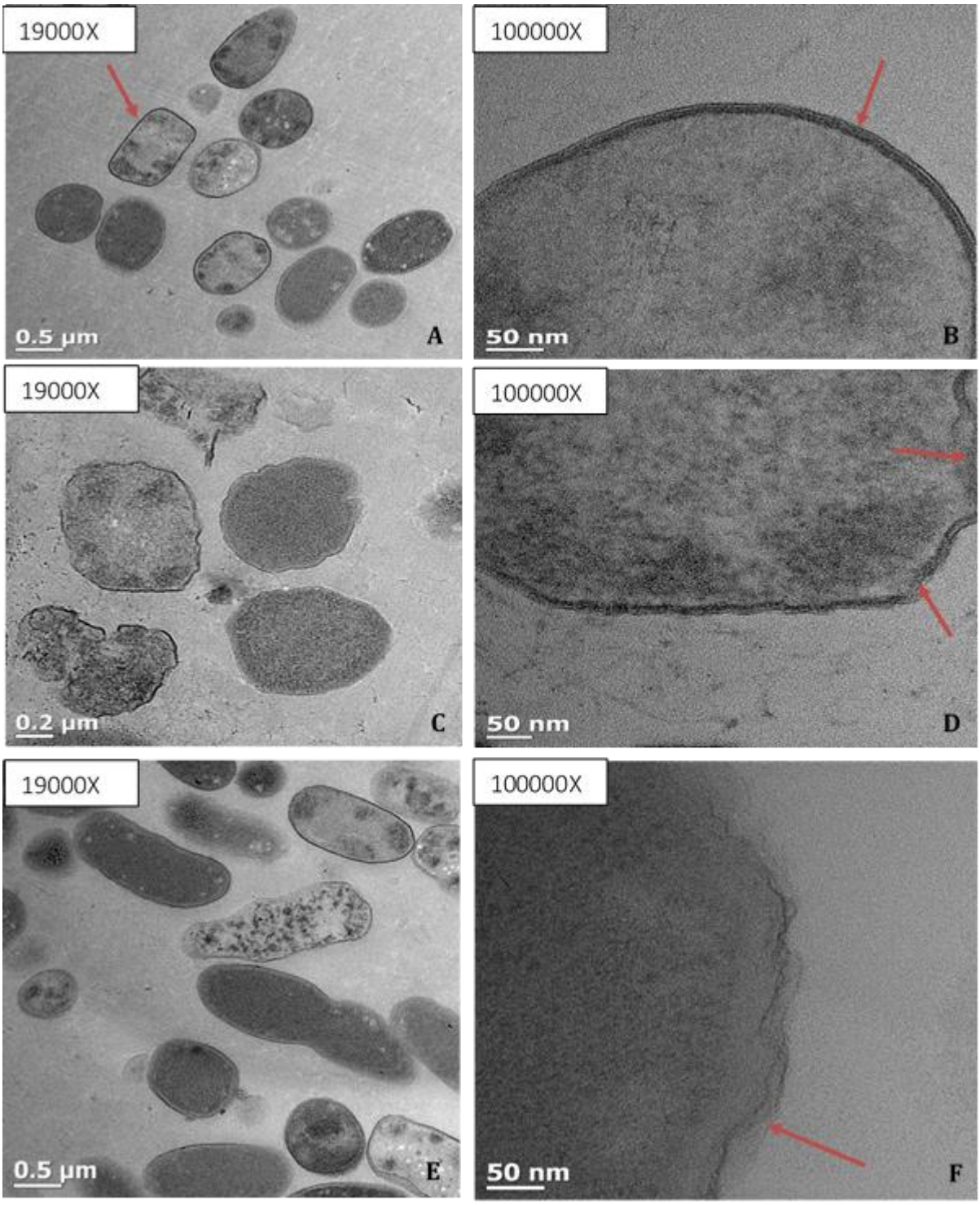

Figure 5:3: Transmission Electron Microscopy images of strains ciprofloxacin resistant EPEC (A and B), ciprofloxacin susceptible EPEC (C and D) and STEC (E and F).

The red arrows show the entire margin for the ciprofloxacin-resistant isolate as well as disrupted margins for the ciprofloxacin susceptible strains.

The STEC and ciprofloxacin susceptible EPEC isolates showed uniform (all cells examined) disruption of the cell envelope at 19,000X and 100,000X magnification. The ciprofloxacin resistant EPEC showed an intact cell envelope. The ciprofloxacin-resistant strain also showed significantly greater cell envelope thickness (mean cell envelope thickness of 64 nm (±1.4) while the STEC strain had a mean cell envelope thickness of 39.4 (±3.1) nm and the ciprofloxacin susceptible EPEC had a mean cell envelope thickness of 45.16 (±2.2) nm (**Figure 5:4**).

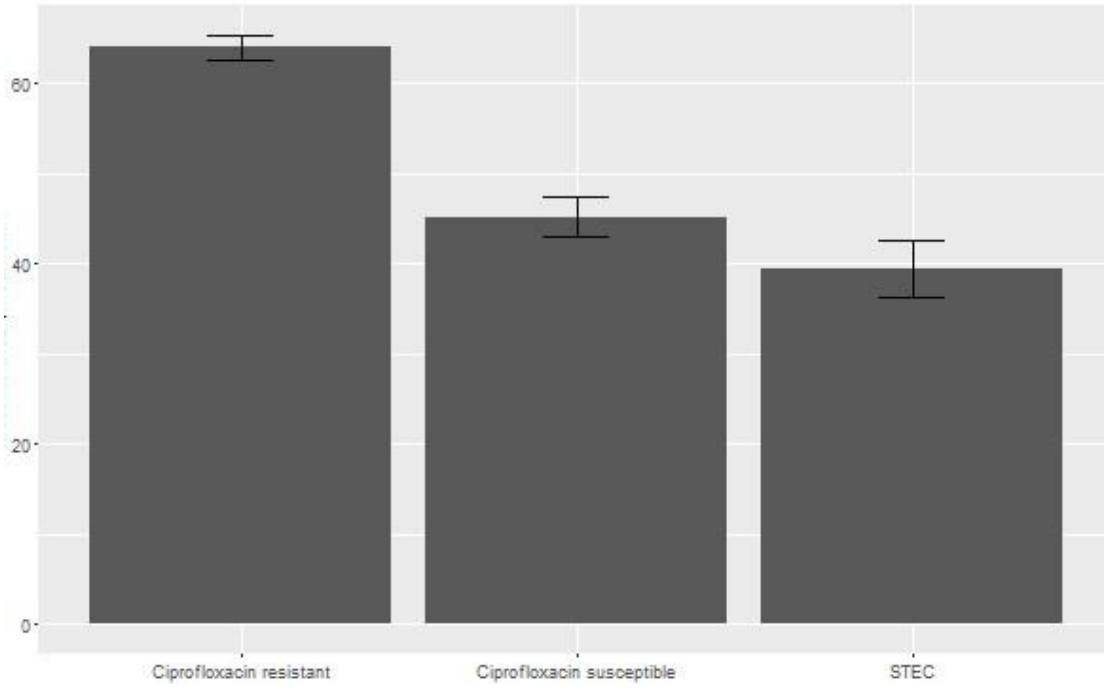

Figure 5:4 Mean cell thickness of the Ciprofloxacin-resistant EPEC and the Ciprofloxacin susceptible strains.

The ciprofloxacin susceptible EPEC showed sections of invagination of the cell envelope, which were not present in other strains. The outer membrane proteins OmpF and OmpC were significantly lower in the ciprofloxacin-resistant isolates, as compared to the ciprofloxacin susceptible isolates. Our preliminary observations establish a link between cell envelope thickness and higher levels of OmpF coupled with lower levels of OmpC. This, however, requires future research.

5.4.2 Global proteomic analysis

LC-MS/MS analyses generated nine raw spectra (one for each triplicate- three for the STEC isolate, three for the ciprofloxacin-resistant EPEC, and three for the ciprofloxacin susceptible EPEC) and were analyzed in the MaxQuant environment which matched the spectral files to approximately 19197 peptides.

A good peptide read was noted for the STEC isolate triplicates. The relative abundance of peptides eluting from the column during the run showed a good gradient for both the MS and MS2 spectra (**Figure 5:5)**.

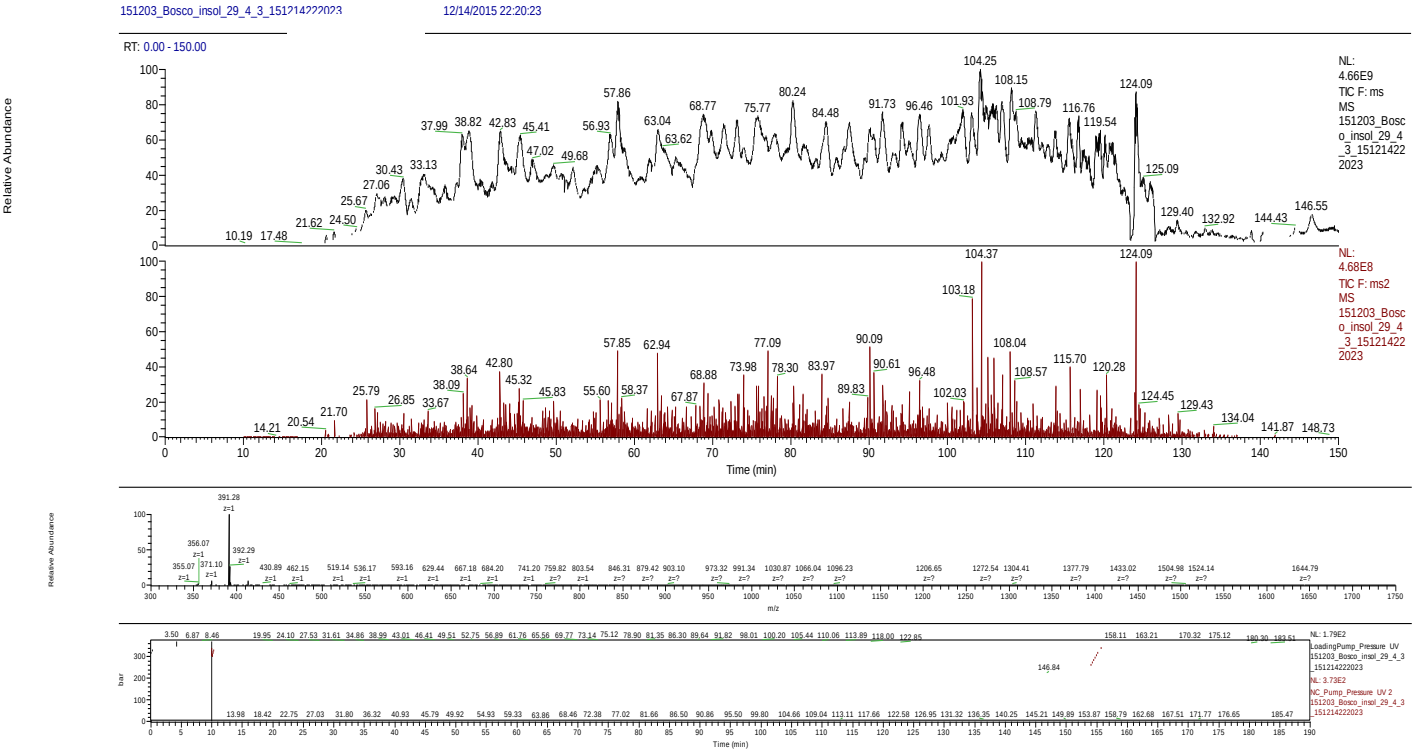

Figure 5:5 Mass spectral files (MS) showing relative abundance of peptides and peptide ions over time for one of the STEC isolate triplicates.

The top panel shows the MS spectra while the second panel shows the MS2 spectra

A good peptide read was noted for the ciprofloxacin resistant EPEC isolate triplicate. The relative abundance of peptides eluting from the column during the run showed a good gradient for both the MS and MS2 spectra (**Figure 5:6**).

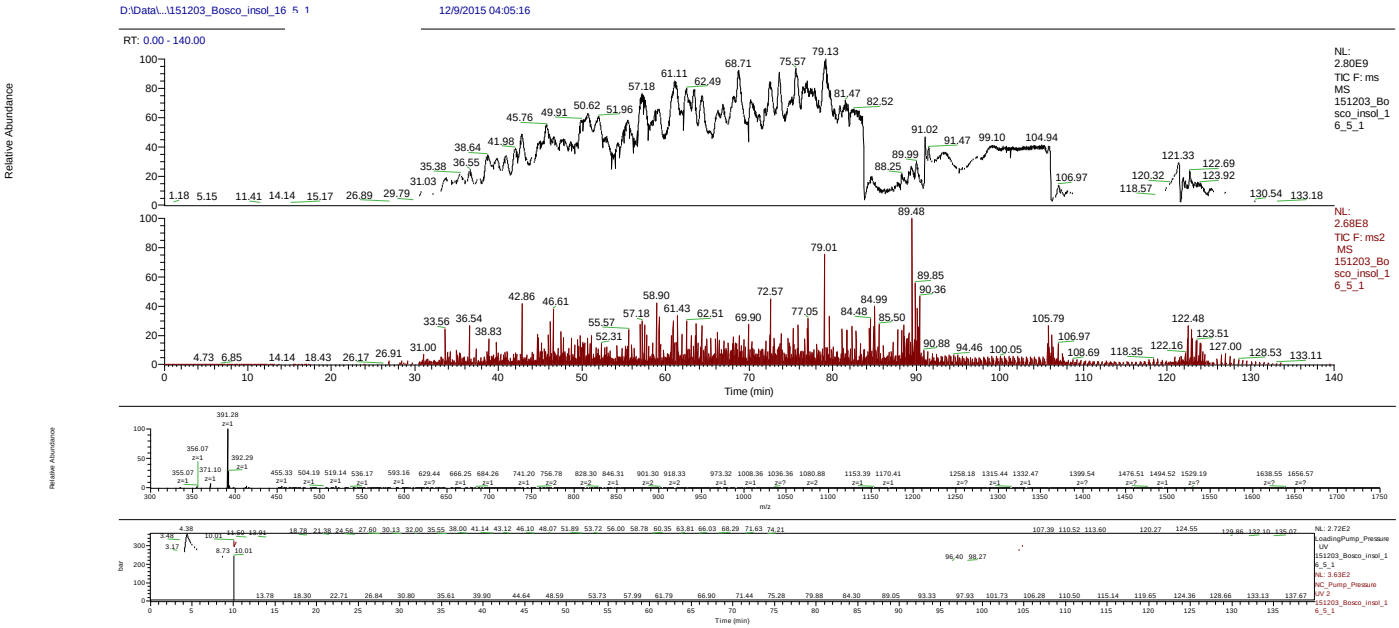

Figure 5:6 Mass spectral files (MS) showing relative abundance of peptides and peptide ions over time for one of the Ciprofloxacin resistant EPEC isolate triplicates

The top panel shows the MS spectra while the second panel shows the MS2 spectra

A good peptide read was noted for the ciprofloxacin susceptible EPEC isolate triplicate. The relative abundance of peptides eluting from the column during the run showed a good gradient for both the MS and MS2 spectra (**Figure 5:7)**.

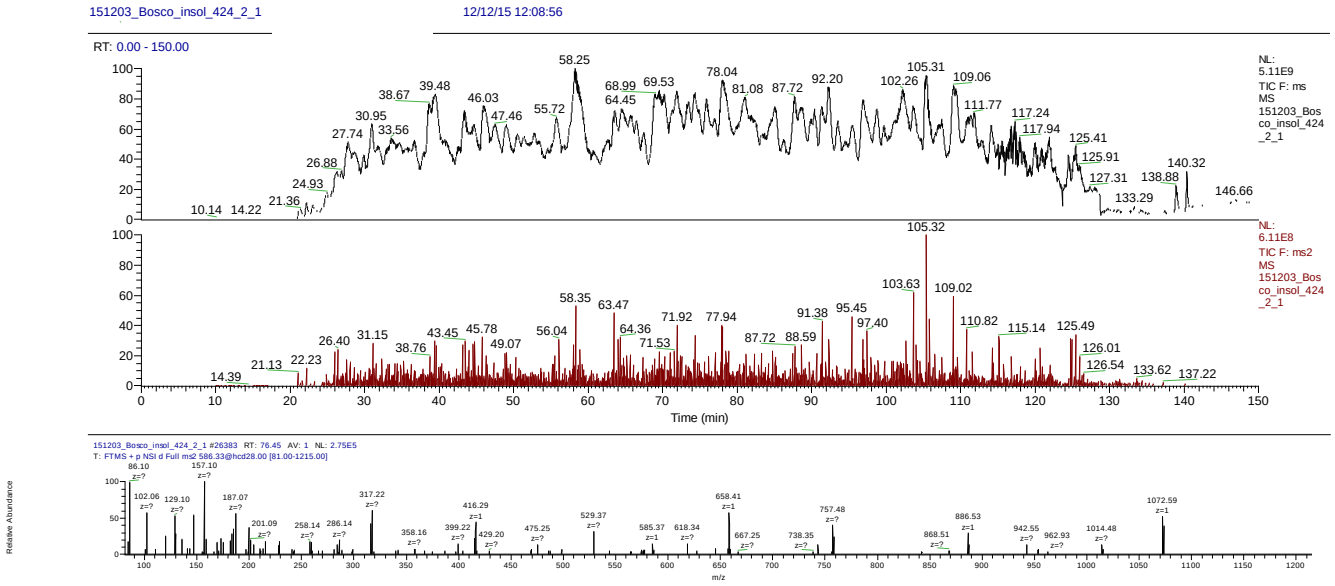

Figure 5:7 Mass spectral files (MS) showing relative abundance of peptides and peptide ions over time for the Ciprofloxacin-susceptible EPEC isolate

The top panel shows the MS spectra while the second panel shows the MS2 spectra

We identified a total of 1630 proteins across all three strains: 1404 protein groups for the ciprofloxacin-resistant EPEC strain; and 1438 proteins groups for the ciprofloxacin susceptible EPEC strain; and 1440 proteins groups for the STEC strain. In order to improve proteome coverage and increase the number of proteins identified in our discovery analysis we prepared three separate stage tip preparations and analysed technical triplicates for each of the 3 clinical *E. coli* strains. Based on the LFQ intensities, the principle component analysis of the proteomics data (**Figure 5:8**) revealed that technical replicates were closely related, to each other, whereas the three different EPEC/STEC clinical *E. coli* strains were well separated.

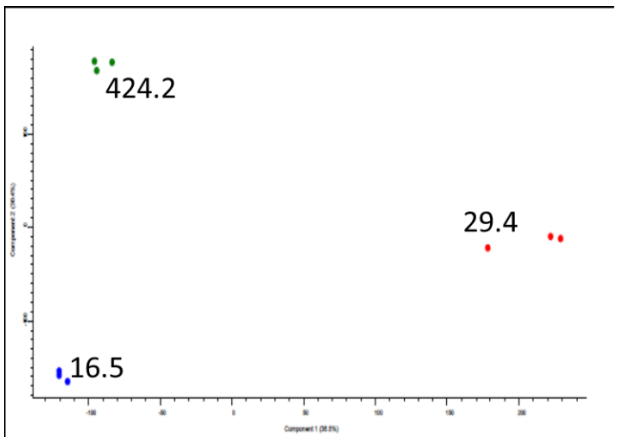

Figure 5:8: Principle component analysis (PCA) component1 versus component 2 from proteome data of 3 clinical E. coli strains.

This PCA analysis reveals good reproducibility amongst the technical replicates of these clinical isolates clustered separately from each other.

The overlap in protein groups amongst the three strains is shown in **Figure 5:9**. A total of 1238 protein groups, accounting for approximately 85% of proteins, were identified in all three strains (common in all the three strains); the remaining protein groups being present in only one or two of the isolates.

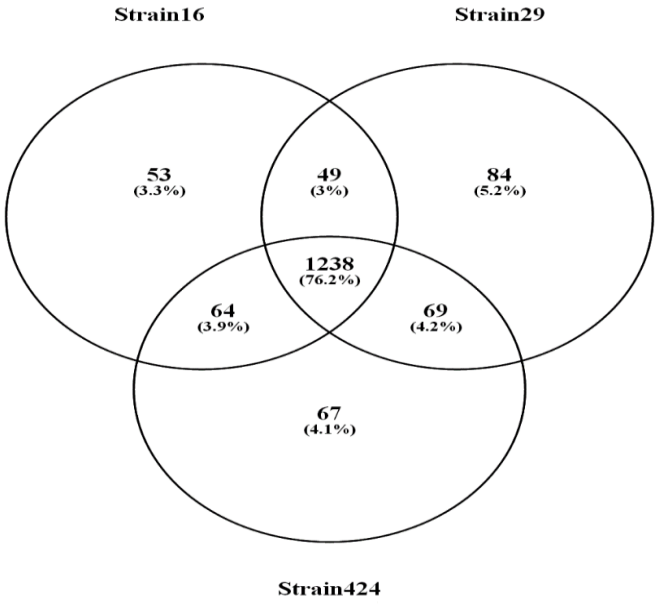

Figure 5:9: Comparison of proteomes of clinical E. coli strains. Demonstrating the unique proteins in each strain as well as the overlap of proteins across all three strains.

5.4.3 Overall comparison of *E. coli* strains.

The strains possessed distinct protein profiles, with each producing a set of unique proteins that was not identified in the other two. Using strict criteria of unique protein identification, (absent in the other two isolates, present in two or more replicates in the single isolate based on LFQ intensity values, two ≥ peptides identified per protein group) we identified 28, 56, and 41 unique protein groups for the ciprofloxacin-resistant EPEC, STEC, and the ciprofloxacin susceptible EPEC strain, respectively (**The ProteomeXchange accession number is PXD005546 and password for access in the interim is <StecEpec2017!>**). We then compared Label-Free Quantification (LFQ) intensities of 1627 proteins by using ANOVA with a permutation-based FDR correction for multiple hypothesis testing (cutoff FDR 1%). In this comparison, we identified 943 proteins with statistically significant quantitative differences in abundance across the three strains. We also applied a two-tailed t-test with an FDR of 1% when comparing each stain to each other.

We further analysed the proteome profiles of each strain and identified 41 virulence factors in the ciprofloxacin-resistant EPEC strain; 35 virulence proteins in the STEC strain and 46 virulence proteins in the ciprofloxacin susceptible EPEC strain (LFQ- intensity values in 2 or more replicates) (**Table 5.4**).

Table 5.4 Virulence factors identified in each strain

Virulence factor/strain	**16_5**	**29_4**	**424_2**
Attachment	13	13	13
Cell Lysis/ Cell invasion	4	4	4
Invasion/ Iron acquisition	9	8	9
Motility	1	1	6
Proteolysis	5	0	0
Type III secretion system	9	9	9
Total	**41**	**35**	**41**

5.4.4 Comparing the ciprofloxacin-resistant EPEC strain to the STEC strain

We identified 498 proteins with significant differential abundance between the ciprofloxacin-resistant EPEC and STEC strains. A total of 220 proteins were significantly abundant in the STEC strain while 278 proteins were significantly more abundant in the ciprofloxacin-resistant EPEC strain (**Figure 5:10a**).

Amongst these, we identified virulence factors and drug resistance associated proteins such as YeiP, Fdx, LolA, YaeT, OmpA as well as a number of putative lipoprotein family proteins which were significantly more abundant in the ciprofloxacin-resistant EPEC strain. We found the virulence factors including serine kinase family protein; OmpC; OmpF and OmpX were significantly more abundant in the STEC strain. OmpC, OmpF, and OmpX are outer membrane proteins central to the YebF export mechanism which play key roles in antimicrobial resistance.[31,30] We also identified the virulence factors FepB, YbhA, amidohydrolase family of proteins, and Nudix family of proteins which were only in the ciprofloxacin-resistant EPEC strain.

The silent hemolysin (SheA), which was differentially abundant in the STEC strain has been shown to have a cytocidal and apoptotic activity like the Shiga toxin but is prevalent in both pathogenic and non-pathogenic *E. coli*.[32] Proteins involved in the formation of attaching and effacing lesions, that were significantly more abundant in the STEC strain, and are coded by genes located on a chromosomal pathogenicity island called the Locus of Enterocyte Effacement (LEE), this is discussed further in the text. The gene ontology (GO) analysis of the comparison of ciprofloxacin resistant EPEC to the STEC strain showed an enrichment of GO terms specifically for biological processes including iron regulation, transport and homeostasis while no significant GO enriched terms were enriched for the ciprofloxacin-resistant EPEC strain.

5.4.5 Comparison of the STEC strain to the ciprofloxacin susceptible EPEC strain.

We identified 383 proteins with significant differential abundance between the STEC strain and the ciprofloxacin-susceptible EPEC strain. We found that 191 proteins were significantly abundant in the STEC strain and 192 proteins significantly more abundant in the ciprofloxacin susceptible EPEC (**Figure 5:10b**). We found the siderophore-interacting protein, enterobactin synthetase component F and putative lipoprotein implicated in virulence to be significantly more abundant in the ciprofloxacin-susceptible EPEC strain. Virulence factors - LolA, BamD, BamC, OmpC, OmpX, putative lipoprotein, serine kinase family protein, OmpA family protein and ion protease were significantly more abundant in the STEC strain. Further, we identified virulence factors - flagellin, serine protease, EatA, FlgH, YbhA, FepB, CheY and hydrolase, NUDIX family protein only present in the ciprofloxacin-susceptible strain. Gene Ontology analysis of the ciprofloxacin-susceptible strain was based only on significantly abundant proteins and showed that five biological processes were enriched. These included regulation of glycogen metabolic processes, generation of precursor metabolites and energy, glucose metabolism, chemotaxis, and taxis. No metabolic processes were significantly enriched for the STEC strain when compared to the ciprofloxacin susceptible EPEC strain.

5.4.6 Comparison of the ciprofloxacin-resistant and the ciprofloxacin susceptible EPEC strains

We identified a total of 543 proteins with significant differential abundance between the ciprofloxacin-resistant EPEC and the ciprofloxacin susceptible EPEC strain: 295 proteins were significantly more abundant in the ciprofloxacin-resistant EPEC strain, and 248 proteins were significantly more abundant in the ciprofloxacin susceptible EPEC strain (**Figure 5:10c**). We identified virulence factors OmpX, OmpC, EntF and a number of putative lipoproteins to be significantly more abundant in the ciprofloxacin susceptible EPEC strain. We identified 13 virulence factors - LolA, YeiP, putative lipoprotein, OmpA family protein, BamC, BamA, DegQ, Lon, FepB and Fdx which were significantly more abundant in the ciprofloxacin-resistant strain. Furthermore, we identified four virulence factors, flagellin, EatA, FlgH, and SppA, uniquely identified in the ciprofloxacin-susceptible EPEC; and one virulence factor, the amidohydrolase family of proteins to be uniquely present in the ciprofloxacin-resistant strain.

We found six biological processes enriched in the ciprofloxacin-resistant EPEC strain. These included: cellular response to an antibiotic (including proteins that mediate antibiotic resistance, e.g. TolB, PhoU, and DsbA); phosphate starvation; regulation of glucose and glycogen metabolic processes; generation of precursor metabolites and energy and protein folding. Biological processes enriched in the ciprofloxacin-susceptible EPEC strain were chemotaxis and taxis.

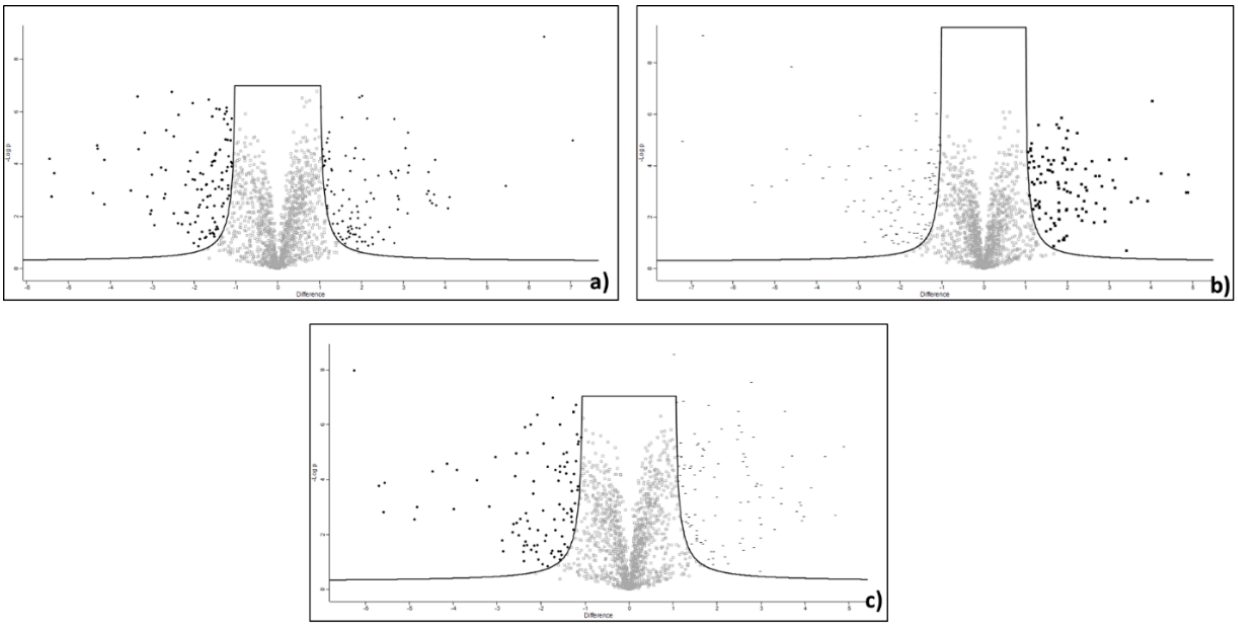

Figure 5:10: Volcano plots demonstrating significant abundance with P-value of 0.01 and 2-fold change.

a) Volcano plot of ciprofloxacin resistant EPEC and Shiga toxin producing *E. coli* strains, b) Volcano plot of Shiga toxin producing and ciprofloxacin susceptible *E. coli* strain, c) Volcano plot of ciprofloxacin resistant and susceptible EPEC strains.

5.5 Discussion

Growth on solid media followed by proteomic analysis enable identification of important virulence factors and antimicrobial resistance mechanisms.

To date, nearly all large-scale bacterial proteomics studies have been done on strains cultured in liquid growth media.[33-35] However proteomic analysis of bacterial isolates grown on solid media are of particular interest; this provides an opportunity to study the proteomes of minimally passaged isolates as used in primary culture plates. For instances previous studies that employed similar proteomic approach to characterize the global response of exponential phase *E. coli* O157:H7 Sakai grown under four steady-state conditions revealed a universal response to all tested condition that included the activation of the master regulator RpoS and the RcS phosphorelay system involved in the biosynthesis of the exopolysaccharide colanic, as well as down regulation of elements involved in chemotaxis and motility.[36] Consistently, chemotaxis genes and proteins were amongst the heaviest down regulated in osmotically adapted cells.[37] The authors advanced that chemotaxis and motility systems are most dispensable functions during steady-state growth at high osmolarity.[37] Unlike liquid media, bacterial cells grown on solid media experience particular environmental conditions including high osmotic strength and dessication that ultimately results in a physiological and morphological transformation of the bacteria.[38] We have detected a number of proteins implicated in cell motility and virulence more abundantly expressed in the ciprofloxacin susceptible EPEC.

Of those, it is worthy to highlight proteins such as flagellar L-ring protein (FlgH), flagellin, flagellar motor switch protein (FliN), sensor histidine kinase (RcsC) that are all proteins associated with bacterial lateral flagella and inducible surfaces flagellar systems.[39] Additionally, within the proteins unique to ciprofloxacin susceptible EPEC strain we have identified CheA, CheY and methyl-accepting chemotaxis protein III - chemotactic regulators involved in the transmission of sensory signals from chemoreceptors to flagellar motor components. Of interest, we did not observe the expected enrichment of osmoprotective proteins and reduced chemotaxis-associated proteins as expected from solid medium growth.[24] The ciprofloxacin-susceptible strain

displayed a distinguishable motility swarming/chemotactic profile which may be useful for identifying pathogenic *E. coli*.[40]

Among the proteins exclusively identified in STEC is ZapE, a protein associated with cell division that seems not be essential *in vitro* but is required for growth under low-oxygen conditions and during an infectious process.[41] The detection of proteins such as ZapE suggests that proteomic examination of bacteria could be a convenient means to characterise survival determinants with important roles during host invasion. In sum, the data presented here clearly indicates that limited laboratory passage on solid media followed by mass spectrometry-based proteomic analysis presents a valuable avenue to assess proteomic insight into clinical isolates in a quantitative manner, enabling identification of important virulence factors that might otherwise be missed/lost.

CHROMagar™STEC allows the formation of mauve colonies of strains that are tellurite resistant[42]. Resistance to tellurite is encoded by the *terZABCDEF* gene cluster present on the pathogenicity islands of STEC[43,44] and select for strains carrying virulence proteins[45]. Tellurium susceptibility is related to the lack of *ter* genes.[44] We could detect TerA, TerE, TerC, TerB, TerD, TerW, and TerZ from CHROMagar™STEC isolates although it has been suggested that Enterohaemorrhagic *E. coli* (EHEC) O157:H7 clinical isolates lose the ter gene cluster during laboratory passage.[43]

The three clinical *E. coli* strains in this study carried key virulence and antimicrobial resistance proteins. This is in agreement with an earlier study which showed the likely co-occurrence of virulence and antimicrobial resistance in individual isolates.[16] Recovery of *E. coli* from tellurite containing solid media may select for strains carrying tellurite resistance determinants on plasmids (IncHI1,IncHI2, IncP) that are known to harbour multiple antibiotic resistance determinants[46,47]. Also, full or partial deletions of the *Tel*^{R} island (s) diminish the virulence and/or fitness of the EHEC O157:H7.[43] Additionally, the *ter* cluster is significantly less widespread in the *eae*-negative STEC strains as compared to the *eae*-positive ones.[48]

Comparative proteomic analysis reveals differential antimicrobial resistance mechanisms.

Fluoroquinolones are potent antimicrobial agents used clinically for the management of community-acquired and nosocomial infections.[49] It is a critically important antimicrobial for human medicine.[50] Our data showed that the ciprofloxacin-resistant EPEC isolate possesses reduced levels of key virulence proteins (e.g. motility, toxins, outer membrane proteins) compared to the ciprofloxacin susceptible EPEC strain and STEC strain. In particular, apparent losses of OmpF have been associated with increased resistance to multiple antibiotics including quinolones.[51] Specifically, one study reported that the decreased expression of OmpF in a *gyrA* mutant strain did not affect the survival of the bacteria and could be responsible for the spread of high-level quinolone-resistant strains.[51] Additionally, coupled with a lower abundance of OmpC and OmpF, the ciprofloxacin-resistant EPEC strain showed a significantly higher abundance of metallo-beta-lactamase family protein compared to the other two isolates. However, the detected MICs to cephalosporins and carbapenems reflect susceptibility to these antibiotics. Since in this study we used a quantitative approach, the levels of transcription and translation of genes coding for the metallo beta-lactamase may be insufficient for the resistance phenotype to be manifest. Furthermore, this strain showed higher levels of multidrug resistance proteins and of the ABC superfamily of efflux pump proteins that are chromosomally encoded and can cause resistance to multiple drugs when overexpressed.[52] The detection of the dihydropteroate synthase protein could explain the high level of resistance to trimethoprim-sulfamethoxazole in all the three strains; this agrees with previous studies investigating resistance to sulphonamides.[53,54] SXT resistance has been associated with resistance determinants such as *sul* (*sul1* and *sul2* which encodes the dihydropteroate synthase) and *dfrA* (encodes the dihydrofolate reductase protein), class 1 integrons and mobile genetic elements.[55] Overall, the ciprofloxacin resistant (multidrug resistant) strain carried more heavy metal and antibiotic efflux pump proteins than the susceptible strains, consistent with previous findings.[56]

Correlation between levels of key cell envelope proteins , ompF, ompC and ompA with the observed cell envelope thicknesses

The ciprofloxacin resistant isolate which is also multidrug resistant showed a thicker cell wall envelope as compared to the ciprofloxacin susceptible isolates. This strain also had higher levels

of OmpA and lower levels of OmpF and OmpC as compared to the ciprofloxacin susceptible isolates. Interestingly, previous research has shown an association between high levels of OmpF and resistance to fluoroquinolones.[51] OmpF and OmpC have also been shown to play a role in acidic resistance.[57] Further research is needed to determine levels of other key components such as lipids and how changes in their levels affect cell thickness.

The virulence profiles of STEC and EPEC strains

For the STEC strain, we could detect key LEE virulence proteins such as intimin gamma, serine proteases, superoxide dismutase, toxin B and translocated intimin receptor (Tir) proteins. These were either totally absent or present at lower levels in the EPEC strains. ToxB is a nontoxic homopentameric protein responsible for which toxin binding and internalisation into target cells by interacting with the glycolipid, globotriaosylceramide (Gb3).[9] The ToxB protein is located on a large plasmid found only in STEC strains associated with the most severe form of the disease.[58] Intimin type γ and FeoB are implicated in attachment and iron transport respectively, responsible for the colonisation of enterohaemorrhagic *E. coli* O157:H7.[59]

Unlike the EPEC strains, the STEC strain had significantly higher levels of the silent haemolysin A responsible for cytolysis and for inducing apoptosis. This haemolysin can play the same role as haemolysin A (*hlyA*) in the pathogenesis of STEC disease.[32] We detected higher LFQ values for outer membrane proteins such as OmpC, OmpX and the outer membrane assembly factor protein in the STEC strain associated with attachment and immune evasion.[60,61]

Overall the STEC strain yielded more virulence factors compared to the EPEC strains. However, EatA, an immunogenic serine protease, was identified exclusively in the ciprofloxacin-susceptible EPEC strain and contributes to virulence by degrading MUC2, the major protein present in the small intestinal mucous layer, to accelerate access of the enterotoxin to the enterocyte surface. EatA appears to modulate both adherence to epithelial cells and intestinal colonisation in part by digesting EtpA, a novel exoprotein adhesin molecule that is secreted by ETEC.[62] Another example, OmpA a well-described virulence factor among pathogenic *E. coli*[63] was significantly more abundant in the ciprofloxacin-resistant EPEC strain. Our findings indicate that the three isolates display distinct patterns of virulence factor protein abundance.

5.6 Conclusions

This study demonstrates the utility of tellurite containing screening agar for the detection of EPEC and STEC, and the application of tandem MS coupled with UHPLC to characterise the proteome of *E. coli* from solid media.

This study demonstrates that solid medium aids detection of virulence and antimicrobial resistance proteins and that mauve colonies on CHROMagar™STEC all secreted tellurite resistance proteins, with the selected strains all carrying different arsenals of virulence and antimicrobial resistance proteins. The significant abundance of proteins in antimicrobial resistance such as the Metallo-beta-lactamase and multidrug efflux pumps and heavy metal efflux pumps sheds light on the molecular mechanisms of antimicrobial resistance in this setting. The workflow described here may facilitate further research into bacterial pathogenicity from low passage clinically isolated strains.

Further research is needed to characterise the link between cell envelope thickness and levels of key cell envelope proteins. Levels of other key cell envelope components like lipids and sugars should be assessed in relation to differences in cell thickness.

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Chapter Six

6 Prevalence and characterization of food and waterborne bacterial pathogens in Lotus River surface water, meat from informal abattoirs, and stool from children in Nyanga Township, Cape Town, South Africa.

6.1 Summary

Introduction: Approximately 19% of child deaths globally are due to diarrhoea. Food and waterborne bacterial pathogens such as STEC cause diarrhoea or dysentery especially among children under the age of five, resulting in malnutrition, dehydration, and/or death. The Nyanga Township has one of the highest infant mortality rates in Cape Town and yet little is known about the non-human reservoirs and characteristics of food and waterborne pathogens in this area. This study aimed to achieve the following objectives:

i. Determine the epidemiologic characteristics and clinical features of children presenting with diarrhoea at the Nyanga Community Health Center (NCHC).
ii. Determine the prevalence of STEC, and other food and waterborne bacterial pathogens in the stool of children with diarrhoea, Lotus River surface water, and meat from informal abattoirs in Nyanga.
iii. Determine the mean colony forming units of coliforms per millilitre of Lotus River surface water in the Nyanga Township.
iv. Determine the antimicrobial resistance patterns of food and waterborne bacterial diarrhoeal pathogens isolated from the stool of children with diarrhoea, Lotus River surface water and meat from the informal abattoirs in Nyanga.

Methods: Stool from children with diarrhoea, raw and processed meat, and Lotus River surface water were collected from the Nyanga Township between June 2015 and March 2016. Real – time PCR was done to detect *stx* in TSB enrichment of the samples and in the *E. coli* isolates that formed mauve colonies on CHROMagar™STEC while endpoint PCR was used to detect *eaeA*, *ipa*, *LT*, *ST*, *daaC*, and *aat* in TSB enrichment and from *E. coli* isolates that formed mauve colonies on CHROMagar™STEC. The antimicrobial susceptibility patterns of the pathogens were determined using the VITEK® 2 automated system and the broth microdilution method.

Results: The commonest form of diarrhoea in children presenting to the health centre in Nyanga over the study period was acute diarrhoea affecting children less than 2 years (mean age =14.9 months) of age, with an average duration of 2.5 days.

STEC was isolated from 2% of the stool and water samples but not from meat. We detected one or more diarrhoeal pathogens (including detection of diarrheic *E. coli* virulence marker genes) in 73% (48/66) of the diarrhoea cases. The most prevalent diarrhoeal pathogens among children were diffusely adherent *E. coli* (DAEC), and *Shigella* (18% and 17% respectively) and these were much more prevalent in stool as compared to meat (no *Shigella* and 2% for DAEC) and water (5% for *Shigella* and 3% for DAEC). STEC isolated in this study showed resistance to ampicillin (AMP) and trimethoprim – sulfamethoxazole (SXT) only. SXT resistance was most commonly observed amongst all pathogens, irrespective of their source.

Conclusions: There was a high prevalence of bacterial pathogens in the stool of children with diarrhoea. The Lotus River and meat from the informal abattoirs are potential environmental reservoirs for food and waterborne bacterial pathogens. Further public and environmental health interventions are needed to ensure food safety in the Nyanga Township.

6.2 Background

Twenty-five percent of deaths among children aged 1-59 months of age in Africa are attributable to diarrhoea.[1] Even though large-scale studies like the Global Enteric Multicenter Study (GEMS)[2], provided relevant information on the common causes of paediatric infectious diarrhoea in sub-Saharan Africa, they did not consider the distribution of such pathogens in the non-human environment of the patients. Studying the non-human environment of patients with diarrhoea is important because most of the bacterial causes of infectious diarrhoea can be transmitted from non-human reservoirs such as food of animal origin, animals, and surface water. According to Karen *et* al, 2016, the GEMS demonstrated that the poorest of the poor might succumb to different pathogens at different rates in different parts of the world, and thus potentially altering public health intervention strategy.[3] For instance, while most attributable cases of moderate-to-severe diarrhoea in the GEMS (considering all study sites in sub-Saharan Africa and South Asia) were due to four pathogens (Cryptosporidium, heat stable toxin producing *Escherichia coli*, Rotavirus and *Shigella*), other pathogens (*Aeromonas*, *Vibrio cholerae* O1, and *Campylobacter jejuni*) were important in selected sites.[2] Essentially, this infers that public health interventions targeting diarrhoeal pathogens should be based on locally generated data.

Food and waterborne bacterial pathogens cause infectious diarrhoea especially among children less than five years of age, the elderly, and the immunocompromised.[4] Transmission of these pathogens is favoured by factors such as inadequate environmental and personal hygiene, as well as a lack of access to clean water. Such factors have been reported to be associated with increased health vulnerability especially among inhabitants of low-cost housing settlements.[5] Food and waterborne bacterial pathogens include diarrhoeagenic *E. coli*[6], *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Aeromonas*, *Vibrio*, and *Plesiomonas shigelloides*.[7,8] Among the diarrhoeagenic *E. coli*, Shiga-toxin producing *E. coli* (STEC), has been associated with the most severe clinical sequelae in the form of haemolytic uraemic syndrome (HUS), especially among children.[9] Outbreaks of STEC often occur as a result of consumption of contaminated foods of animal origin such as fermented sausages[10] and hamburgers[11], contact with animals[12], and consumption of contaminated water.[13]

The environmental reservoirs of STEC that have been investigated in Africa include water, animal faeces, animal carcases, and processed meat products.[14-18]

Among food and waterborne bacterial pathogens, some, such as *Campylobacter* and *Salmonella* are primarily pathogens of domestic animals like poultry, and only accidentally infect the human host as a result of meat contamination at slaughter.[19] The major food animal reservoirs for *Yersinia enterocolitica*[20] and *Vibrio*[21-23] are swine and the molluscan shellfish respectively. *Aeromonas* and *Plesiomonas* have been shown to cause choleraic diarrhoea[24] and can be transmitted to humans from cattle[25] or surface water[26] respectively.

Studying the environmental antibiotic resistome (the density of antibiotic resistance determinants in the environment) is important because there is evidence to suggest a link between antibiotic resistance among bacterial pathogens in the environment and resistance to antibiotics, such as aminoglycosides, extended-spectrum beta-lactams and quinolones in a clinical setting.[27,28] The use of treated human and animal waste (slurry) from wastewater treatment plants, for agricultural purposes (as manure), leads to contamination of the environment with antibiotic resistance genes. Also, the exposure of animals to levels of antibiotics that are less than the minimum inhibitory concentration as a result of anthropogenic contamination (the disposal into the environment of antibiotics due to human activities such as agriculture) selects for antibiotic-resistant bacteria in the animal.[29] According to the Advisory Group on the Integrated Surveillance of Antimicrobial Resistance (AGISAR) of the World Health Organisation (WHO), a monitoring system should be set up only if there is a recognised public health burden of enteric illness due to a specific foodborne etiological agent.[30] To this end, WHO has recommended country level development of antimicrobial surveillance programs that integrate data from bacterial isolates originating from humans, food-producing animals, and retail meats.[30] There are three categories of such bacteria namely; (1) indicator bacteria, for example, *E. coli*, (2) zoonotic bacterial pathogens such as *Salmonella*[31] and (3) strictly animal pathogens like *Salmonella gallinarum*.[32] The three categories of bacteria are common in the gastro-intestinal tract of man and animals.

Diarrhoea and foodborne antimicrobial resistance in Nyanga township

The Nyanga township (GPS coordinates: 33.9931S, 18.5822E) was established in 1946 and is in the City of Cape Town Municipality, in the Western Cape. It has a population density of 18,775 persons / km^2 and a total population of 57,996 people with 27.3% of these younger than 14 years. A burden of disease workgroup under the Child Health Services section of the Provincial Government of the Western Cape reports that the infant mortality rate per 1000 live births (60 in 2001 and 42 in 2004) in Cape Town was highest in Nyanga in the study period 2001-2004.[33] Since then, this work group has not reported the most recent infant mortality rate in the region.

The Nyanga Community Health Center (NCHC) is a primary health care facility managed by the City of Cape Town, which serves the Nyanga area. Here, diarrhoea is syndromically managed, and stool samples are seldom collected for laboratory testing. According to a caregiver survey conducted in the Nyanga health district, 80% of the caregivers that attended primary health care centres had been advised by health workers to manage diarrhoea from home using Oral Rehydration Salts (packets) and home-made sugar-salt solutions. In this survey, only children that presented with severe dehydration and or fever were referred to the Red Cross Children's War Memorial Hospital.[34] This is in line with the Integrated Management of Childhood Infections (IMCI) guidelines. Therefore, there is no routine or systematic laboratory-based surveillance for foodborne diarrhoeal bacterial pathogens and foodborne antimicrobial resistance in this area. The antimicrobial resistance national policy framework (2014-2024) for South Africa focuses on the optimisation of monitoring and early detection of antimicrobial resistance, strengthening, coordinating and institutionalising interdisciplinary efforts. The strategy involves enhancing infection prevention and control and ensuring appropriate use of antibiotics in human and animal health.[35] Whilst the formal food production industry is addressed in this framework, there is no component to tackle foodborne antimicrobial resistance in the informal abattoir setting.

The Lotus River

The Lotus River flows from the Zeekoevlei catchment area and has large and small tributaries. It flows through three townships namely; Nyanga, Khayelitsha, and Crossroads. The Lotus River is made up of storm-water, and its level fluctuates depending on rainfall amounts and waste disposed into it. Domestic animals are often seen grazing along the Lotus River banks and are

occasionally slaughtered here (see **Figure 6:1A and Figure 6:2B**). The extent of use of water from the Lotus River is not clear. However, there is an extensive human settlement on its banks (**Figure 6:3**). Generally, in South Africa, water collected from rivers is used for irrigation and domestic purposes.[36] Also, water in this river ends up in the municipal waste water, and sewerage treatment system, and therefore the level of contamination of this river affects the cost of treatment and recycling of the waste water.

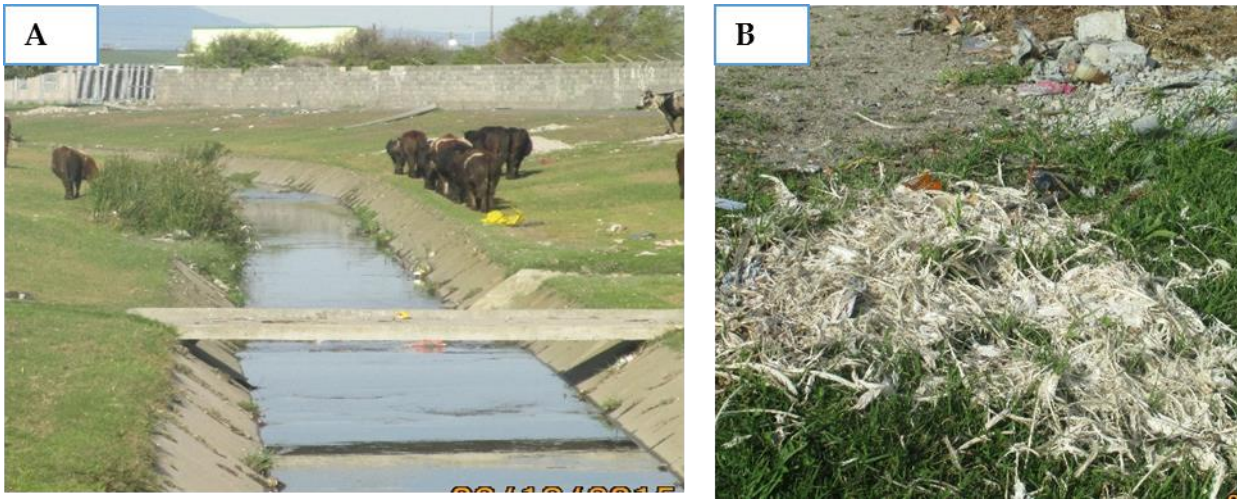

Figure 6:1A-Photo showing cattle grazing along the Lotus River.
Figure 6:2B-Chicken feathers on the Lotus River bank indicating possible use of the Lotus River water for carcas cleaning or discarding of the slaughter waste into the river.

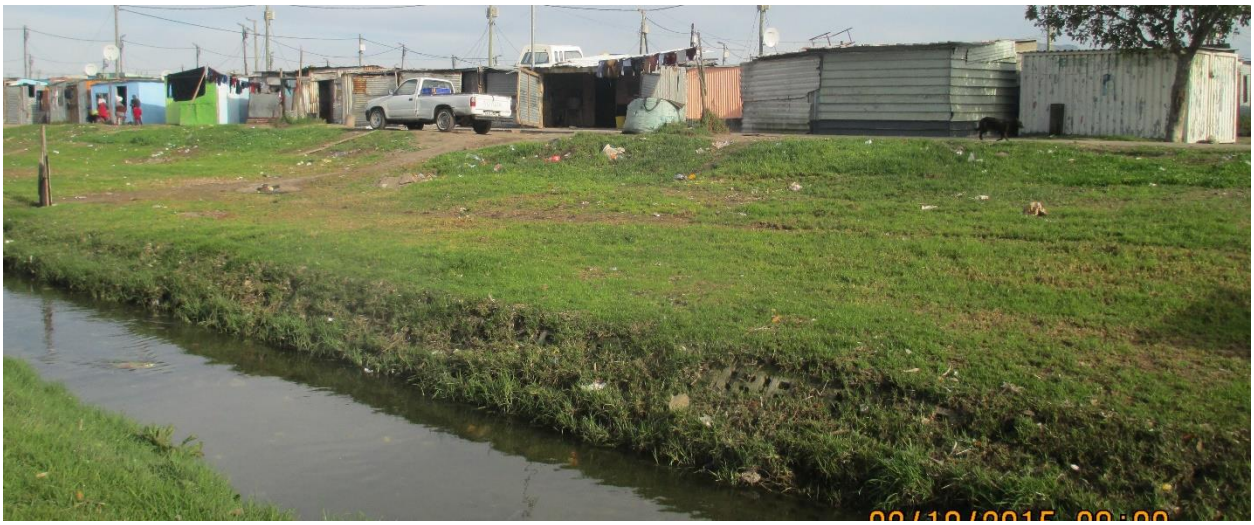

Figure 6:3 Informal settlements ("Shacks") located on the banks of the Lotus River in Nyanga, Cape Town.

The Water and Sanitation Services Department of the City of Cape Town monitors coliform counts in the Lotus River monthly to determine water safety. It follows the South African National Standard 241:2011 on drinking water quality. However, the incubation of thermotolerant *E. coli* at 44.5℃ in the water microbiology laboratory at this centre, does not favour growth and detection of STEC O157:H7.[37] Coliform counts are made and water is passed as safe for use if it has a low count, but coliforms such as *E. coli* are never tested for the possession of virulence genes. Furthermore, at this laboratory, there is no culture for bacterial pathogens. Therefore, a water sample with low coliform count but with pathogenic *E. coli* of public health importance such as *E. coli* O157:H7 may be passed as safe for distribution, use for irrigation or recreational purposes. For example, per the annual report published by the City of Cape Town Water and Sanitation Services for the period 2012/13, two samples in the Cape Metropole area had an *E. coli* count of greater than one cell per 100ml and were therefore deemed unfit for consumption. In this same report, microbiological monitoring in the informal settlements was often hindered by the fact that the communal water standpipes were near the communal toilets and were often dirty and leaking.[38]

6.2.1 Aims and objectives

We aimed to use a One - Health approach to study the prevalence and characteristics of STEC and other bacterial diarrhoea pathogens isolated from the stool of children with diarrhoea, meat from informal abattoirs and surface water from the Lotus River in the Nyanga Township. We also noted the epidemiologic and clinical data relating to patients with diarrhoea attending the Nyanga Community Health Center (NCHC). The aims of this study were:

i. Determine the epidemiologic characteristics and clinical features of children presenting with diarrhoea at the Nyanga Community Health Center (NCHC).
ii. Determine the prevalence of STEC, and other food and waterborne bacterial pathogens in the stool of children with diarrhoea, Lotus River surface water, and meat from informal abattoirs in Nyanga.
iii. Determine the mean colony forming units of coliforms per millilitre of Lotus River surface water in the Nyanga Township.

iv. Determine the antimicrobial resistance patterns of food and waterborne bacterial diarrhoeal pathogens isolated from the stool of children with diarrhoea, Lotus River surface water and meat from the informal abattoirs in Nyanga.

6.3 Methods

6.3.1 Selection and enrollment of children with diarrhoea

Procedures for enrollment and administration of questionnaires: The target population was children under twelve years of age with diarrhoea who attended the NCHC on Monday, Wednesday, and Friday of every week during the study period from October 2015 to April 2016. At enrolment, in the general waiting area, guardians of patients under 12 years of age, were approached by the study clinical research worker and presented with an informed consent form in isiXhosa (see **9.3**), or English (see **9.2**) for those for whom isiXhosa was not their first language (Ethical Clearance number: HREC 2015/140). Only those that consented were recruited in this study. A questionnaire designed using Epi Info 7™ (CDC, USA) (see **9.1**) was piloted before it was administered to the consenting guardians by the study clinical research worker, to collect demographic and clinical data, as well as data on exposure to environmental factors associated with diarrhoea.[39] Medical staff in the unit managed the diarrhoea cases as is done routinely.

Collection of clinical data: A trained nurse examined these children for clinical signs associated with diarrhoea and collected the relevant clinical data. Diarrhoea was defined as three or more loose stools within the past 24 hr period.

Collection of stool samples from patients older than three years of age: The toilet seat was lifted and sample collection tissue paper placed across the toilet bowl (A triple layer of sample collection tissue paper was used). The toilet seat was then lowered. The patient was advised to urinate first before the sample was collected. Alternatively, three layers of tissue paper roll were placed on a clean floor surface, and the patient positioned to pass stool in the middle of the triple tier. The scoop on the lid of the screw capped stool collection container was then used to collect a pea-sized amount of stool which was then placed into the container. The triple layer tissue was then discarded into the toilet.

Collection of stool from children younger than three years: A freshly passed stool sample was collected by scooping from the diaper but with care to avoid contact or scrapping from the diaper surface as it has bactericidal chemical compounds. Using a plastic scoop, we transferred at least

a pea-sized amount of stool into a screw-capped container. Samples were then transported to the laboratory within 12h after collection using a temperature-monitored cool-box.

6.3.2 Meat sample collection

Retail meat samples were collected in accordance with the recommendations by the World Health Organisation (WHO) on Integrated Surveillance of Foodborne Antimicrobial Resistance.[30] Sampling was active and population based. This was done on the first two Fridays of every month for eight months between October 2015 and May 2016.

Forty-nine raw and 36 ready–to-eat meat samples were collected from 53 local meat retail stalls (stalls were selected based on convenience) along main access roads in the Nyanga Township (see **Figure 6:4**). Both prepared (**see Figure 6:5**), and fresh specimens were received in the original packaging and delivered to the laboratory within 12h after collection using a temperature-monitored cool-box. Reserve samples were held at -80°C for purposes of retesting. Meat categorization was done in line with the food categorization scheme recommended by the United States' Interagency Food Safety Analytics Collaboration, with modifications to suit the informal meat trade. On this basis, we categorised meat as raw or processed, including chicken, pork, beef and mutton as categories.[40]

Figure 6:4 The local retail meat stall in the informal slaughter setting in Nyanga.
Sheep are slaughtered on the dirty floor, slaughter waste scattered on the stallside, and meat sold close to the slaughter area.

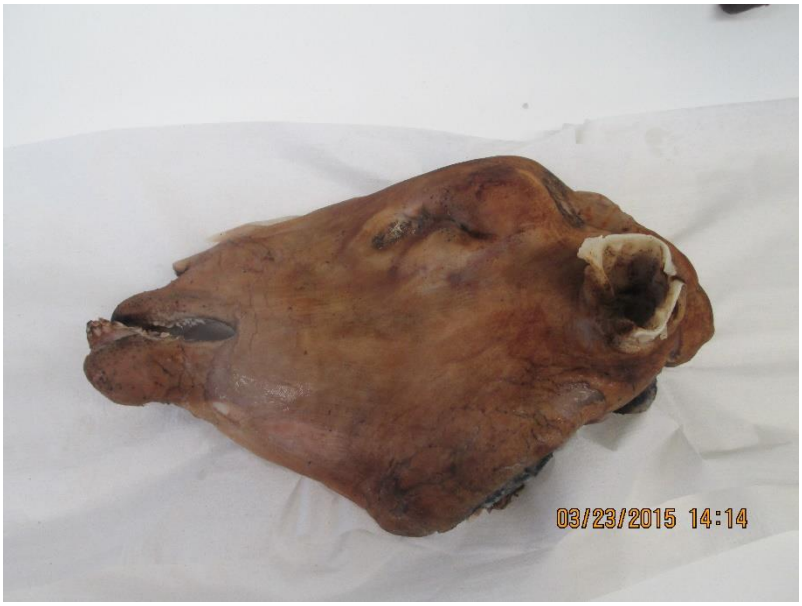

Figure 6:5 Ready -to – eat sheep head that has been processed by scalding in hot water and roasting.

6.3.3 Surface water sample collection from the Lotus River

Sixty-four water samples were collected over a period of ten months between 7 a.m and 12 noon from July 2015 to March 2016 using the depth integrated grab sampling method with samples being collected at a depth of approximately 30cm below the surface of the river towards the centre of the stream.[41] Samples were collected using clean, sterile containers tied to a long pendant rope with the first collection released back into the river and then a volume of 100ml poured into a 100ml sterile collection bottle (see **Figure 6:6** A.). The sample was then immediately transported at 4 ℃ to the City of Cape Town Water and Sanitation microbiology laboratory where it was processed within 24hr of collection.

Figure 6:6 A. The Depth Integrated Grab sampling of Lotus River water using a rope tied to a bucket with water collected from the centre of the stream. B. Water collection point showing the nature of surface water at one of the collection points along the Lotus River (LR16).

Water was collected from four points (LR13, LR14, LR15, and LR16) located along the section of the Lotus River traversing the Nyanga area. These points were previously used by the City of Cape Town Water and Sanitation Division as surveillance points (see **Figure 6:7**).

Figure 6:7 The water collection points along the Lotus River in Nyanga.
LR13, LR14, LR15, and LR16 are water collection points along the Lotus River section that drains the Nyanga informal settlement.

6.3.4 Microbiological analysis of stool, meat and water samples for STEC and other bacterial pathogens.

6.3.4.1 Enrichment for STEC and other foodborne bacterial pathogens

Stool: A pea-sized amount of stool sample was inoculated in 90ml of TSB (Oxoid, Basingstoke, UK) and incubated at 35-37℃ for 24 hours as described in **3.3.3.**

Meat: Twenty-five grammes of tissue in a sterile stomacher bag was thoroughly pummeled using a sterile mortar and pestle, and inoculated into 225ml of Tryptic Soy Broth (TSB; Oxoid, Basingstoke, UK) and incubated at 35°C for 24 hours as previously described but with slight modifications[42] (see **Table 6:1**).

Table 6:1 The food sample processing and enrichment protocol.

Sample type	Portion size	Volume of TSB	Incubation conditions
Ready-to-Eat Foods	25 ± 0.5 g	225 ± 0.5 ml TSB	35 ± 2°C for 18-24 h
Raw Poultry Products	25 ± 0.5 g	225 ± 0.5ml TSB	35 ± 2°C for 18-24 h
Raw Meat	25 ± 0.5 g	225± 0.5 ml TSB	35 ± 2°C for 18-24 h
Whole bird rinsate	25 ± 0.5 ml sample rinsate	225± 0.5 ml TSB	35 ± 2°C for 18-24 h
Sheep heads	25 ± 0.5 ml sample rinsate	225± 0.5 ml TSB	35 ± 2°C for18-24 h

Water: One hundred millilitres of surface water (without dilution) was filtered through a 0.45µM membrane filter (Pall Corporation, Port Washington, USA). The membrane filter was then placed in 90ml of TSB and incubated for 18h at 35℃. This was in accordance with the International Organization for Standardisation guidelines on microbiology of water (ISO 9308-3: 1998).[43]

Enumeration of faecal coliforms in water samples: The mTEC agar enumeration method which provides a direct count of coliforms growing on the surface of the filter membrane was used.[44] This approach is routinely used by the City of Cape Town, Water and Sanitation Division, Athlone. Briefly, six ten-fold serial dilutions of 10ml of surface water sample in 90ml of distilled water were made. A 0.45 µM nitrocellulose membrane (Pall Corporation, Port Washington, USA) was placed in a filter funnel (Pall Corporation, Port Washington, USA) and fixed on a filter funnel manifold (Pall Corporation, Port Washington, USA) (see **Figure 6:8**). The water (100ml at each of

the six dilutions) was poured over the membrane, and the vacuum pump turned on until the water was completely drained. Using sterile forceps, the layer was placed on modified mTEC agar (Difco, Detroit, USA) and incubated at 35°C for two hours to revitalise stressed cells. This was followed by incubation at 44°C for 22 hours as per the routinely used protocol in the environmental water microbiology laboratory at the City of Cape Town microbiology laboratory in Athlone, Cape Town, and according to the ISO guidelines on water processing with slight modifications.[43] Pink coloured colonies were counted and the number of coliforms per 100ml of water sample calculated.

Figure 6:8 Water filtration unit used for the filtration of the water samples, the City of Cape Town Water and Sanitation Division.
The five funnels with blue sterile paper covers are connected to a vacuum pump

6.3.4.2 Isolation and characterization of STEC from TSB

To isolate STEC from TSB enrichments of stool, food and surface water, we streaked a 3mm loopful of TSB on CHROMagar™STEC following overnight incubation and incubated overnight at 37℃ (**Figure 6:9, Figure 6:10, Figure 6:11**). Subsequently, up to five mauve colonies were sub-cultured separately on MacConkey agar with crystal violet and sorbitol MacConkey agar plates. Lactose and sorbitol fermentation properties were determined for each colony after incubation at 37℃ for 24h. Isolate characterization was then done as described in **3.3.5**.

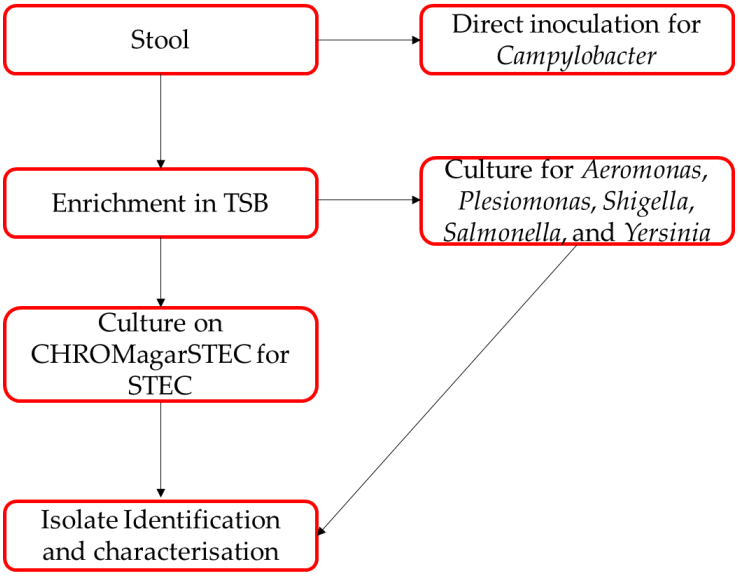

Figure 6:9 Workflow scheme for stool processing

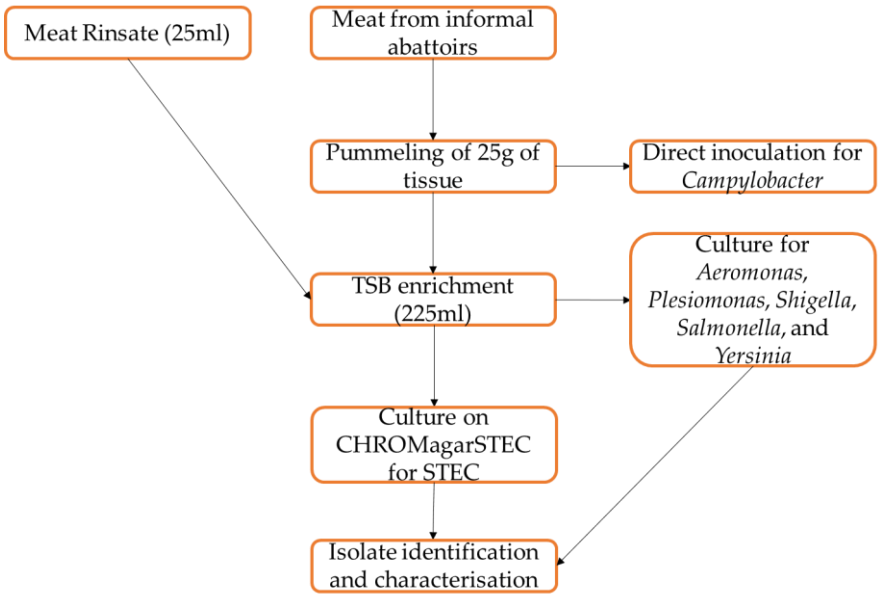

Figure 6:10 Workflow scheme for meat sample processing

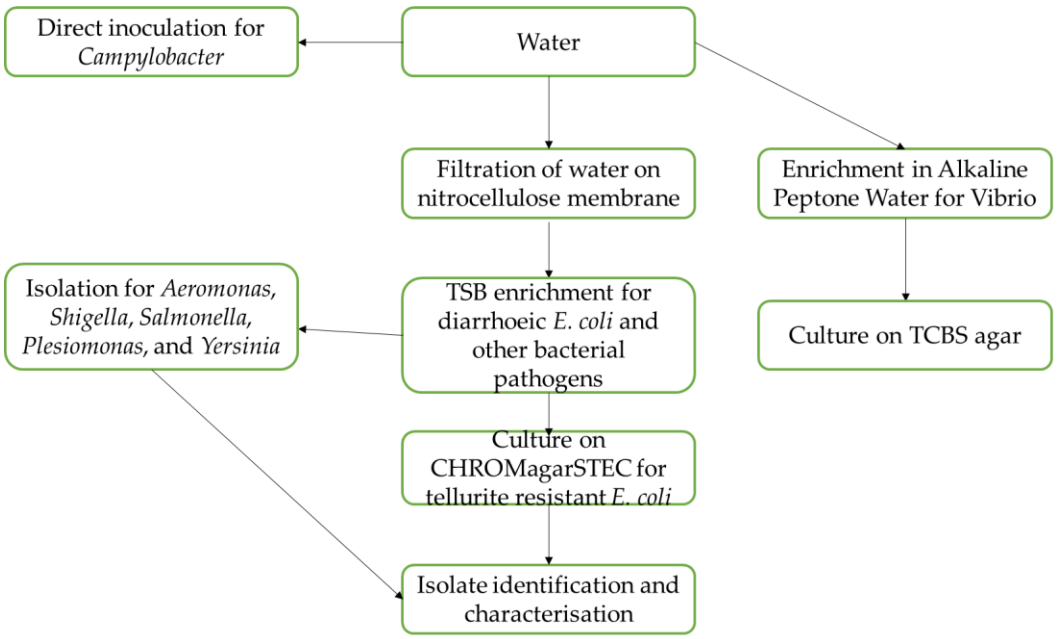

Figure 6:11 Workflow scheme for processing Lotus River surface water.

6.3.4.3 *Detection of serotype-specific O-antigen genes and other DEC virulence genes in TSB*

We screened the TSB for serotype-specific O-antigen genes (*rfbE* for *E. coli* O157 and *wbdl* for *E. coli* O111) and other diarrheic *E. coli* (DEC) virulence genes. The detection of the DEC virulence genes was performed as described earlier in **3.3.5**.

DNA extracted from ATCC strains, *E. coli* ATCC43887 (Positive for the *wbdl*, a marker gene for *E. coli* O111) and *E. coli* C4193-1 (Positive for the *rfbE*, a marker gene for *E. coli* O157) using the boiling lysis method were used as positive controls in the PCR reactions. The following primers were used as previously described (see **Table 6:2**).[45]

Table 6:2 The primers used for the amplification of serotype-specific O-antigen genes in TSB enrichments of stool, water, and meat samples.

Target Gene	PCR primer	Primer sequence	PCR product size
rfbE	O157-F	CGGACATCCATGTGATATGG	259bp
	O157-R	TTGCCTATGTACAGCTAATCC	
wbdl	O111-F	TAGAGAAATTATCAAGTTAGTTCC	406bp
	O111-R	ATAGTTATGAACATCTTGTTTAGC	

6.3.4.4 *Isolation and characterization of Salmonella, Shigella, Vibrio, Plesiomonas, Aeromonas, Yersinia, and Campylobacter*

Salmonella enterica* and *Shigella spp: Following overnight incubation, a 3mm loopful of TSB was used to inoculate an XLD (Xylose Lysine Deoxycholate) agar plate. Red colonies with a black centre (for *Salmonella enterica*) or red colonies (for *Shigella spp*. or *Salmonella* Typhi) were sub-cultured on MacConkey agar with crystal violet and 2% blood agar. The Triple Sugar Iron (TSI), oxidase, indole, urease, and citrate tests (Greenpoint Media Laboratory, NHLS, Albertynshof) were done to determine the biochemical properties of the suspect colonies. The VITEK® 2 system was used to confirm the identification. Speciation of *Shigella* was done using the Wellcolex* colour *Shigella* Rapid latex agglutination test (Oxoid, Basingstoke, UK) which consists of two reagents comprised of coloured latex particles coated with antibodies that are specific for each of the *Shigella* species.

Salmonella enterica were sent to the NICD and serotyped (for the common serotypes) using antiserum specific to the 46 different O antigens and the 119 H antigens (including the Phase 1 and Phase II).

Vibrio spp: For *Vibrio spp.*, 10ml of surface water sample was inoculated in 90ml of Alkaline Peptone Water and incubated at 42 ℃ for eight hours. Subsequently, a 3mm loopful of the surface pellicle of Alkaline Peptone Water (APW) (Greenpoint Media Laboratory, NHLS, Albertynshof) was sub-cultured onto a dried Thiosulfate – Citrate – Bile salts – Sucrose (TCBS) agar plate (Greenpoint Media Laboratory, NHLS, Albertynshof). The plate was incubated for 24h at 35 ℃ with yellow or green colonies sub-cultured on MacConkey agar with crystal violet and incubated for 18h at 35˚C before identification using the VITEK® 2 system.[46]

***Plesiomonas_spp*:** A 3mm loopful of TSB was used to inoculate MacConkey agar with crystal violet and Cefsulodin – Irgasan - Novobiocin (CIN) agar (Greenpoint Media Laboratory, NHLS, Albertynshof) and incubated at 35°C for 24 hours. Non- lactose fermenting colonies on MacConkey agar with crystal violet and opaque colonies with a pink centre on CIN agar were tested for oxidase activity. Further, biochemical testing was possessed out on the oxidase positive

colonies using the spot indole, urea slants, citrate agar slants and TSI slants with confirmatory identification done using the VITEK® 2 system.

Aeromonas spp: A 3mm loopful of TSB was inoculated on Aeromonas-Yersinia agar (Greenpoint Media Laboratory, NHLS, Albertynshof) and incubated at 37°C for 24 hours. Pale colonies with a rose to red centre were tested for oxidase activity. Oxidase positive colonies were then streaked on MacConkey agar with crystal violet and incubated overnight at 35°C before confirmatory identification using the VITEK® 2 system.

Yersinia enterocolitica: Following overnight incubation in TSB, a 3mm loop was used to inoculate a Cefsulodin – Irgasan - Novobiocin (CIN) agar plate. The CIN plate was then incubated overnight at room temperature. Characteristic "bull's eye" colonies (colourless colonies with a red centre) were subjected to further biochemical testing. Catalase positive, non-lactose fermenting, urease positive colonies were identified using the VITEK® 2 system.

Campylobacter spp: Twenty-five millilitres of carcas rinsate was centrifuged at 3000 r.p.m, and the resultant pellet streaked on a Charcoal Cefoperazone Deoxycholate modified Agar (CCDA) plate and incubated under microaerophilic conditions for 72 hours at 35°C. Suspect colonies were Gram stained for characteristic "birds-in-flight" appearance. Oxidase positive colonies with the characteristic Gram appearance were then identified using the VITEK® 2 system.

6.3.4.5 Quality control strains used during isolation of STEC and other foodborne bacterial pathogens

ATCC controls strains for *Yersinia spp.*, *E. coli*, *S. enterica* and *Campylobacter,* were used (see **Table 6:3**). In-house control clinical isolates of *Vibrio spp.*, *Plesiomonas spp.*, and *Aeromonas spp.*, whose identification was initially confirmed using the VITEK® 2 automated system were used. For each run of samples, a known negative sample was spiked with a positive control and run in the same batch and the same way as the test food and water samples, depending on the pathogen being screened for.

Table 6:3 Quality control strains used in the processing of stool, meat, and water samples.

Organism	ATCC strain
STEC	*E. coli* O157 ATCC 35150
Yersinia enterocolitica	*Yersinia enterocolitica* subs. enterocolitica
Campylobacter spp.	*Campylobacter jejuni* ATCC 33292D-5
Salmonella enterica	*S. enterica* subsp. enterica serovar Pomona strain B (ATCC® BAA-2653™)
Aeromonas hydrophila	In – house, positive control isolates used. Identified using VITEK 2® automated system
Plesiomonas shigelloides	In – house, positive control isolates used. Identified using VITEK 2® automated system
Vibrio vulnificus	In – house, positive control isolates used. Identified using VITEK 2® automated system
Vibrio metchnikovii	In – house, positive control isolates used. Identified using VITEK 2® automated system

6.3.4.6 Antimicrobial Susceptibility Testing of STEC and other bacterial pathogens

Gram-negative bacterial pathogens were subcultured on MacConkey agar with crystal violet and sent for identification and antimicrobial susceptibility testing using the GNI card on the VITEK® 2 automated system as described in **3.3.3**. For all the bacterial pathogens, species-specific break points, as per the CLSI 2015 guidelines (U.S.A), were used. The WHONET software has these breakpoints built-in.

6.3.5 Data Analysis

Questionnaire information was filled into Epi Info 7™ (CDC, USA) for analysis. Data analysis was done using Epi Info 7™ (Centre for Disease Control, Atlanta, USA) and STATA (STATA Corp LLC, College Station, Texas, USA). Comparison of proportions and statistical significance were calculated using a two-tailed Chi-square test or Fischer's exact test, with $p<0.05$ considered significant.

Antimicrobial susceptibility MIC data were analysed using the WHONET version 5.6 software. Within the software, the CLSI interpretation guidelines were used to categorise isolates as resistant, intermediate or susceptible to selected antimicrobials. The types of analysis undertaken with this software included determination of the percentage of isolates that were Resistant or Susceptible and generation of resistance profiles.

6.4 Results

6.4.1 Epidemiological and clinical and data of children with diarrhoea attending Nyanga Community Health Center.

6.4.1.1 Area of origin of diarrhoea cases

Of the 66 patients recruited in this study, 56 (84.8%) were residents of the Nyanga township (Area B), 6 (9.1%) were from the Khayelitsha township (Area A) while 4 (6.1%) were from the Crossroads township (Area C) (see **Figure 6:12**).

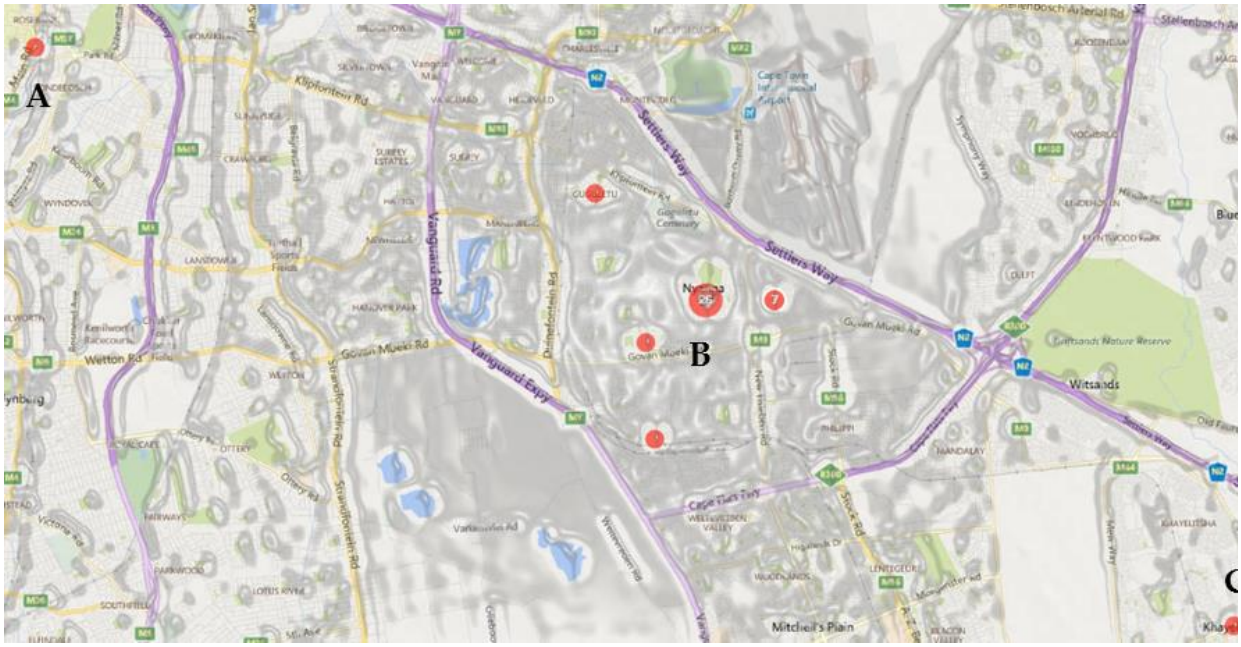

Figure 6:12 Map showing the areas of origin of patients that attended the Nyanga Community Health Center.
Area **A=Crossroads, Area B= Nyanga, Area C= Khayelitsha**

Red dots represent diarrhoea cases -the larger dots correspond to a higher number of cases.

6.4.1.2 Temporal distribution of diarrhoea cases over the study period

Every patient that presented with diarrhoea during the enrollment days was recruited into the study. Most cases were recruited in February 2016 and the lowest number in October 2015 (see **Figure 6:13**). There was a bimodal peak (November 2015 and February 2016) in the number of patients recruited over the study period.

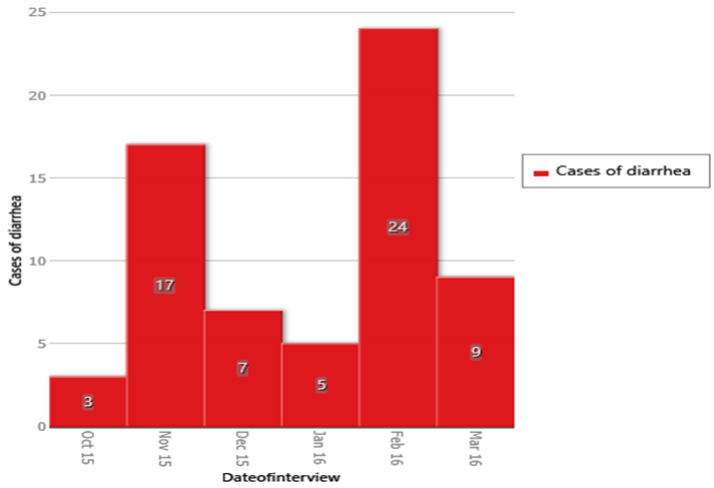

Figure 6:13 The number of cases recruited per month for the study period.

6.4.1.3 Clinical data collected at enrollment

The mean duration of diarrhoea was 2.5 days. The most common symptom was weakness and dehydration (30/66, 46%), and was noted among patients infected with DECs, *Plesiomonas shigelloides*, and *Shigella*. Of all the cases, 5%, 20%, and 17% presented with bloody stool, vomiting, and fever respectively. Fever was noted among patients with *Salmonella*, *Plesiomonas shigelloides*, and *Shigella*. Blood in stool was observed only in a single patient for each of STEC and *Aeromonas* infections. Blood in stool was not noted among any of the patients from whom *Shigella* was cultured. Only 2% (1/66) of the recruited children had a history of immediate prior antibiotic use before recruitment into this study (see **Table 6:4**). The proportion of children that were using antibiotics immediately prior to visiting the health centre was significantly higher (p=0.024) among those that presented with fever 9% (1/11) compared to those that didn't 0% (0/55).

Table 6:4 Clinical characteristics of enrolled participants, stratified by bacterial pathogen.

Characteristic	All Cases (n=66)	STEC (n=1)	DECs (n=15)	Campylobacter (n=3)	*Aeromonas* (n=2)	*Salmonella* (n=4)	*Plesiomonas*(n=6)	*Shigella* (n=11)
Duration of diarrhoea								
Mean duration of diarrhoea (±SD) days	2.4 (1.5)		1.7 (0.98)	1.67 (1.52)	3 (1.4)	2.5 (1.7)	2.5 (1.37)	1.2 (0.82)
Number presenting with symptom								
Blood in stool	3	1	1	-	1			
Vomiting	13	-	5	-	1	-	1	2
Fever (>38℃)	11	-	4	-		2	1	2
Weakness and dehydration	30	-	3	-	-	-	2	1
Cough	6		1	-			1	2
Belly pain	3	-	-	-	-	-	1	2
Antibiotic use								
Antibiotic use	1	-	-	-	-	-	-	1

***N/A= Not applicable; A bacterial pathogen was not isolated from some of the patients that presented with the symptoms.**

6.4.1.4 *Epidemiological data acquired using questionnaires from patients attending Nyanga Community Health Center*

Of the 66 outpatients with acute diarrhoea that were recruited in the study, 36 were female while 30 were male. The mean age in months of the cases was 14.9 months (±10.9). The mean number of children under 5 years of age per household was two while the mean household size was 4 persons.

Thirty percent of the patients had domestic animals in their households. The commonest animal kept was the dog (kept by 17% of the households).

Most of the children were brought to the clinic by their mothers as the primary caregivers (85%), and only 24% of the mothers were still breastfeeding their children. Of the primary caregivers, 11%, 74%, and 6% had a primary, secondary and tertiary level of education respectively.

Seventeen percent of the study recruits were brought to the clinic for immunisation and routine health checks and not for purposes of illness (see **Table 6:5**).

Of the study recruits, 39% reported having received rotavirus vaccination and 85% of the households disposed of the child's faecal waste into the domestic refuse bin.

Seventy-nine percent of the households (of the enrolled patients) used the communal standpipes as the primary source of water for daily use while 21% used in-house piped water.

Table 6:5 Epidemiologic data from patients attending the Nyanga Community Health Center.

	Number (%)	95% Confidence Interval (CI)	
		Lower CI	Upper CI
Sex			
Male	30 (45%)	34	57
Female	36 (55%)	43	66
Animals kept at home			
Dog	11 (17)	7.4	25.9
Cat	5 (8)	1.0	14.1
No animal	46 (70)	58.3	81.1
Other	4 (6)	2.4	14.6
Primary caregiver			
Mother	56 (85)	74.3	91.6
Grand Mother	2 (3)	1.2	7.3
No relation	1(2)	0.3	8.0
Aunt	1 (2)	0.3	8.0
Sister	2 (3)	1.2	7.3
Other	2 (3)	1.2	7.3
Level of education of primary caregiver			
Primary	7 (11)	5.2	20.3
Secondary	49 (74)	62.6	83.3
Tertiary	6 (9)	4.2	18.5
Religious Education	4 (6)	2.4	14.6
Purpose of visit to the clinic			
Immunization	5 (8)	3.3	16.5
Sickness	55 (83)	72.6	90.4
Routine Health check	6 (9)	4.2	18.45
Breastfeeding status			
Breast feeding	16 (24)	13.6	34.9
Rotavirus immunization			
Rotavirus immunized	26 (39)	27.3	51.49
Disposal of soiled nappies			
Bin	56 (85)	76	93.73
Source of water for domestic use			
House piped water	14 (21)	9.1	33.4
Communal stand pipe	52 (79)	66.6	90.86

6.4.2 Microbiological analysis results

6.4.2.1 *Foodborne bacterial pathogens and DEC virulence genes identified from stool of children with diarrhoea and from environmental samples*

A summary of the foodborne bacterial pathogens and diarrheic *E. coli* virulence genes detected in the stool of children with diarrhoea, Lotus River surface water, and meat from the informal abattoirs in Nyanga, Cape Town is given in **Table 6:6**.

Table 6:6 Prevalence of foodborne bacterial pathogens and diarrheic E. coli virulence genes in the stool, Lotus River surface water, and meat from the informal abattoirs in Nyanga.

Pathogens isolated per sample type n (%)			
Pathogen	**Human (n=66)**	**Meat (n=85)**	**Water (n=64)**
Salmonella enterica	4 (6)	4 (5)	-
Shigella flexneri.	11 (17)	-	3 (5)
Plesiomonas shigelloides	6 (9)	1 (1)	11 (17)
Aeromonas sorbria.	2 (3)	3 (3)	4 (6)
Campylobacter jejuni.	3 (5)	6 (7)	-
DAEC	8 (12)	2 (2)	2 (3)
EaggEC	2 (3)	-	1 (2)
STEC	1 (2)	-	1 (2)
EPEC	1 (2)	1 (1)	-
EIEC	1 (2)	-	-
Vibrio vulnificus	-	-	6 (9)
Yersinia enterocolitica	-	-	-
Genes			
aat	9 (14)	-	-
daaC	23 (35)	6 (7)	24 (38)
stx_1	4 (6)	17 (20)	19 (30)
stx_2	2 (3)	4 (5)	-
Ipa	4 (6)	-	5 (8)
LT	5 (8)	1 (1)	21 (33)
rfbE	1 (2)	5 (6)	9 (14)
wbdl	1 (2)	-	5 (8)
aat	9 (14)	-	-
daaC	23 (35)	6 (7)	24 (38)
eaeA	-	6 (7)	-

6.4.2.1.1 Bacterial pathogens and DEC virulence genes detected in stool

A bacterial pathogen was isolated from 36 (55%) of the 66 patients with diarrhoea. From 6 (6/36, 17%) of these, more than one bacterial pathogen was isolated (*Shigella flexneri* and STEC-one patient, *Shigella flexneri* and *Campylobacter jejuni jejuni*-one patient, *Aeromonas sorbria* and DAEC-

one patient, *Shigella flexneri* and DAEC-one patient, DAEC and EAEC-one patient, DAEC and EaggEC-one patient). Of the 66 stool samples that were cultured on CHROMagar™STEC, the formation of mauve colonies was observed for thirteen of the samples (20%). All the mauve colonies were confirmed to be *E. coli.* Of these 13, eight were DAEC, two were EAggEC, one was STEC, one was EPEC, and one was EIEC. The single STEC isolate from stool belonged to serotype O106. Of the eight DAEC isolates, six were non-typeable while two belonged to serotype O153. Of the two EAggEC, one was non-typeable while one belonged to serotype O143. The single EPEC belonged to serotype O49, while the single EIEC was non-typeable.

Of the 66 stool samples, 23 samples (35%) tested positive for the *daaC* gene, a virulence marker for DAEC (compared to the 8 samples (12%) from which tellurite resistant DAEC were isolated- these 8 stool samples also tested positive for the *daaC*). Four stool samples (6%) possessed the *ipa*, a virulence marker for EIEC- but which can also be detected in *Shigella* (compared to only one sample from which tellurite resistant EIEC were isolated- this one sample also tested positive for the *ipa*). Five stool samples (8%) possessed the *LT* (see **Figure 6:14***)*, a virulence marker for ETEC (no tellurite resistant ETEC were isolated from any of the stool samples).

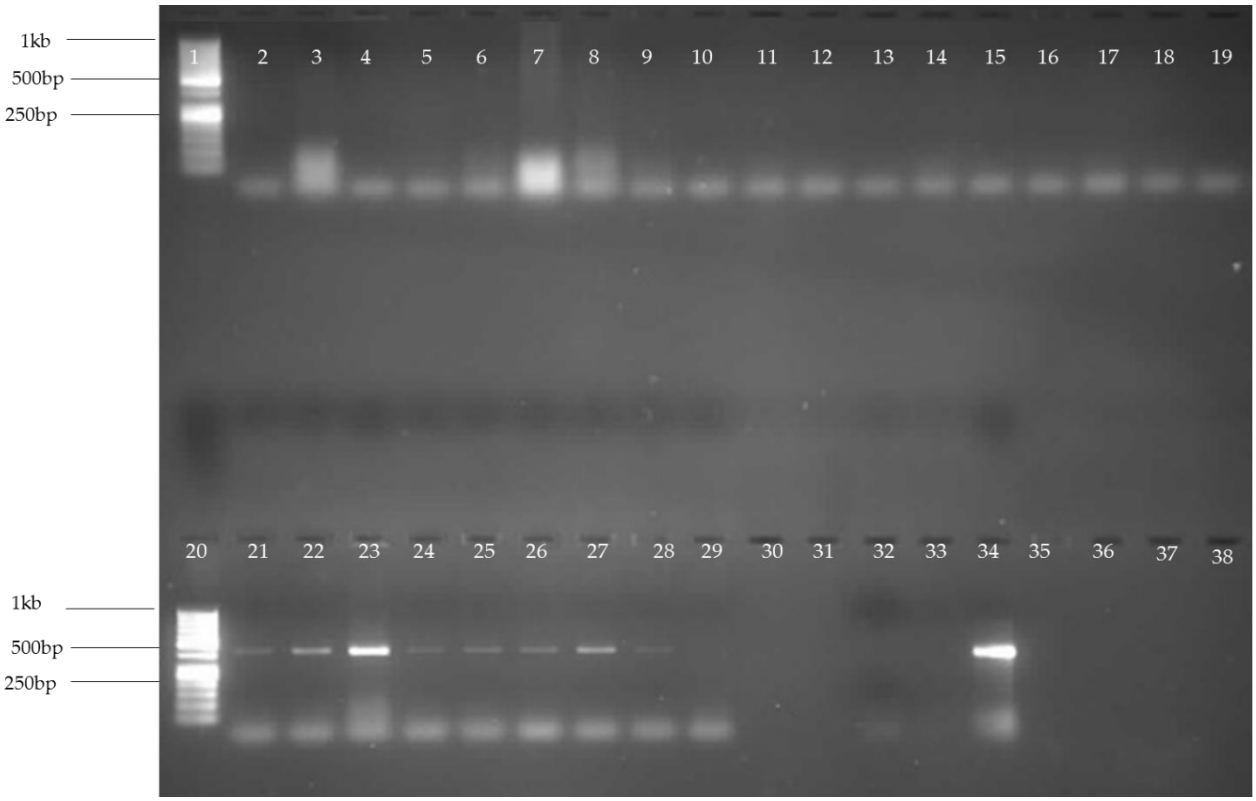

Figure 6:14 Electrophoresis gel image obtained after running a PCR to detect the LT gene (size 440bp).
Lane 1 and 20 contains the 1kb ladder (GeneRuler™ DNA molecular weight ladder -ThermoFisher Scientific Inc., MA, USA). Lanes 2-38 contained samples except for lane 20 (contained the 1kb ladder), lane 32 which was the negative control and lane 34 which was the positive control. Samples in lanes 21-28 were positive while the rest of the samples were negative.

Nine stool samples (14%) possessed *aat* (see**Figure 6:15**), a virulence marker for EAggEC (compared to 2 samples from which tellurite resistant EAggEC were isolated- these two samples also tested positive for the *aat*).

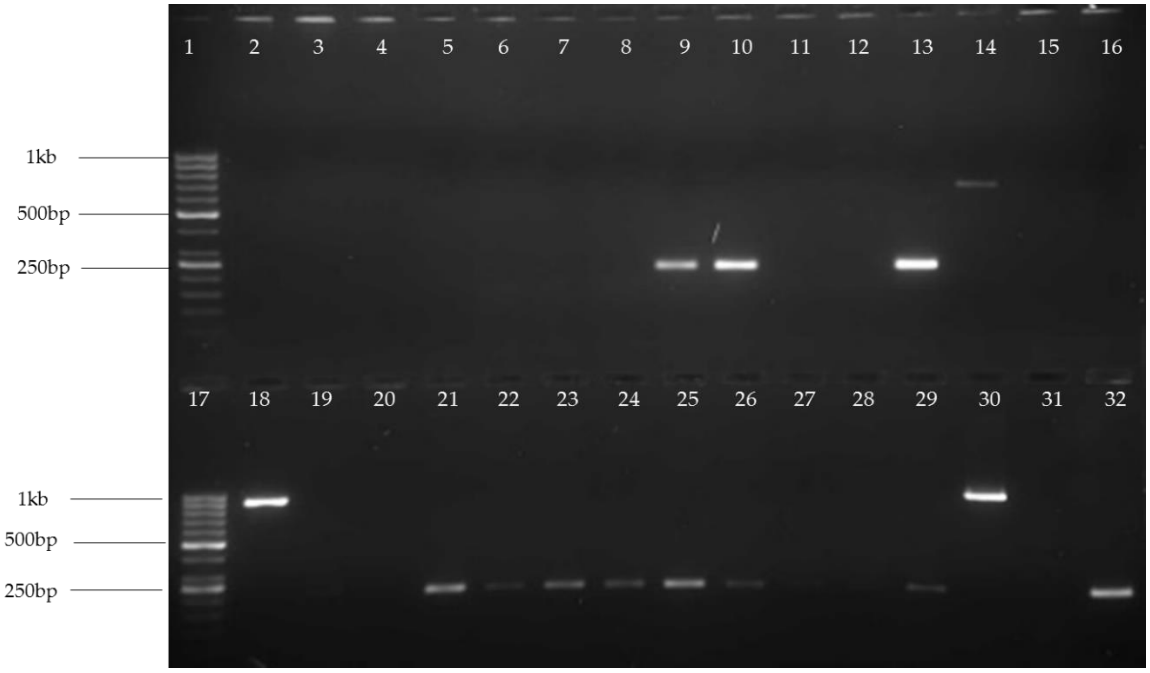

Figure 6:15 Electrophoresis gel image for PCR product following multiplex PCR amplification for aat, ipa, and daaC. **Lanes 1 and 17 contained the 1kb ladder (GeneRuler™ DNA molecular weight ladder -ThermoFisher Scientific Inc., MA, USA). Lanes 2-32 contained samples except for lanes 14 (positive control for *aat*),18 (positive control for *ipa*), 19 (negative control), 32 (positive control for *daaC*). Samples in lanes 9, 10, 13, 21, 22, 23, 24, 25, 26, and 29 were positive for *daaC*. The sample in lane 30 was positive for *ipa*. The rest of the samples were negative for *aat*, *ipa*, and *daaC*.**

The *wbdl* gene (for *E. coli* serotype O111) and the *rfbE* gene (for *E. coli* serotype O157: H7) were detected in a single specimen each (none of the tellurite resistant diarrheic *E. coli* isolated from stool belonged to serotype O111 or serotype O157).

Upon screening TSB from clinical samples for *stx* genes, stx_1 and stx_2 were detected in 4 (6%) and 2 (3%) specimens respectively (compared to one sample from which a tellurite resistant STEC carrying stx_1 was isolated – no stx_2 carrying tellurite resistant STEC was isolated; the one sample from which STEC was isolated also tested positive for stx_1).

Overall, a bacterial pathogen was isolated, or a DEC virulence marker gene detected in 48 (73%, 48/66) stool samples. For all the pathotypes, more of the virulence marker genes were detected in TSB enrichment of stool as compared to the actual number of diarrheic *E. coli* pathotypes isolated on CHROMagar™STEC. This could be explained by the fact that not all diarrheic *E. coli* are tellurite resistant and so would not form colonies on CHROMagar™STEC.

6.4.2.1.2 Bacterial pathogens and diarrheic *E. coli* virulence genes in meat

Of the 85 meat samples, mauve colony formation on CHROMagar™STEC was noted for 32 (38%). From the 32 samples, 32 mauve colonies were picked all of which were identified to be *E. coli*. Only three of the 32 *E. coli* possessed virulence genes. Of these three, two possessed the *daaC* genes (DAEC) while one possessed the *eae* gene (EPEC). None of the mauve colonies had *stx* genes. The two DAEC and one EPEC were non-typeable.

Following screening of the 85 TSB enrichments of meat samples using real-time and gel-based PCR, *stx*$_1$ (20%, 17/85), *stx*$_2$ (5%, 4/85), *eae* (7%, 6/85), *aat* (1%, 1/85), *daaC* (7%, 6/85), *rfbE* (6%, 5/85), and *lt* (1%, 1/85) were detected. Comparatively, no tellurite resistant STEC was isolated from any of the meat samples while one tellurite resistant EPEC (the meat sample from which the EPEC strain was isolated also tested positive for *eae*), and two tellurite - resistant DAEC (the two meat samples from which the DAEC were isolated also tested positive for the *daaC*) were isolated from the meat samples. The most commonly detected virulence gene in all the meat samples was the *stx*$_1$ genes. These were detected in 17 (20%) of the meat samples (see **Table 6:7**).

For all sample types, more of the virulence marker genes were detected in TSB enrichments as compared to the actual number of diarrheic *E. coli* pathotypes isolated.

In addition, the following bacterial pathogens were isolated: *Salmonella enterica Idikan* (5%, 4/85), *Plesiomonas shigelloides* (1%, 1/85), *Aeromonas sorbria* (3%, 3/85), *Campylobacter jejuni jejuni* (7%, 6/85), EPEC (1%, 1/85) and DAEC (2%, 2/85).

6.4.2.1.2.1 Foodborne bacterial pathogens and DEC virulence gene detection rates per meat type

Raw meats: Mainly the raw beef cuts possessed the *stx*$_1$ genes (53% of the beef cuts, n=17) while 14% (3/22) of the raw pork samples possessed the *stx*$_1$ genes (see **Table 6:7**). The *rfbE* gene, which signifies the presence of *E. coli* O157, was detected only in the raw meats including beef cuts, mutton, pork, and chicken. Only one of the raw pork samples possessed the *aat* genes.

Ready-to-eat meats: Among the ready to eat meats, 12% (3/26) of the roast beef possessed *stx*$_1$ while 13% (1/8) and 13% (1/8) of the roast pork samples possessed the *stx*$_1$ and *stx*$_2$ genes respectively. The *daaC* genes were detected only in the roast beef. Of all the ready-to-eat meats,

the *LT* gene was detected in only one pork sample while the *ipa* gene was not detected in any meat sample.

Table 6:7 Distribution of DEC virulence genes and bacterial pathogens in the different meat types from the informal trade in Nyanga.

		Number of samples with virulence gene/ bacterial pathogen (%)											
Food type (n)	**Category**	***stx*$_1$**	***stx*$_2$**	***eaeA***	***aat***	***daaC***	***RfbE***	***LT***	***Salmonella***	***Campylobacter***	***Plesiomonas***	***Aeromonas***	**DEC**
Beef cuts (17)	Raw	9 (53)	1 (6)	2 (12)	-	3 (18)	1 (6)	-	1 (6)	-	1 (6)	2 (12)	2 (12)
Mutton (9)	Raw	1 (11)	-	-	-	-	1 (11)	-		1 (11)	-	1 (11)	-
Pork (22)	Raw	3 (14)	2 (9)	3 (14)	1 (5)	-	1 (5)	1 (5)		-	-	-	1 (5)
Chicken (11)	Raw	-	-	-	-	-	2 (18)	-	3 (27)	5 (45)	-	-	-
Roast beef (26)	Processed	3 (12)	-	1 (4)	-	3 (12)	-	-		-	-	-	-
Roast pork (8)	Processed	1 (13)	1 (13)	-	-	-	-	-		-	-	-	-
Roast chicken (3)	Processed	-	-	-	-	-	-	-		-	-	-	-
Total		**17**	**4**	**6**	**1**	**6**	**5**	**1**	**4**	**6**	**1**	**3**	**3**

DEC= Diarrheic *E. coli*, *rfbE*=putative perosamine synthetase gene, *daaC*= fimbrial adhesion gene, *LT*=gene for the heat-labile toxin of ETEC, *aat*= an anti-aggregation protein transporter gene, *stx*=Shiga toxin gene, *eae*= intimin encoding gene." – "means that the gene was not detected or the pathogen was not isolated.

6.4.2.1.3 Bacterial pathogens and DEC virulence genes detected in TSB enrichments of Lotus River surface water

From the 64 water samples, 16 formed mauve colonies (all the 16 were identified as *E. coli*) on CHROMagar™STEC. Of the 16 *E. coli*, only four isolates had virulence genes. Overall, from the 64 surface water samples, the following bacterial pathogens were isolated: STEC (2%), DAEC (3%), EPEC (2%), EaggEC (2%), *Shigella flexneri* (5%), *Plesiomonas shigelloides* (17%), *Aeromonas sorbria* (6%), and *Vibrio vulnificus* (9%). The proportion of all bacterial pathogens from the Lotus River was highest at collection point LR13 ($p<0.001$), and lowest at collection point LR16. *Aeromonas sorbria* was isolated from point LR15 and LR16 only while *Vibrio vulnificus* and *Shigella flexneri* were isolated from all points except LR16. *Plesiomonas shigelloides* was isolated from all the water collection points (**Table 6:8**).

Table 6:8 Diarrhoeal Pathogens isolated from the water collection points along the Lotus River.

Site	**Diarrhoeal pathogens detected**									
	STEC	*Salmonella*	EPEC	EAggEC	*Aeromonas*	DAEC	*Vibrio* spp.	*Shigella*	*Plesiomonas*	Total
LR13	1	ND	ND	1	ND	1	4	1	2	10
LR14	ND	ND	ND	ND	ND	ND	1	1	4	6
LR15	ND	ND	ND	ND	3	ND	1	1	4	9
LR16	ND	ND	ND	ND	1	1	ND	ND	1	3
Total	1	-	-	1	4	2	6	3	11	28

ND= not detected, LR13, LR14, LR15, and LR16 are surface water collection points along the Lotus River.

Stx1 genes were detected in 30% (19/64) of the water samples (compared to only one STEC isolated from the water samples- the one sample from which the single STEC was isolated also tested positive for *stx1*) (**Figure 6:16**).

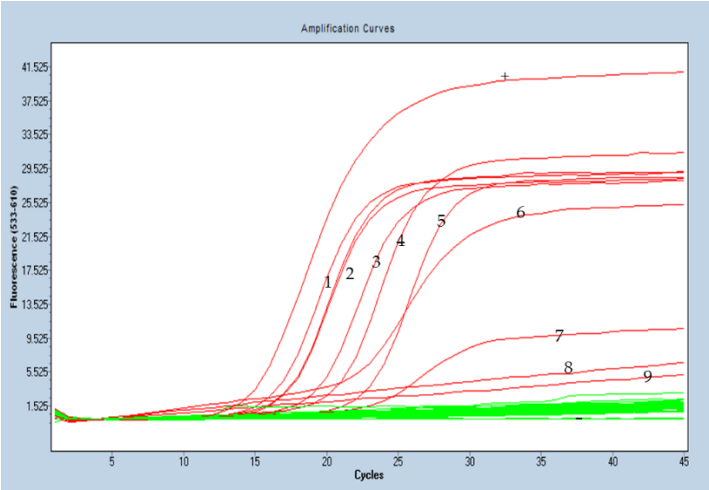

Figure 6:16 Amplification curves following Real-time PCR for stx_1.

Curves 1-9 represent test samples. The curve labelled + represents the positive control while the curve labelled – represents the negative control. Water samples represented by curves 1-6 were considered positive while samples represented by curves 7-9 were considered negative.

The *rfbE* gene for STEC O157 and the *wbdl* gene for STEC O111 were detected in 14% (9/64) and 8% (5/64) of the water samples respectively (none of the diarrheic *E. coli* isolated from the Lotus River surface water was *E. coli* serotype O157 or *E. coli* serotype O111).

The *eaeA* gene was detected in 14% (9/64) of the surface water samples (No EPEC was isolated from any of the Lotus River surface water samples). The most commonly detected gene in the surface water samples was the *daaC* which was detected in 38% (24/64) of the surface water samples (compared to the two tellurite-resistant DAEC that were isolated from the Lotus River surface water. These two DAEC were positive for the *daaC*). The *LT* and *ipa* were detected in 33% (21/64) and 8% (5/64) of the surface water samples respectively (no ETEC or EIEC were isolated from any of the surface water samples). The prevalence of stx_1 genes was highest at site LR13 compared to other sites along the Lotus River ($p = 0.020$) (**Table 6:9**). For all the pathotypes, more of the virulence marker genes were directly detected from TSB-enriched surface water as compared to the actual number of diarrheic *E. coli* pathotypes isolated on CHROMagar™STEC. This could be explained, in part, by the fact that not all diarrheic *E. coli* are tellurite resistant and so will not grow on CHROMagar™STEC or the presence of non-viable bacteria in the water.

Table 6:9 Number of E. coli virulence genes at water collection points along the Lotus River.

Site	Number of *E. coli* virulence genes (n=64)						
	IpaH	***eaeA***	***stx*$_1$**	***rfbE***	***wbdl***	***daaC***	***LT***
LR13	0	6	8	3	2	5	6
LR14	2	3	3	3	1	7	5
LR15	2	0	1	1	1	4	3
LR16	1	0	7	2	1	8	9
Total	5	9	19	9	5	24	21

***rfbE*=putative perosamine synthetase gene, *daaC*= fimbrial adhesion gene, *LT*=gene for the heat-labile toxin of ETEC, *aat*= an anti-aggregation protein transporter gene, *stx*=Shiga toxin gene, *eae*= intimin encoding gene, *wbdl* = O111 antigen gene.**

6.4.2.2 Lotus River surface water faecal coliform counts per millilitre of surface water

Overall, the water collection point LR16 had the highest mean coliform CFU/ml of surface water while LR13 had the lowest (see **Table 6:10**). There was no significant difference in the mean CFU/ml for the different collection points (p=0.5908). The month of February 2016 had the highest CFU/ml as compared to the other months (p= < 0.001) while March 2016 had the lowest mean CFU/ml.

Table 6:10 Faecal coliform counts (CFU/ml) for water collection points LR13, LR14, LR15, and LR16.

Site	Mean Faecal coliform counts (CFU/ml)											
Month	**Jun./15**	**Jul./15**	**Aug./15**	**Sept./15**	**Oct./15**	**Nov/15.**	**Jan./16**	**Feb./16**	**Mar./16**	**Apr./16**	**Mean**	**Std. Dev.**
LR13	5.E+04	5.E+05	9.E+04	8.E+04	2.E+05	5.E+05	4.E+05	7.E+05	2.E+04	2.E+05	2.74E+05	234908.9
LR14	1.47E+06	8.71E+05	1.02E+05	1.01E+05	1.02E+06	2.01E+05	5.00E+05	5.30E+06	6.00E+03	8.70E+05	1.04E+06	1571143.8
LR15	1.36E+05	1.77E+06	8.60E+04	9.90E+04	5.65E+05	4.26E+05	7.80E+05	3.85E+06	3.00E+03	1.60E+05	7.88E+05	1198343
LR16	8.72E+05	1.22E+06	8.00E+04	4.60E+04	5.25E+05	1.25E+05	1.60E+05	7.32E+06	3.30E+04	6.90E+05	1.11E+06	2220536
Mean	6.32E+05	1.09E+06	8.95E+04	8.15E+04	5.78E+05	3.13E+05	4.60E+05	4.29E+06	1.55E+04	4.80E+05		
Std. Dev	669473	540173	9291.5	2548.6	3372 56.1	1785 36.6	256645	2785837.6	1382 0.3	3544 94.9		

Std.Dev.= Standard deviation

6.4.3 Antimicrobial susceptibility of STEC, and other bacterial foodborne pathogens from stool, water, and meat from the informal abattoirs in Nyanga

6.4.3.1 Antimicrobial susceptibility patterns of STEC and other diarrheic E. coli from the stool, water, and meat.

Diarrheic *E. coli* from the environment (Lotus River surface water and meat from informal abattoirs) and children with diarrhoea may be considered both as major foodborne pathogens and sentinel organisms (an indicator organism used for monitoring antimicrobial resistance in faecal bacteria because they are ubiquitous in a wide range of hosts, acquire resistance easily, and are reliable indicators of resistance in other foodborne pathogens).[47] We isolated 3, 13 and 4 DECs from meat, stool, and water respectively. Overall, amongst these 20 isolates, there was resistance to the following antibiotics: ampicillin (8/20, 40%), amoxicillin-clavulanate (3/20, 15%), cefoxitin (2/20, 10%), cefuroxime (2/20, 10%), cefotaxime (2/20, 10%), cefepime (1/20, 5%), ciprofloxacin (1/20, 5%), trimethoprim-sulfamethoxazole (20/20, 100%), and nitrofurantoin (1/20, 5%).

Two isolates were ESBL-producing (resistant to cefotaxime and confirmed using the ESB1F sensititre plate).

Only three isolates were multi-drug resistant and were isolated from meat (one isolate) and stool (two isolates).

Of the two STEC isolated, the isolate from water was susceptible to all the antimicrobials tested except SXT while the other, from a child, was resistant to AMP and SXT.

There was no significant difference between resistance rates of diarrheic *E. coli* from children with diarrhoea and those isolated from the environment (see**Table 6:11**).

Table 6:11 Differences in the rates of resistance to selected antibiotics for isolates from human (stool from children), and non-human sources (water and meat) sources in Nyanga.

Percentage resistant to antibiotic per source (Child, N=13, Environment, N=7)			
Antibiotic	**Children n (%)**	**Environment n (%)**	**p-value**
AMP	5, (38)	3 (43)	0.84
AMC	2, (15)	2 (29)	0.45
FOX	2 (15)	- (0)	0.28
CXM	2 (15)	1 (14)	0.95
CXA	2 (15)	1 (14)	0.95
CTX	2 (15)	-(0)	0.28
FEP	1 (8)	- (0)	0.44
NIT	1 (8)	- (0)	0.44
CIP	- (0)	1 (14)	0.17

Notably, resistance to ciprofloxacin was only noted for one environmental isolate. AMP=ampicillin, AMC=amoxicillin-clavulanate, FOX=cefoxitin, CXM=cefuroxime, CXA=cefuroxime-axetil, CTX=cefotaxime, FEP=cefepime, NIT=nitrofurantoin, CIP=ciprofloxacin.

6.4.3.2 *Antimicrobial susceptibility patterns of the Shigella, Salmonella, Aeromonas, Plesiomonas shigelloides, Campylobacter, and Vibrio from the stool, meat and Lotus River surface water in Nyanga.*

Shigella: *Shigella* showed resistance to ampicillin (67%, 10/15), amoxicillin-clavulanate (67%, 10/15), cefuroxime (47%, 7/15), cefuroxime axetil (47%, 7/15), cefoxitin (53%, 8/15), amikacin (47%, 7/15), and gentamicin (47%, 7/15)**.** (see **Table 6:12**).

Table 6:12 Resistance profiles of Shigella spp. from meat, water, and stool in Nyanga (N=15).

Resistance profile	Number of isolates	Percentage of Isolates
FOX	1	7
AMP AMC	3	21
CXM CXA FOX	2	14
AMP AMC FOX	1	7
AMP AMC CXA	2	14
AMP AMC CXM FOX	1	7
AMP AMC CXM CXA FOX	3	21

FOX-cefoxitin, AMP-ampicillin, AMC-amoxicillin clavulanate, CXM-cefuroxime, CXA-cefuroxime-axetil.

The antibiotic susceptibility patterns for *Salmonella enterica*, *Aeromonas sorbria*, *Plesiomonas shigelloides*, *Vibrio vulnificus*, and *Campylobacter jejuni* from the different sources are as shown in the table below (**Table 6:13**).

Table 6:13 Percentage Resistant per specimen type for Salmonella, Shigella, Plesiomonas, Vibrio, and Aeromonas.

	***Salmonella* n (%R)**		***Shigella* n (%R)**		***Plesiomonas* n (%R)**		***Aeromonas* n (%R)**		**Vibrio n (%R)**
Antibiotic	**Meat (n=4)**	**Stool (n=4)**	**Water (n=4)**	**Stool (n=11)**	**Water (n=11)**	**Stool (n=6)**	**Meat (n=2)**	**Stool (n=1)**	**Water (n=6)**
AMP	-	-	3 (75)	7 (64)	11 (100)	6 (100)	2 (100)	1 (100)	-
AMC	-	-	3 (75)	7 (64)	-	-	-	-	-
CXM	NT	NT	-	6 (55)	-	-	-	-	-
CXA	NT	-	1 (25)	6 (55)	-	-	-	-	-
FOX	4 (100)	1 (25)	-	8 (73)	2 (18)	-	-	-	6 (100)
CAZ	-	-	-	-	-	1 (17)	-	-	-
ETP	-	-	-	-	-	-	1 (50)	1 (100)	6 (100)
AMK	NT	-	-	7 (64)	-	-	-	-	1 (17)
GEN	NT	NT	-	7 (64)	2 (18)	-	-	-	-
CIP	4 (100)	4 (100)	-	-	-	-	-	-	-
TGC	-	-	-	-	-	-	--	1 (100)	-
SXT	3 (75)	4 (100)	4 (100)	11 (100)	11 (100)	6 (100)	2 (100)	1 (100)	6 (100)

***NT= Not Tested for. *Campylobacter* from water, stool, and the meat were all susceptible to ciprofloxacin and thus not included in this table. *Salmonella* not tested against amikacin, gentamicin, cefuroxime and cefuroxime axetil as they are inactive against the agent in-vivo. Expert interpretation rules embedded in the WHONET software were used to classify as resistant or susceptible. The CLSI clinical guidelines and breakpoints were used to analyse the data sets. AMP=ampicillin, AMC=amoxicillin-clavulanate, CXM=cefuroxime, CXA=cefuroxime axetil, FOX=cefoxitin, AMK=amikacin, GEN=gentamicin, CIP=ciprofloxacin, SXT=trimethoprim-sulphamethoxazole, NIT=nitrofurantoin.**

6.5 Discussion

The key findings of this study were; (1) the patients that presented with diarrhoea at the Nyanga Community Health Centre were less than two years old and presented with an acute form of diarrhoea, (2) for a high number of patients (73% - 48/66), we detected at least one bacterial pathogen in stool- the most prevalent bacterial pathogen being *Shigella*, (3) the number of diarrheic *E. coli* virulence genes detected in the TSB enrichments was higher than the actual number of diarrheic *E. coli* isolated from human stool, Lotus River surface water, and meat from the informal abattoirs, (4) meat from the informal abattoirs and surface water from the Lotus River carried bacterial pathogens that could cause diarrhoea, (5) surface water from the Lotus River surface water possessed a high number of faecal coliforms indicating faecal contamination., (6) there was no significant difference in the prevalence of resistance to selected antibiotics among human and environmental diarrheic *E. coli* isolated in Nyanga.

Globally, in 2010, 22 foodborne diseases resulted in two billion diarrhoea cases and 25.2 million DALYs (Disability Adjusted Life Years) in children. Of the 22 foodborne diseases, sixteen were bacterial and included the following; heat-stable toxin producing enterotoxigenic *E. coli (*ST-ETEC), STEC, *Campylobacter*, *Shigella*, *Salmonella*, and typical enteropathogenic *E. coli* among others.[48] In the Global Enteric Multi-centre Study (GEMS), a longitudinal study, EPEC and ST-ETEC were significantly associated with an increased risk of death in children less than one year of age with moderate-severe-diarrhoea as compared to age-matched controls, in sub-Saharan Africa.[2] These pathogens occur at different rates in different regions, and therefore public health interventions should be based on locally generated data.[49] Therefore, this study focused on determining the prevalence of STEC and other selected food and waterborne bacterial pathogens in human and non-human sources in an informal settlement setting- Nyanga Township, Cape Town. Furthermore, this study aimed to determine the clinical and epidemiological characteristics of children under the age of 12 years with diarrhoea that attended the Nyanga Community Health Centre (NCHC) The faecal coliform counts per millilitre of Lotus River surface water were determined as indicators of faecal contamination.

Food and Waterborne bacterial pathogens in stool of children with diarrhoea

In this study, we detected one or more diarrhoeal pathogens in 73% (48/66) of the diarrhoea cases. This is lower than the 83% reported by the GEMS study.[2] This can be explained by the fact that we focused on only the bacterial pathogens while the GEMS study considered the bacterial, viral and parasitic causes of diarrhoea. Importantly, carriage of the pathogens or their virulence genes does not necessarily infer their role in causation[50,51]. Normal carriage of most bacterial diarrhoea pathogens has been well documented in the previous research.[2]

Among the four key pathogens highlighted in the GEMS study as being significantly abundant in children with moderate-to-severe diarrhoea as compared to age-matched controls (the highest number of attributable cases of moderate-to-severe diarrhoea were due to these four pathogens) at all the study sites in sub-Saharan Africa and Asia (*Shigella*, Cryptosporidium, Rotavirus, and heat stable toxin producing enterotoxigenic *Escherichia coli*), we investigated only for *Shigella and* tellurite-resistant ETEC. Tellurite-susceptible ETEC was not investigated because they could not be isolated on CHROMagar™STEC-given that the main focus of this study was tellurite resistant STEC. Other pathogens like *Aeromonas* and *Campylobacter jejuni* were significant (had more attributable cases of moderate-to-severe diarrhoea) at some sites (for example in Bangladesh) but not in the other sites. *Aeromonas* and *Campylobacter* were also investigated in this study.

Temporal distribution of the diarrhoea cases

The peak of the summer (dry) season is February or March each year.[52] At around this time, the City of Cape Town reports the highest diarrhoea related mortalities every year.[52] In this study, a bimodal distribution of cases was noticed with high numbers reported in November 2015 and February 2016 (In the same months, we noted the highest concentration of faecal coliforms in the Lotus River surface water). Several other studies have already reported the association between summer months and a high number of diarrhoea cases. For instance, in the Western Cape, research has shown that the peak in diarrhoea cases admitted to the Red Cross War Memorial Children's Hospital's Rehydration Unit, has shifted (in tandem with the peak of the dry season) from January (1976) to March (2015) over a period of four (1976-2015) decades.[52]

Clinical characteristics of cases

The average duration of diarrhoea in all patients was 2.5 days. Per the World Health Organization (WHO) scheme on the classification of diarrhoea[53], the commonly reported form of diarrhoea in the Nyanga community was acute diarrhoea, which was observed to affect children under the age of five mainly.

The most common symptom among children with diarrhoea was weakness and dehydration (moderate) (30/66, 46%), and was noted among patients infected with DECs, *Plesiomonas shigelloides*, and *Shigella flexneri*. Some of the patients that possessed *Shigella flexneri* and *Plesiomonas shigelloides* in the stool, also presented with a fever (>38˚C). Where as infections with *Shigella flexneri* and EIEC have commonly been associated with fever, *Plesiomonas shigelloides* infections have rarely been associated with development of fever.[53]

Of all the cases, 5% (3/66), 20% (13/66), and 17% (11/33) presented with bloody stool, vomiting, and fever respectively. Blood in stool was noted among the patients that had STEC, DECs, and *Aeromonas* infections. In the absence of screening for other possible causes of blood in stool such as *Entamoeba histolytica*, it cannot be assumed that dysentery was related to the pathogens detected in the stool sample. It would be expected that blood in stool be observed for stool samples from which *Shigella flexneri* was isolated, which was not the case in this study. However, recent research has shown that *Shigella* infection is increasingly associated with watery diarrhoea as opposed to dysentery and that many cases of *Shigella* would be untreated if only stool samples with blood are screened.[2,3]

On the other hand, all the patients that possessed *Salmonella enterica* (non-typeable) in stool presented with a fever. Invasive non-typhoidal salmonellosis has previously been shown to be on the increase in sub-Saharan Africa.[54]

Antibiotic use was significantly higher among patients with fever compared to those without ($p=0.024$). Therefore, antibiotic prescription appears to have been appropriate in this study population.

Epidemiological characteristics of diarrhoea cases that attended the Nyanga Community Health Centre

Unlike the GEMS study, we did not have controls and thus could not conduct a risk factor analysis. The inclusion of a control group would have enabled us to conduct a risk factor analysis comparing the cases and the controls.

The average age of the children recruited in this study was 14.9±10.9 months. In agreement with earlier studies, children under five years of age are more vulnerable to diarrhoeal disease. [55,56] The mean age in this study is similar to that described in the GEMS study (16.9 months in the 11-23-month old age strata). [39]

The male: female ratio was 1:0.83. There was no significant difference in the occurrence of the different bacterial pathogens between the sexes.

Only 83% (55/66) of the children recruited in the study visited the clinic due to diarrhoeal sickness, 8% (5/66) had visited for immunisation, while 9% (6/66) had visited for a routine health check. This means that in approximately 17% (11/66) of the diarrhoeal patient's diarrhoea was incidentally identified. This may be an indicator of the health care-seeking behaviour of patients with diarrhoea in Nyanga. In the Gambian site of GEMS, it was reported that only 49% of the diarrhoea patients visited a health care setting.[57]

Of the recruited patients, none kept domestic ruminants at home such as goats, sheep or cattle. The ruminants are the common reservoirs of STEC infection[58]. Instead, dogs were the most common household animals (17%-11/66).

Of the recruited patients, mothers were the primary caregivers in 85% (56/66) of the patients, and 24% (16/66) of the children were still breastfeeding. Breastfeeding confers protection against diarrhoeal disease as protective antibodies are passed on to the child via breast milk.[59]

Only 39% (26/66) stated they had received rotavirus vaccination (likely to have been under-reported-medical records were not available for some of the patients). Rotavirus has been reported as an important cause of acute gastro-enteritis in children.[60] In the GEMS, it was confirmed that rotavirus was the most common cause of moderate to severe diarrhoea during the first year of life and therefore that immunisation against rotavirus would substantially reduce the burden of moderate-to-severe diarrhoea. It will be important to determine the ongoing

contribution of rotavirus to the burden of acute diarrhoea in this setting, post implementation of routine rotavirus immunisation.

Most the diarrhoea patients in this study (79% - 52/66) used the communal standpipe as the primary source of water for daily use. In Nyanga, 93% of the population uses the regional water scheme where water is provided via community water standpipes and in-house piped water, while 2% use the services of a water vendor.[61] In informal settlements, like Nyanga, there have been reports of illegal connections to the municipal water supply network. Such connections are possible avenues for contamination of communal standpipe water. Lack of access to clean water is linked to high incidences of diarrhoeal disease.[62] According to the City of Cape Town Water and Sanitation Department, the communal standpipe nozzles in the informal settlements like Nyanga often are contaminated.[38] The GEMS study reports a lack of access to clean water as a key risk factor for diarrhoea in such settings.[2]

Prevalence of STEC, DECs, and DEC virulence genes in stool, water and meat

We found a 2% (1/66, 1/64) prevalence of tellurite resistant STEC isolates in both stool and water; no STEC was isolated from meat. Compared to the prevalence of *stx* in the stool (6%-4/66 for stx_1 and 3%-2/66 for stx_2), water (30%-19/64 for stx_1 and no stx_2), and meat (20%-17/85 for stx_1 and 5%-4/85 for stx_2), the STEC isolation rate was very low. This could be because not all STEC are tellurite resistant and as such did not grow on CHROMagar™STEC. Alternatively, the *stx* genes detected could represent non-viable STEC or free floating phages. A similar study conducted in Amathole district, South Africa, on meat and meat products found a 3% prevalence of STEC.[63] The STEC isolate from stool belonged to serotype O106, while that from the Lotus River surface water was non-typeable. STEC O106 has not previously been reported in South Africa.

The 30% (19/64) prevalence of stx_1 genes in Lotus River water was higher than the 15% previously detected in the Berg River system.[36] There was a significant difference in the levels of detection of the stx_1 genes between the different collection points located along this river ($p = 0.02$) with LR13 (13%) and LR16 (11%) having the highest stx_1 detection rates. Surprisingly, LR13 had the lowest mean CFU/ml of coliforms and yet had a high stx_1 detection rate. This is in agreement with

previous research which showed the absence of correlation between levels of microbial indicators (coliform counts per millilitre of surface water) and the carriage of *stx* genes.[64] This could also point towards possible different water use trends at the different points along the Lotus River, differences in the rate of flow of the river at the various points, and the level and type of contamination of the water runoff joining the river at these points.

The high number of *stx*$_1$ genes compared to *stx*$_2$ genes in the environmental samples could mean that there is a lesser risk of severe STEC disease associated with the non-human samples. This is because strains carrying *stx*$_2$ alone or with *stx*$_1$, have been more commonly associated with the development of HUS in human patients.[65] Therefore, the detection of *stx*$_2$ genes in meat (beef and pork) from the informal slaughter setting could mean that meat is a possible source of STEC capable of causing the more severe form of STEC infection.

Serotype-specific marker genes (*rfbE* for *E. coli* O157 and *wbdl* for *E. coli* O111) were detected more in Lotus River surface water as compared to meat from the informal abattoirs and stool from children with diarrhoea. There was an 8% (5/64) and 14% (9/64) prevalence of STEC O111 *wbdl* genes and STEC O157:H7 *rfbE* genes in the Lotus River surface water (none of the diarrheic *E. coli* from water belonged to serotype O157 or O111). The failure to isolate these serotypes could be explained by the fact that not all *E. coli* O157 or O111 are tellurite resistant and as such, they did not grow on CHROMagar™STEC. This highlights the Lotus River surface water as a potential reservoir of STEC O157:H7 and STEC O111 for human and animal transmission in Nyanga. This is of clinical and epidemiological significance because STEC O157 and STEC O111 are key serotypes associated with STEC infection.[66]

The prevalence of DAEC isolated from stool, meat, and water was 12% (8/66), 2% (2/85), and 3% (2/64) respectively (compared to a prevalence of 35% (23/64), 7% (6/85), and 38% (24/64) of *daaC* genes in stool, meat, and surface water respectively). This difference can be explained by the fact that not all DAEC are tellurite resistant while others may be unviable. There was a higher prevalence of DAEC isolated from stool as compared to the environmental samples.

EAggEC was only isolated from stool (3%-2/66) and water (2%-1/64) respectively (compared to a 14% -9/66 prevalence of *aat* genes in the stool, and none in water). No EAggEC was isolated from meat. There was generally a low prevalence of EAggEC in this setting.

The prevalence of EPEC isolated from stool and meat was 2% (1/66) and 1% (1/85) respectively (compared to 0% and 7%- 6/85 of *eae* in stool and meat). No EPEC was isolated from the Lotus River surface water. EIEC was only isolated from a single stool sample (2% of all stool samples) and was not isolated from meat and water.

We noted higher levels of the *LT* genes in the water (21/64 – 33%) as compared to stool (5/66 – 8%). Water also possessed several other DEC virulence genes. This could be explained by the fact that the Lotus River is used as a drinking water source for various ruminants. Ruminant excreta get washed into the Lotus River surface water. The water would then act as a common source of contamination for human (if used for domestic chores and for recreational activity- slaughter of chicken was observed along the banks of this river), cattle (drink water from the Lotus River) and other domestic animals such as stray dogs (drink water from the Lotus River). Even though humans may not directly consume water from this river, contamination of hands of food handlers and carcases is a potential route of transmission. On this basis, the Lotus River can be considered a potential "environmental common" for both human and animal STEC infection. An environmental common is defined as an ecosystem from which humans and animals derive livelihood.

Aeromonas, Shigella, Salmonella, Plesiomonas, and Campylobacter, in stool, water, and meat

The most prevalent diarrhoeal pathogen among the children with diarrhoea was *Shigella* (17%, 11/66), which was more common in stool as compared to meat (no *Shigella*) and water (5%, 3/64 for *Shigella*). Since *Shigella* was significantly more abundant in human stool, it's possible that stool is the likely source of contamination of the environment with *Shigella*. Invasive shigellosis has been reported to be more prevalent in children less than one year of age in South Africa, especially those that are HIV-positive.[67] High prevalence of *Shigella* in stool can also be explained by secondary *Shigella* infections in households, nurseries and daycare centres.[68,69]

Salmonella enterica causes 93.8 million cases of gastro-enteritis and 155,000 deaths globally each year- of which 80.3 million cases are foodborne.[70] *Salmonella enterica* (non-typeable) was detected in only 6% (4/66) of the stool samples and all the children that possessed *Salmonella* presented with a fever. The fevers in the patients that had *Salmonella* could be due to *Salmonella* undetected invasive infection.

Salmonella enterica Idikan was not isolated from the Lotus River surface water but was isolated from 5% (4/85) of the meat samples. *Salmonella enterica* Idikan is less commonly associated with infectious disease but has previously been isolated from food producing animals, animal feeds, meat from abattoirs, water, and associated environments in South Africa at a prevalence of 6% (between 2012 and 2014).[71] An earlier study in South Africa showed broiler chickens to be the environmental reservoirs of multi-drug resistant *Salmonella*.[72]

Plesiomonas shigelloides was detected in 9% (6/66) of the stool samples, 17% (11/64) of the water samples, and was not detected in meat. Some of the patients that possessed *Plesiomonas shigelloides* presented with bloody diarrhoea. Previous research has associated bloody diarrhoea with *Plesiomonas shigelloides* infection.[73] Several other reports have indicated its role in causing diarrhoea with varying severity.[74-76] However, this pathogen is not routinely screened for in South Africa.

Campylobacter jejuni was isolated from 5% (3/66) of the stool samples, and 7% (6/85) of the meat samples. Related studies in South Africa have reported a prevalence of 10% for *Campylobacter* in the stool of diarrhoea patients in the Venda region, Limpopo, South Africa.[77] *Campylobacter jejuni* in South Africa is commonly isolated from poultry and is increasingly becoming resistant to antibiotics.[78-80] In this study, it was not isolated from Lotus River surface water.

Aeromonas sorbria was isolated from only 3% (2/66) of the stool samples and 4% (3/85) of the meat samples and was not isolated from the Lotus River surface water. *Aeromonas* has been reported to cause fatal invasive disease in West Africa.[81] It was reported among diarrhoea patients in the Venda region of South Africa[82] and has been isolated from treated wastewater effluents in Durban.[83]

Non-cholera *Vibrio (Vibrio vulnificus)* was only isolated from the Lotus River surface water and was not isolated from meat and stool. Non-cholera *Vibrio* has rarely been associated with diarrhoea in South Africa.

Yersinia was not detected in any sample. There have only been a few reports of *Yersinia* in Cape Town, mostly before 1990.[84,85] *Yersinia enterocolitica* has previously been reported in a food handler and a child with diarrhoea.[86,87]

Faecal coliform contamination of the Lotus River

In February 2016, the colony forming units of faecal coliforms per millilitre of Lotus River surface water peaked at all water collection points, and the water levels were visibly low. Coincidentally, in the same month, the number of diarrhoea cases reported at the Nyanga Community Health Center was highest. The mean CFU/ml over the study period was 1.11E+06 -2.74E+05. As per the Department of Water and Forestry of South Africa regulations for microbial quality of recreational surface water (it should not have more than 1000 CFU/ml), the water in the Lotus River is not fit for drinking or recreational use. The highest mean CFU/ml for the Lotus River was observed for collection point LR16, and the lowest was noted for LR13. However, most of the bacterial pathogens were isolated from sampling point LR13. It is not clear as to the use habits of the Lotus River surface water at these two points. However, LR13 is located upstream of LR16, and it appears that LR13 is situated close to a dense concentration of semi-permanent shelters. Since point LR16 is located downstream of LR13, there could be a higher concentration (due to a reduced flow rate of surface water) of faecal coliforms downstream.

Eventually, storm water in this channel ends in the urban sewerage treatment system. Contamination of storm water, therefore, leads to highly contaminated sewage and waste water which becomes difficult to treat and recycle by the urban sewage treatment systems and thus affecting the water quality of the urban aquatic system.

Bacterial pathogens per meat type

Among the raw meats, *Salmonella* was isolated from beef, chicken, and mutton, while *Campylobacter* was isolated from chicken and mutton. DAEC was isolated from beef and mutton.

As per the Food Safety Inspection Services of USDA, 25g of raw meat should not possess detectable levels of foodborne pathogens such as *Salmonella* and *Campylobacter*. This means that raw meat from the informal slaughter setting is unsafe for human consumption. A similar study conducted in the Eastern Cape found only *Aeromonas* and not *Salmonella* or *E. coli*.[88]

Raw beef and pork samples mainly possessed the *stx*$_1$ gene (53% - 9/17 of the beef cuts and 14% - 2/22 of the raw pork samples). The *rfbE* gene which signifies the presence of *E. coli* O157 was detected only in the raw meats including beef cuts, mutton, pork, and chicken. *Stx*$_1$ was more prevalent among the different meat types as compared to *stx*$_2$. Notably, *stx*$_2$ has been associated with the more severe disease. Thus, there may be less risk of severe STEC disease linked to meat from the informal abattoirs.

Among the ready to eat foods, 12% (3/26) of the roast beef possessed *stx*$_1$ while 13% (1/8) and 13% (1/8) of the roast pork samples possessed the *stx*$_1$ and *stx*$_2$ genes respectively. The genes detected could represent dead bacteria, free floating phages, or viable organisms. If they represent viable organisms, this could mean that there is a risk of STEC infection associated with consumption of ready to eat foods vended in the informal abattoirs.

As per the South Africa Bureau of Standards (SABS) and the regulations governing the microbiological standards for foodstuffs in South Africa, section 15 (1) of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972), 25g of ready to eat meat should not contain organisms of the genera *Salmonella*, *Shigella*, and *E. coli*.

Antimicrobial resistance patterns of STEC and other diarrheic E. coli isolated from clinical and environmental samples.

STEC isolated in this study showed resistance to only ampicillin. Antibiotics are generally contraindicated incase of STEC infections because of the elevated risk of fatal complications such as Haemolytic Uraemic Syndrome.[89] Antibiotic resistance, however, is easily transferred among the gram-negative bacteria in the intestinal tract.

The multidrug resistant diarrheic *E. coli* from the chicken was resistant to ampicillin, ciprofloxacin, amoxicillin-clavulanate, cefotaxime, cefuroxime-axetil, and trimethoprim-

sulphamethoxazole. Antimicrobial resistance among zoonotic pathogens is of clinical significance. Importantly, ciprofloxacin is an important antibiotic used in the treatment of invasive *E.coli* infection.

There was no significant difference in the levels of resistance for isolates from human and non-human sources. The single isolate that was ciprofloxacin resistant was isolated from chicken. However, further investigations would help ascertain the use of quinolone antibiotics in poultry traded in the informal abattoirs.

The two ESBL-producing *E. coli* isolates reported in this study were both isolated from the stool of children. No ESBL producing strain was isolated from water or meat. In this study, we did not evaluate the antibiotic use history of the cases. The ESBL-producing strain could have been acquired from another source. Alternatively, previously non-ESBL-producing *E. coli* strains acquiring the ESBL phenotype due to horizontal gene transfer or due to antibiotic selection pressure.

Since some of diarrhoeagenic *E. coli* might have been missed (tellurite-susceptible diarrhoeagenic *E. coli*), the actual incidence of antimicrobial resistance for human and non-human sources might have been underreported. An adjunct approach including screening for the presence of antimicrobial resistance genes (for instance, the ESBL genes) in human and non-human sources would provide a better understanding of antimicrobial resistance in this setting.

Antimicrobial susceptibility patterns of Aeromonas, Shigella, Salmonella, Plesiomonas, Campylobacter, and Vibrio from human and non-human sources.

Given the high prevalence of Shigellosis among the children with diarrhoea in Nyanga, we noted with concern the resistance to multiple antibiotics. *Shigella* showed resistance to ampicillin (67%, 10/15), amoxicillin-clavulanate (67%,10/15), and trimethoprim-sulfamethoxazole (100%, 15/15). Notably, ampicillin and trimethoprim-sulfamethoxazole are the first line antibiotics used for the treatment of shigellosis.[90] On this basis; there is a need for an alternative treatment strategy for Shigellosis in Nyanga. Also, 13 of the 15 *Shigella* isolates from all sources were resistant to at least one antibiotic. Specifically, resistance to cefoxitin, amikacin, and gentamicin for *Shigella* was only

seen among the human isolates and not the water isolates. A related study showed ESBL production by *Shigella* isolates screened between 2003 and 2009. [91]All isolates from water and stool were resistant to SXT. The most common resistance profile (in 21% -14/66 of the isolates) was AMP, AMC, CXM, CXA and FOX.

Worryingly, we noted that all the *Salmonella* isolates from meat and stool were resistant to ciprofloxacin, a choice antibiotic for the treatment of invasive salmonellosis.

Even though *Aeromonas* has not frequently been reported as a serious cause of diarrhoea in South Africa, with a few exceptions, we noted resistance to tigecycline in the one isolate obtained. Tigecycline resistance has rarely been reported in *Aeromonas* but was reported in a single isolate from a clinical sample in Taiwan.[92]

6.5.1 Limitations of this study

For us to be able to attribute bacterial pathogens to diarrhoea among the children presenting with diarrhoea at the NCHC, we would ideally have matched cases with controls (case-control study design). However, this was not done due to logistical and administrative reasons.

We used TSB enrichment media mainly targeting STEC which is the primary focus of the study. However, this medium is not the most suited for other bacterial pathogens such as *S. enterica*.

In this study, we only report on the tellurite resistant STEC but not the tellurite susceptible STEC which fail to grow on CHROMagar™STEC.

6.6 Conclusions

This study provides information on the epidemiology and characteristics of food and waterborne bacterial pathogens in children from Nyanga, Cape Town, who presented to primary care with diarrhoea, as well as from potential sources of infection in their physical environment.

There were high detection rates for bacterial pathogens or DEC virulence genes in stool from patients attending the Nyanga Community Health Centre. There was a higher prevalence of *stx*$_1$ in both human and environmental samples as compared to *stx*$_2$.

The Lotus River surface water was pathogen-rich (high prevalence of *stx*$_1$ and ETEC) and highly contaminated with faecal coliforms (mean CFU/ml =1.11E+06 -2.74E+05). Therefore further research needs to be done to establish water use habits and activity along this river to prevent transmission of bacterial pathogens like STEC to users.

The raw and ready-to-eat beef and pork meat types possessed high prevalence of *stx*$_1$, and *stx*$_2$ as compared to the other food types and therefore are a more likely source of STEC infection in Nyanga. The Codex Alimentarius public health guidelines for the hygiene of street vended meats in Africa (CAC/GL 22R-2013), for the control of *Campylobacter and Salmonella* in chicken (CAC/GL 78-2011), for the control of non-typhoidal *Salmonella* in beef and pork (CAC/GL/87-2016), and for the general control of food hygiene to control foodborne parasites (CAC/GL89-2016) need to be enforced to ensure consumer safety. The South African National Standards (SANS 885:200X) on processed meat should also be enforced in the informal abattoir setting in the Nyanga Township. This means that further measures need to be put in place to ensure the safety of meat from the informal slaughter in Nyanga, Cape Town, South Africa.

These results suggest the need for a larger scale (over at least 12 months) age-matched case-control study to better understand the role of these bacterial pathogens in the causation of diarrhoea in children in this (and similar) settings. Given the high rates of bacterial contamination of meat and water, a detailed study of risk factors for bacterial diarrhoea would be useful to determine the role of these potential sources of infection.

6.7 References

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Chapter Seven

7 Multi-Locus Variable Number of Tandem Repeats Analysis (MLVA) to determine genetic relatedness of STEC and other diarrheic *E. coli* from stool, meat from the informal abattoirs, and Lotus River surface water in the Nyanga Township, Cape Town.

7.1 Summary

Introduction: Multi Locus Variable number of Tandem repeats Analysis (MLVA) of STEC and other diarrheic *E. coli* is important for source tracing and outbreak identification.

Objectives: To determine the MLVA profiles of diarrheic *E. coli* from Lotus River surface water, meat from the informal abattoirs, and stool of children with diarrhoea in Nyanga Township.

Methods: MLVA typing of tellurite-resistant diarrheic *E. coli* from the stool of children with diarrhoea, meat from the informal abattoirs, and the Lotus River surface water was done. Conventional PCR using dye-labelled primers was done to amplify seven Variable Number Tandem Repeats Loci (VNTR). Capillary electrophoresis was done to determine the amplicon sizes per locus which were then used to determine the allele (number of tandem repeats per locus). A dendrogram was generated using the allelic string in BioNumérics version 7.6 software; isolates that showed 100% similarity of MLVA profiles were considered a clone.

Results: The diarrheic *E. coli* from human and non-human sources in Nyanga were genotypically diverse and showed 19 distinct MLVA genotypes. The two DAEC isolates (NY60 and NY28) that were isolated from children with diarrhoea had identical MLVA genotypes and were assumed to be clonal. Overall, the dendrogram showed that tellurite resistant diarrheic *E. coli* from human and non-human sources were intermingled (genotypically close to strains from different sources). The two STEC (NY29 and 710) showed a 71.4% level of genotypic homology and belonged to different serotypes. The two extended-spectrum beta-lactamase (ESBL)-producing DAEC from stool belonged to the same serotype (O153) but had different MLVA genotypes.

Conclusions: Diarrheic *E. coli* from human and non-human sources in Nyanga Township were genotypically diverse. Two DAEC isolates formed a clone and could have been transmitted between the children or acquired from the same source. Further larger scale studies are needed to fully understand the epidemiology of STEC and other diarrheic *E. coli* in the Nyanga Township.

7.2 Introduction

STEC is an environment-induced zoonosis which is emerging due to changes in livestock farming habits, food processing, and consumer food habits.[1] It can be transmitted through different routes

including person to person, contaminated food, animal contact, and through contact with recreational water.[2] To determine the likely sources (human and non-human) of STEC, isolates from the different sources must be genotyped to determine their genetic relatedness. In cases of an outbreak, this would assist with source tracing. In the absence of an outbreak, subtyping would help establish an epidemiological linkage between STEC isolates from different sources, identify previously unidentified outbreaks, and determine the dominant subtypes in the different human and non-human sources. Bacterial subtyping is also useful for monitoring the impact of product recalls in response to outbreaks.[3] In many cases, MLVA is used in addition to other subtyping tools such as Pulse Field Gel Electrophoresis (PFGE) to increase discriminatory power between closely-related strains. MLVA is based on detection of varying numbers of tandem repeats at distinct variable-number tandem repeat loci along the bacterial genome.[4] It has previously shown to be highly discriminatory and gives concordant results to Whole Genome Sequencing (WGS).[5,6]

Conventional MLVA protocols require gel electrophoresis to determine the band sizes. Improved MLVA assay approaches involved the use of multiple dye colours (analysed by multi-coloured capillary electrophoresis) and enhanced PCR multiplexing targeting the various loci with the MLVA patterns based on allele sizes, thus eliminating the need to analyse band patterns on gel images.[5-7] Several MLVA protocols have already been developed for all STEC serotypes including both O157 and non-O157 STEC strains,[8,9] while others are only discriminatory and applicable among the O157 STEC.[10] However, in non-outbreak scenarios (no single STEC strain is targeted), and due to the emerging trend of hybrid STEC strains, a more generic MLVA protocol targeting both the O157 and non-O157 STEC serotypes (plus other pathotypes in the case of hybrid strains) would be suited for public health investigations to establish epidemiological linkage between strains from different sources. It has been argued that the discriminatory power at serotype level is likely to be diminished if multiple serotypes are targeted.[3] However, there are over 450 serotypes of STEC with new serotypes continually being reported as STEC serotypes.[11] The generic MLVA protocol that facilitates discrimination of both O157 and non-O157 STEC (plus other diarrheic *E. coli* pathotypes) was developed using the ECOR (*Escherichia coli* reference

collection) strains to target seven genomic Variable Number of Tandem Repeats (VNTR) loci and proved to be highly discriminatory for O157 and non-O157 STEC.[5-7] As an improvement to this protocol, additional three loci were included to improve its discriminatory power.[9] The extra three loci would particularly be useful when dealing with closely similar strains as would be the case in an outbreak situation.

Despite the high burden of diarrhoea that is characteristic of informal settlements such as the Nyanga Township, the poor hygiene and sanitation standards in the informal settlements, and unhygienic slaughter practice in the informal abattoirs, there is no information on the epidemiological linkage (or MLVA genotypes) between STEC and other diarrheic *E. coli* from human and non-human sources.

7.2.1 Aims and objectives

In this study, we aimed to determine the MLVA genotypes of tellurite-resistant STEC and other diarrheic *E. coli* isolated on CHROMagar™STEC from human and non-human sources in Nyanga Township.

7.3 Methods

Meat, stool, and water were collected from informal abattoirs, children with diarrhoea, and the Lotus River in Nyanga (**Figure 7:1**).

7.3.1 Meat ,stool, and water collection, processing, and microbiological analysis.

The samples were processed and diarrheic *E. coli* isolated on CHROMagar™STEC. Confirmed *E. coli* were screened for *eae*, *aggr*, *daaC*, *ipa*, *stx*$_1$, *stx*$_2$, *ST*, and *LT* genes as described in **3.3.5.** Serotyping was done to determine the O-antigens using specific antisera as described in **3.3.5.** Antimicrobial susceptibility testing was done using the VITEK® 2 automated system with the minimum inhibitory concentrations (MIC) confirmed using the broth microdilution method as described in **4.3.3.**

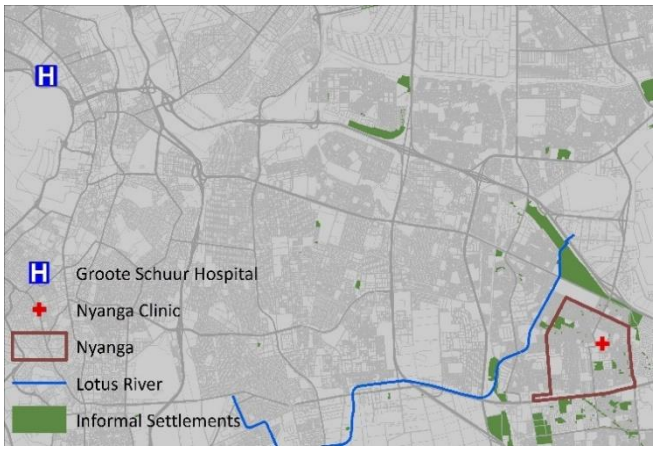

Figure 7:1 Street map showing the location of Groote Schuur Hospital and the Nyanga community.

In Nyanga, it shows the Location of the Lotus River, the Nyanga Community Health Centre and the Informal Settlements. The informal slaughter places are located within the informal settlement

7.3.2 Selection of isolates for MLVA typing

All the diarrheic *E. coli* isolated from the different sources were genotyped using the generic MLVA protocol.

7.3.3 MLVA typing

7.3.3.1 DNA template preparation for PCR

DNA was extracted from the *E. coli* isolates using the MagNApure bacterial/fungal DNA extraction kit on a MagNApure LC machine.

7.3.3.2 *Gel-based PCR amplification of VNTR gene targets*

The following dye labelled primers were used as previously described with a minor modification (**Table 7.1**). The NED dye was used instead of TET for suitability with the available 16-capillary fluorescence-based capillary electrophoresis system 3130*xl* Genetic Analyser (Applied Biosystems, Forster City, California, U.S.A).[8]

Table 7.1 Primers and dye colours used to detect the seven loci.

Primer /Probe Name	Dye-Sequence (5'-3')	Primer Tm
CVN001-F	HEX-AACCGGCTGGGGCGAATCC	57.6℃
CVN001-R	GGCGGCGGTGTCAGCAAATC	57.9℃
CVN002-F	**NED**-AACCGTTATGAARGRAAGTCCT	49.2℃
CVN002-R	TCGCCCAGTAAGTATGAAATC	50.5℃
CVN003-F	6FAM-AAAAATCCGGATGAGWTGGTC	50.5℃
CVN003-R	TTGCGTTGTCAGTAATTTGTTCAG	52.3℃
CVN004-F	HEX-MGCTGCGGCRCTGAAGAAGA	53.8℃
CVN004-R	CCGGCAGGCGAAGCATTGT	57.9℃
CVN007-F	6FAM-ACCGTGGCTCCAGYTGATTTC	54.4℃
CVN007-R	ACCAGTGTTGCGCCCAGTGTC	58.3℃
CVN014-F	6FAM-TCCCCGCAATCAGCAAMACAAAGA	55.7℃
CVN014-R	GCAGCRGGGACAACGGAAGC	57.9℃
CVN015-F	**NED**-TAGGCATAGCGCACAGACAGATAA	55.7℃
CVN015-R	GTACCGCCGAACTTCAACACTC	56.7℃

Subsequently, three gel-based PCR reactions targeting seven VNTR loci were done involving two multiplex PCR reactions and one singleplex PCR reaction.[8] The multiplex PCR reactions were run with the QIAGEN PCR-multiplex kit (QIAGEN GmbH, Hilden, Germany). 10pmol each of CVN003 and CVN014 primers were included in one multiplex PCR reaction, while 10pmol each of CVN001, CVN004, CVN007 and CVN015 primers were included in the second multiplex PCR. CVN002 was run separately with 10pmol of each primer, 1 unit of Amplitaq polymerase (Applied Biosystems, Forster City, California, U.S.A), 200μM dNTP and 1x polymerase buffer (Applied Biosystems, Forster City, California, U.S.A). The multiplexes were run under the following conditions: 95℃ for 15 min, 94˚C for 30 s, 63℃ for 90 s, 72℃ for 90 s, and 72˚C for 10 min. The singleplex PCR reaction for CVN002 was run at 94˚C for 5 min, 30 cycles of 94˚C for 30 s, 50˚C for 30 s and 72℃ for 50 s, and 72℃ for 7 min.

As a positive control, we considered an in-house strain (NY50) that possessed all the seven VNTR loci. PCR grade water was used as the negative control for the PCR.

To confirm PCR amplification for each of the seven VNTR loci, and to estimate the amount of PCR product prior to pooling, gel electrophoresis was run at 80 volts for 45 minutes on a 1.5% agarose gel. A Bioline hyperladder IV was run as a DNA ladder marker.

7.3.3.3 Dilution of PCR fragments by pooling PCR amplicons from the three PCR runs

After the PCR amplifications, 20µl of the multiplex which had CVN003 and CVN014 amplicons, 3.5µl of the multiplex which had CVN001, CVN004, CVN007, and CVN015 amplicons, and 10µl of the reaction which had CVN002 amplicon were pooled together to make a total volume of 33.5µl. Water (66.5µl) was then added to make 100µl.

7.3.3.4 Fragment Analysis using capillary electrophoresis

Subsequently, 1µl of the mixture was added to 1µl of the ILS600 internal size standard (Promega, Madison, Wisconsin, USA) and 12µl of formamide (Promega, Madison, Wisconsin, USA) and loaded onto the 16-capillary fluorescence-based capillary electrophoresis system 3130*xl* Genetic Analyser (Applied Biosystems, Forster City, California, U.S.A). The electrophoresis was run at 60 ℃ for 35 min using POP4 polymer (Applied Biosystems) with an injection voltage of 15kV.

7.3.3.5 Data analysis

Analysis of isolate characteristics data

Serotype, virulence genes, and Minimum Inhibitory concentration related data was analysed as described in **6.3.5**.

Raw data files from the Genetic Analyser were first analysed using GeneMapper® software Version 4.0 (Applied Biosystems, Forster City, U.S.A) to generate data on the peak sizes for each of the seven VNTR loci and to confirm amplification. Information on the dye used, sample name, and the size of the fragments was exported from the GeneMapper® software in tab-delimited format.

The electropherogram files from the Genetic Analyser were directly imported as curves into BioNumerics version 7.6 software (Applied Maths, bioMérieux, N.V, U.S.A). Data on the

fingerprint dyes used for the different VNTR loci was acquired along with the importation of the ABI sequencer trace files to create a new fingerprint type. The raw chromatograms were normalised as per the reference system which was based on the marker peaks defined in the reference dye (ILS 600 internal size standard). All the VNTR peaks of interest were identified based on colour and size and each new multiple of repeats assigned to a distinct allele number using the MLVA plug-in in for BioNumerics v 7.6. The Internal Lane Standard 600 (Promega®, Madison, Wisconsin, USA) was used as a reference to assign sizes to DNA fragments separated by capillary electrophoresis. Using the sizes of the DNA fragments, the VNTR copy numbers or allele numbers were determined using an amplicon size - allele number conversion table previously described by Linstedt *et* al. 2007. A dendrogram was then constructed based on the allelic string using the categorical coefficient and the Unweighted Pair Group Method (UPGM) algorithm.

7.4 Results

7.4.1 Diarrheic *E. coli* from human and non-human sources and their characteristics

Of the 20 diarrheic *E. coli* isolates, 13 were from the stool of children with diarrhoea who attended the Nyanga Community Health Centre while seven were from the non-human sources (3 from meat and 4 from surface water). The pathotypes detected included; EPEC (2/20, 10%), STEC (2/10, 10%), DAEC (12/20, 60%), EIEC (1/20, 5%), and EAggEC (3/20, 15%). Fourteen isolates could not be serotyped using the antisera available (were non-typeable). The serotypes detected varied widely (**Table 7.2**). Of the 20 isolates, eight were resistant to at least one antibiotic while two isolates from stool were resistant to multiple antibiotics.

Table 7.2 STEC and other diarrheic E. coli from human and non-human sources in Nyanga that were included in the MLVA analysis.

Isolate Number	Source	Pathotype	Serotype	Resistance profile
777	Mutton	DAEC	Non-Typeable	AMP AMC CXM CXA
LR16	Lotus River water	DAEC	O101	AMP
NY29	Child	STEC	O106	AMP
789	Beef	EPEC	Non-Typeable	-
NY3.2	Child	DAEC	Non-Typeable	-
NY1.2	Child	DAEC	O153	AMP AMC CXM CXA FOX CTX
NY50	Child	EPEC	O49	-
767	Lotus River water	DAEC	Non-Typeable	AMP AMC TZP
NY1.1	Child	DAEC	O153	AMP AMC CXM CXA FOX CTX
NY13.1	Child	EAggEC	Non-Typeable	-
NY43	Child	EAggEC	O143	-
NY58	Child	DAEC	Non-Typeable	-
710	Lotus River water	STEC	Non-Typeable	-
NY4	Child	EIEC	Non-Typeable	-
E101.1	Lotus River water	EAggEC	Non-Typeable	-
NY60	Child	DAEC	Non-Typeable	AMP
NY28	Child	DAEC	Non-Typeable	AMP
E33.1	Child	DAEC	Non-Typeable	-
E34.1	Child	DAEC	Non-Typeable	-
PK-STEC	Pork	DAEC	Non-Typeable	-

The pathotypes included: diffusely adherent *E. coli* (DAEC), Enteroinvasive *E. coli* (EIEC), Shiga-toxin producing *E. coli* (STEC), and Enteroaggregative *E. coli* (EAggEC). Resistance profiles include the following antibiotics: ampicillin (AMP), amoxicillin-clavulanate (AMC), cefuroxime (CXM), cefuroxime-axetil (CXA), cefoxitin (FOX), and cefotaxime (CTX).

7.4.2 Amplification of the different genomic VNTR loci genes.

For each of the VNTR loci, the amount of amplicon was roughly assessed by gel-based PCR and gel electrophoresis on a randomly selected isolate (**Figure 7:2**).

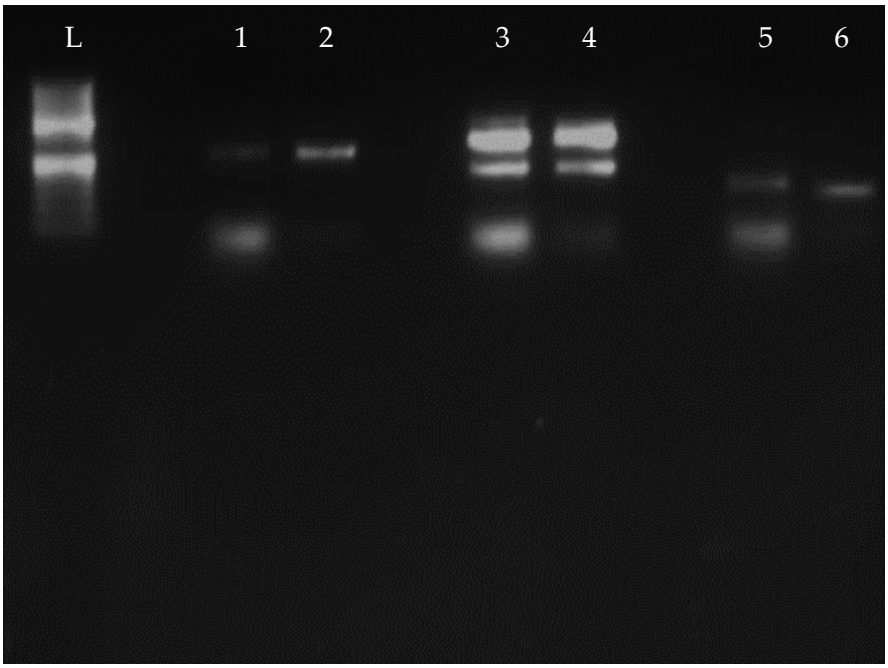

Figure 7:2 Electrophoresis Gel image for pooled PCR product for the two multiplex and one singleplex PCR for two randomly selected diarrheic E. coli isolates

Lanes 1 and 2= reaction 1 showing amplification of the VNTR loci CVN002 (450bp) for isolate 1 and 2 respectively, lanes 3 and 4=both showing amplification of VNTR loci CVN001 (484bp), and CVN004 (322bp) for isolate 1 and 2 respectively and lanes 5 and 6= both showing the amplification of the CVN014 locus (140bp) for isolate 1 and 2 respectively. Lane L= 1 kb DNA ladder.

7.4.3 Fragment data analysis using capillary electrophoresis

Capillary electrophoresis was done on the pool of the amplicon product from the three PCRs that were run per isolate (two multiplexes and one singleplex). The resultant electropherograms (viewed using the GeneMapper® software) showed distinct peaks (different sizes and dye colours) for each VNTR locus. PCR amplification of the seven VNTR loci showed different amplicon sizes (in base pairs) (**Table 7.3)**. Considering the 20 diarrheic *E. coli* isolates from human and non-human sources, some of the genomic VNTR loci were amplified, while others were not (**Figure 7:3**). For example, the VNTR locus CVN003 was not amplified (It was a null allele) in 70% (14/20) of the isolates.

Table 7.3 Examples of amplicon sizes observed for each of the VNTR loci

VNTR loci	CVN014	CVN015	CVN002	CVN007	CVN001	CVN004	CVN003
Amplicon size (base pairs)	104-159	210-300	362-470	342-359	433-499	300-392	413-488

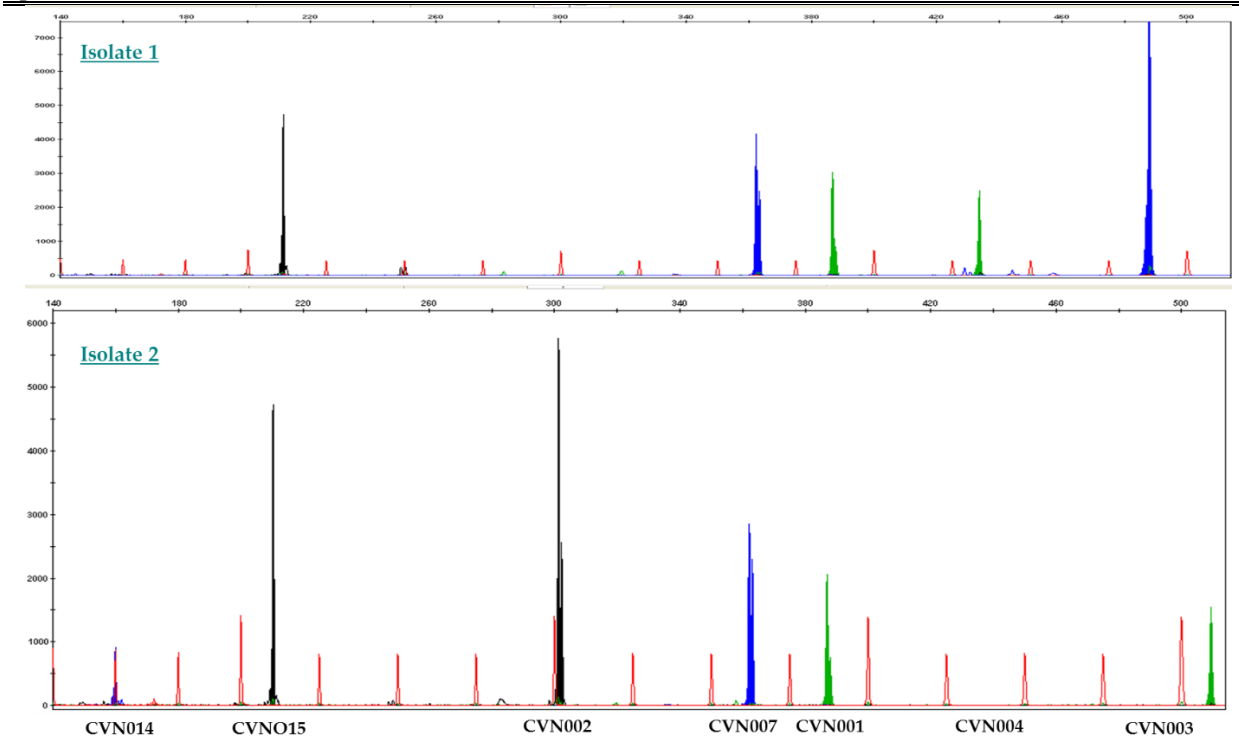

Figure 7:3 Electrophoregram showing the difference in the VNTR loci detected for two isolates (Isolate 1 and Isolate 2).

The red (size standard), blue (FAM), black (NED), and green (HEX) peaks of different sizes represent the different VNTR loci. VNTR **loci represented by the NED dye= CVN002 and CVN015, FAM dye= CVN003, CVN014, and CVN007, HEX dye= CVN001 and CVN004. The vertical axis shows relative fluorescence while the horizontal axis (at the top of each electropherogram) is the size in base pairs.**

7.4.4 Alleles detected per VNTR locus

The number of repeats that were detected per genomic VNTR locus ranged from none to twelve (**Table 7.4**).

Table 7.4 Alleles detected by MLVA: distribution per isolate, serotype and locus.

Isolate	Serotype	Allele number per genomic VNTR Locus						
		CVN001	CVN002	CVN003	CVN004	CVN007	CVN014	CVN015
777	NT	6	NA	NA	7	2	3	1
LR16	O101	6	6	NA	7	2	3	1
NY29	O106	5	8	4	7	2	2	3
789	NT	1	8	4	7	3	1	3
NY3.2	NT	6	NA	NA	6	1	6	3
NY1.2	O153	5	NA	5	7	2	2	1
NY50	O49	5	6	5	7	2	3	1
767	NT	NA	NA	NA	7	2	3	3
NY1.1	O153	6	NA	NA	7	2	7	3
NY13.1	NT	6	10	NA	6	2	6	1
NY43	O143	5	8	NA	7	2	4	1
NY58	NT	6	NA	NA	2	NA	NA	NA
710	NT	6	8	NA	7	2	2	3
NY4	NT	5	NA	NA	4	4	7	3
E101.1	NT	6	6	NA	7	2	4	3
NY60	NT	6	12	NA	7	2	4	3
NY28	NT	6	12	NA	7	2	4	3
E33.1	NT	5	8	5	7	4	3	1
E34.1	NT	5	8	4	6	2	1	3
PK-1	NT	6	NA	NA	8	2	4	1

NA=Null Allele; NT= Non-Typeable

7.4.5 MLVA genotypes of tellurite resistant diarrheic *E. coli* from human and non-human sources in Nyanga Township.

The MLVA genotype for each isolate was represented by the allele string for the seven VNTR loci (for example 6-NA-NA-8-2-4-1 was the allele string for the isolate PK-1). Overall, analysis of the twenty tellurite-resistant diarrheic *E. coli* from human and non-human sources showed nineteen distinct MLVA genotypes. Indeed, it was expected that the different diarrheic *E. coli* pathotypes belonged to different MLVA genotypes. Two diffusely adherent *E. coli* (DAEC) isolates from the stool of children with diarrhoea, NY28 and NY60 had identical MLVA profiles and had the same antibiotic resistance profiles. These two DAEC isolates were therefore assumed to be a clone.

The dendrogram showed that the MLVA genotypes of tellurite resistant diarrheic *E. coli* from Lotus River surface water, meat from the informal abattoirs, and stool of children with diarrhoea were intermingled. Isolates from the different sources did not form a clone (**Figure 7:4**).

The two STEC isolates (710 and NY29) were relatively closely related (71.4% level of genotypic homology), had different antibiotic resistance profiles and belonged to different serotypes.

The two ESBL producing DAEC isolates (NY1.1 and NY1.2) from the stool of children with diarrhoea belonged to the same serotype (O153) but had different MLVA genotypes.

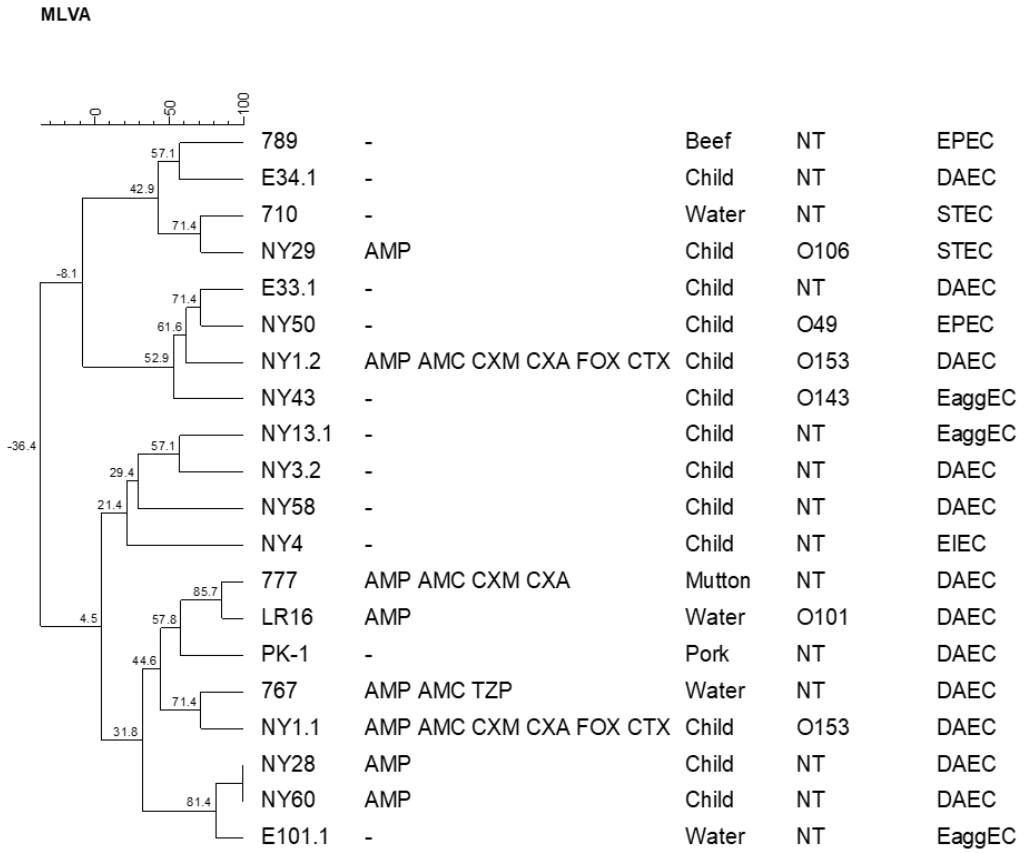

Figure 7:4 Dendrogram of the MLVA results of diarrheic E. coli from human and non-human sources in the Nyanga Township, Cape Town.

STEC= Shiga toxin producing *E. coli*, DAEC= Diffusely adherent *E. coli*, EIEC= Enteroinvasive *E. coli*, EaggEC=Enteroaggregative *E. coli*, EPEC= Enteropathogenic *E. coli*. NT=Non-typeable.

7.5 Discussion

In summary, the main findings of this study were; (1) tellurite resistant diarrheic *E. coli* from human and non-human sources were genotypically diverse and showed 19 distinct MLVA genotypes, (2) the two DAEC isolates (NY60 and NY28) had the same MLVA genotypes and antibiograms and were likely to be clonal, (3) tellurite resistant diarrheic *E. coli* from human sources were intermingled with those from the non-human sources but there were no clear clones, (4) the two STEC (NY29 and 710) showed a 71.4% level of genotypic homology and belonged to different serotypes, (5) the two ESBL producing DAEC from stool belonged to the same serotype (O153) but had different MLVA genotypes.

According to the joint Food and Agricultural Organisation (FAO)/ World Health Organisation (WHO) Core Expert Group Meeting on STEC, Geneva Switzerland, 19-22 July, 2016, the following was decided as regards source attribution (which was defined as the partitioning of the human disease burden of foodborne STEC illnesses to reservoirs and vehicles): (1) the subtyping approach is useful to determine the non-human reservoirs, (2) two approaches should be used to attribute regional and global burden of STEC infections to specific foods, namely; analysis of outbreak data and systematic review of case-control studies of sporadic laboratory-confirmed infections.[12] In this study, for source attribution, we used the MLVA subtyping approach to characterise tellurite resistant diarrheic *E. coli* from human and non-human sources in the Nyanga Township, Cape Town. The MLVA subtyping method that was used in this study is generic for all pathogenic *E. coli* and has been previously used to type both O157 and non- O157 STEC with high accuracy.[13,14]

The diversity of genotypes in this study could be affected by the sample size and sampling strategy, for example the study was conducted during a non-outbreak scenario and in summer months only. It was, however, expected that the different diarrheic *E. coli* pathotypes belonged to different MLVA genotypes.

The two DAEC isolates (NY60 and NY28) had the same MLVA genotypes, serotype and antibiotic resistance profile and were suspected to be a clone. The two children from whom these DAEC were isolated attended the same health care centre (Nyanga Community Health Centre) and were

both residents of the informal settlement in the Nyanga Township. Therefore, there is a possibility of transmission between these two children or a common source. Whole genome sequencing of the two isolates would help to confirm similarity.

MLVA genotypes of the other tellurite-resistant diarrheic *E. coli* from human sources were intermingled with those from the non-human sources but none belonged to matching clones (did not have 100% similar MLVA genotypes). The two STEC (NY29 and 710) showed a 71.4% level of genotypic homology and belonged to different serotypes. As per the Codex Committee on Food Hygiene (CCFH) recommendations for the attribution of STEC infection to reservoirs and vehicles[15], these two isolates are not from the same source.

The two ESBL-producing DAEC from stool belonged to the same serotype (O153) but had different MLVA genotypes and were therefore unlikely to result from a common source or transmission event.

Limitations of the study

The genotypic diversity is likely to have been affected by the small sample size and the short study period. Also, tellurite susceptible STEC and other diarrheic *E. coli* were not detected in this study. MLVA typing is not as discriminatory as Whole Genome Sequencing (WGS). The two DAEC isolates that had 100% similar MLVA genotypes might have differences at whole genome sequence level.

7.6 Conclusions

Diarrheic *E. coli* from Lotus River surface water, meat from the informal abattoirs, and stool of children with diarrhoea in the Nyanga Township were genotypically diverse. Two DAEC isolates from children with diarrhoea formed a clone. STEC detected in this study were genotypically diverse as were the ESBL-producing DAEC.

These results suggest that diarrheic *E. coli* from human and non-human sources in Nyanga are genotypically diverse. Further larger scale studies are needed to fully understand the epidemiology of STEC and other diarrheic *E. coli* in the Nyanga Township.

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Chapter Eight

8 General discussion and conclusion

Over sixty percent of human infectious diseases and newly emerging infectious diseases are transmitted from animals through direct or indirect means globally.[1,2] Mitigation of zoonotic disease threats requires an understanding of the dynamics of infectious diseases in complex multi-host communities.[2] Many foodborne diseases are zoonotic and exemplify the importance of the emerging 'One Health' approach to studying human and non-human infectious disease.[3] Globally, foodborne infections are increasing because of the globalisation of food trade, modernization and intensification of food production systems, and international travel. In South Africa, as is the case in many other countries, foodborne outbreaks are common but are rarely reported.[4] Since STEC is a persistent global pathogen, efforts to control foodborne disease should consider the contribution of STEC in the target population.[5]

This study aimed to use the One-Health approach to determine the prevalence of Shiga-toxin producing *Escherichia coli* in Cape Town and to characterise the epidemiology of STEC and other foodborne bacterial pathogens isolated from stool, meat and Lotus River surface water in Nyanga, Cape Town.

In summary, the main findings of this study were as follows:

The performance of CHROMagar™STEC

For all sample types, the performance of CHROMagar™STEC was poor compared to that of the duplex real-time PCR. This limitation of CHROMagar™STEC is in agreement with previous research which concluded that the use of tellurite-containing media in screening for STEC would allow the detection of STEC O26, O157, O111 and O145 but most strains from other serotypes would be missed.[6] However, the duplex real-time PCR may have detected the *stx* genes from non-viable bacteria (in the absence of live pathogens) or that are carried by free floating phages.

Prevalence of *stx* genes in stool, meat and surface water

There was generally a low prevalence of STEC in the different sources with a dominance of stx_1 as compared to stx_2. This could mean that STEC is a major public health threat in this setting. However, the tellurite susceptible STEC where missed.

Characteristics of diarrheic *E. coli* isolates from stool submitted to Groote Schuur Hospital

This study demonstrated that diarrheic *E. coli* in this clinical setting are resistant to some antibiotics and that solid medium allows proteomic detection of virulence and antimicrobial resistance proteins and that tellurite resistance proteins were detected in mauve colonies from CHROMagar™STEC. Additionally, we showed possible links between ciprofloxacin resistance and cell envelope thickness among pathogenic strains. Therefore these strains could be of clinical relevance and might be considered for routine screening at the tertiary health center level.

Prevalence and characteristics of diarrheic bacterial pathogens in Nyanga

This study was conducted in the absence of an outbreak and investigated the human and non-human sources in an informal settlement setting. Since the environment is highly contaminated with food and waterborne pathogens, it could be an important reservoir of these pathogens for human infection. The MLVA tool used in this study was not so informative especially given that there was no outbreak.

Foodborne antimicrobial resistance

There was no significant difference in resistance to antibiotics between diarrheic *E. coli* from stool (human), and the environment (meat and Lotus River surface water). Alterations of patient treatment regimes may be necessary especially for pathogens like *Shigella flexneri* which were prevalent in this setting.

8.1 Conclusions and future work

These studies demonstrate the importance of a One – Health surveillance approach for foodborne disease, and the need for broader adoption of similar surveillance approaches in South Africa. Specific gaps in this work, which should be addressed by further studies include the following:

(a) Since we were unable to determine whether tellurite-susceptible STEC are circulating, further research is needed using alternative culture media that allow growth of tellurite susceptible STEC. Use of a more sensitive test such as real-time PCR in parallel to enrichment and solid medium culture might be helpful.

More improved techniques such as whole genome sequencing might aid better characterisation of the STEC isolated in this study.

(b) To determine the STEC strains associated with severe disease, comprehensive microbiological testing and strain characterization should be done on patients presenting with HUS.

(c) To determine contribution of diarrheic pathogens (bacterial, viral, and parasitic) to diarrhoea in children in similar settings in South Africa, a larger scale case-control study over at least 12 months needs to be carried out in both primary health care and the community. Larger scale studies of food from informal abattoirs and surface water are needed to determine the generalizability of our findings.

(d) To understand the extent of foodborne antimicrobial resistance in this setting, there is need to screen for antimicrobial resistance genes in the human and non-human samples in addition to determining the antibiograms of specific foodborne pathogens isolated.

(e) To understand the linkage between ciprofloxacin resistance in STEC/EPEC strains and cell envelope thickness (as determined by transmission electron micrography), a larger sample size and carefully controlled experimental design is needed.

8.2 References

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9 APPENDIX

9.1 Questionnaire

Section 1: Demographic and Epidemiological information

1) **Case interviewed by**:

2) Who is the child's primary caregiver?

a. Mother
b. Grandmother
c. No relation
d. Grandfather
e. Aunt
f. Uncle
g. Father
h. Sister
i. Brother
j. Other; specify

3) How far did the primary caregiver go in school?

a. Primary
b. Secondary
c. Tertiary
d. Religious education
e. No formal education
f. Home address:

4) GPS coordinates of address

X:

Y:

5) Age:

6) **Date of birth of patient**:

7) **Sex of patient**:

a. Male
b. Female

8) What is the purpose of your visit to the health Centre?

a. Immunisation of child
b. Child is sick
c. Routine health check

9) How many episodes of stool has your child passed in the past 24hrs?

a. 1
b. 2
c. 3
d. 4
e. More than 4

10) How do you normally dispose of your child's faeces?

a. Flush toilet
b. Latrine
c. Bury
d. Bush/Field/Open sewer/Open stream
e. Other/specify

11) Has the child ever been vaccinated against the Rotavirus?

a. Yes
b. No
c. Not sure

12) During the last two weeks, has your household obtained drinking water from any of the following?

a. Dam
b. Bore hole
c. Protected water spring
d. River
e. Open well
f. House piped water
g. Communal standpipe

13) Do you store water before drinking it?

a. Yes
b. No

14) How many people have been living in your household regularly for the past six months?

a. 1
b. 2
c. 3
d. 4
e. 5
f. 6
g. More than 6

15) How many children under five years live in the household?

a. 1
b. 2
c. 3
d. 4
e. 5
f. 6
g. More than 6

16) What kind of toilet facility does your household use?

a. Flush toilet
b. Traditional pit latrine
c. Ventilated Improved Pit Latrine
d. Bucket system

17) When you wash your hands, what do you usually use?

a. Water
b. Water and soap
c. Other; specify

18) Do the following animals live in the compound where the child lives?

a. Goat
b. Dog
c. Cat
d. Sheep
e. Cow
f. Rodents
g. Fowl
h. No animal
i. Other; specify

Section 2: Clinical Information

19) Did the child have any of the following signs since the illness began?

a. Blood in stool
b. Fever
c. Cough
d. Drank much less than usual

e. Vomiting
f. Decreased activity or weakness
g. Belly pain
h. Unable to drink

20) Is the child currently breast fed?

a. Yes
b. No

21) Was the patient on antibiotics prior to the visit?

a. Yes
b. No

22) For how many day including today has the diarrhoea episode lasted?

a. 1
b. 2
c. 3
d. 4

Section 3: Diagnostic CRF

23) Stool sample appearance

a. Bloody
b. Not bloody
c. Rice waterly
d. Has mucus appearance

24) Isolated pathogens

a. Aeromonas
b. Plesiomonas
c. Campylobacter
d. Shigella
e. STEC
f. DAEC
g. EIEC
h. EAggEC
i. ETEC

25) Broth PCR results:

a. aat
b. daaC
c. LT
d. stx_1
e. stx_2
f. eaeA
g. wbdl
h. rfbE
i. i
j. pa

9.2 Informed consent form in English

1. For ill participants – You/Your child's illness/symptoms

Your child is presenting with diarrhea which could be caused by *Salmonella* and *E. coli* – two germs that are spread by contaminated food and water.

2. What is the *University of Cape Town* and what is this study about?

The University of Cape Town (UCT) works in collaboration with the National Institute for Communicable Diseases (NICD). The NICD is a public health laboratory organization that carries out surveillance and medical research to better understand the spread of germs. This study focuses on germs that cause diarrhea in children under the age of 12 years and their environment to better understand where these diseases come from.

3. What will it involve for me/my child?

Your child may have picked up a germ from food or water, or someone else in your community. To find out where this germ was picked up we are asking your permission for your child to participate in a surveillance study by providing a stool sample. If we find the germ in your child's stool, we will also ask for a sample of food or water from your home that may have been contaminated.

4. Compensation for study participants

If we remove some food from your home for testing, you will immediately be compensated with replacement cost. We will offer a one-off inconvenience compensation worth R50 for each study participant.

5. Are there any risks or disadvantages to me/my child of taking part?

There is no associated risk to collection of a stool sample from your child.

6. **Are there any benefits to me/my child of taking part?**

By taking part you/your child may help us find the sources of food and waterborne germs and improve the health of many other children and adults.

7. What happens if I refuse to participate?

All participation in research is voluntary. You are free to decide if you want your child to take part.

8. What happens to the samples?

Individual names are removed from all samples and replaced by codes, to ensure that samples can only be linked to the participants by people closely concerned with the study.

Most of the tests that will be done on the sample will be done at UCT. However, for some tests that cannot be done in South Africa, part of the samples will be sent to laboratories overseas.

After the research, a small portion of the stool samples will be stored. In the future, new research about food and waterborne germs may be done on these samples. Future research must first be approved by the University of Cape Town Research Ethics Committee to ensure participants' safety and rights are respected.

9. Who will have access to information about me/my child in this study?

All our study documents are stored securely in locked cabinets and password protected computers. Only a few people, pathologists and scientists closely concerned with the research, will be able to view information from participants.

10. Who has allowed this research to take place?

The University of Cape Town Research Ethics Committee have looked carefully at this work and agreed that the study is important, that it will be conducted properly and participants' safety and rights have been respected.

11. What if I have any questions?

You may ask any of our staff questions at any time:

Dr. Lourens Robberts

University of Cape Town, Division of Medical Microbiology

Telephone: *021 406 6727*

I, (being a guardian/next of kin of) _____ (name of child/next of kin), have had the study explained to me. I have understood all that has been read and had my questions answered satisfactorily.

(*Please insert the boxes below or add others where relevant***)**

□ **Yes, please** *tick I* **agree to allow my child/next of kin to take part in this research.**

□ **Yes, please** *tick I* **agree to samples being stored.**

□ **Yes, please** *tick I* **agree to samples being exported.**

I understand that I can change my mind at any stage and it will not affect care in any way.

Parent/Next of Kin/Guardian's: Name: _____

(Please print name)

Signature: _____Date _____ Time _____

I certify that I have followed the study SOP to obtain consent from the participant/guardian. S/he apparently understood the nature and the purpose of the study and consents to the participation [of the child] in the study. S/he has been given opportunity to ask questions which have been answered satisfactorily.

Designee/investigator's signature: _____ Date _____

Designee/investigator's name: _____Time _____

(Please print name)

Only necessary if the parent/next of kin/guardian cannot read:

I attest that the information concerning this research was accurately explained to and apparently understood by the subject/parent/guardian and that informed consent was freely given by the subject/parent/guardian.

Witness' signature: _____ Date _____

Witness' name: **_____ Time _____**

*A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.

Thumbprint of the parent/guardian/next of kin as named above if they cannot write:

THE SUBJECT/PARENT/GUARDIAN SHOULD NOW BE GIVEN A SIGNED COPY TO KEEP.

9.3 Informed Consent Form in IsiXhosa

1. Kubathathi nxaxheba abagulayo-impawu zokugula kwakho nomntwana wakho

Umntwana wakho ubonakalisa urhudo olwenziwa zintsholongwane ezimbini ekuthiwa yi *salmonella* ne *e. Coli* ezisasazwa lungcoliseko lwamanzi nokutya

2. Ingantoni idyunivesiti yasekapa kwakunye noluphando?

Idyunivesity yasekapa isebenzisana ne national institute for communicable diseases (nicd) incd ngumbutho we laboratory yezempilo kawonke-wonke eyenza ngcono uphando lwezempilo kulwazi ngosasazo lwentsholongwane. Oluphando lugxila kwintsholongwane ezibanga urhudo ebantwaneni abangaphantsi kweminyaka eyi-12 nakwi ndawo abahlala kuzo ukuze bazingcono ukuba ezi zifo zivelaphi.

3. Ndibandakanyeka ngantoni mna nomntwana wam?

Umntwana wakho angayifumana intsholongwane ekutyeni okanye emanzini endlwini yakho okanye komnye umntu ekuhlaleni. Ukwazi ukuba intsholongwane uyifumene phi siyakucela imvume yakho ukuba umntwana wakho athathe inxaxheba kolu phando ngokuthi anikezele ngelindle lakhe,ukuba sithe safumana intsholongwane kwilindle lomntwana wakho,siyakucela ukuba sithathe intwana yokutya okanye amanzi endlwini yakho ekunokuthi kanti anongcoliseko.

4.imbuyekezo kumthathi nxaxheba wophando.

Uyakuhlawulwa imali engange r50.00 voucher ngenxa yexesha lakho olichithileyo

Ukuba ngaba sithe sasusa intwana yokutya endlwini yakho ukuze kuvavanyelwe intsholongwane ,uyakufumana kwangoko imbuyekezo engange r50.00 ye voucher.

5.ingaba bukhona ubungozi okanye ukuhleleleka kum nomntwana wam ekuthatheni inxaxheba?

Akho bungozi obayanyaniswa nokuthathwa kwelindle kumntwana wakho.

6.ingaba ikhona imbuyekezo eza kum nomntwana wam ngokuthatha inxaxheba?

Ngokuthatha inxaxheba kwakho nomntwana wakho kunganceda ekufumaneni unobangela wentsholongwane ekutyeni nase manzini kwaye kuncede ukuphucula impilo yabantu nabantwana.

Abaninzi.

7.kwenzeka ntoni ukuba andivumi ukuthatha inxaxheba?
Wonke umntu uthatha inxaxheba ngokuzi thandela,ukhululekile ukuba uthathe isigqibo sokuvuma ukuba umntwana wakho athathe inxaxheba**.**

8.kwenzeka ntoni kwi sampuli ezithathiweyo?
Igama lomntu ngamnye liyakususwa kwilindle elithatyathiweyo kusetyenziswe i codes,ukuqinisekisa ukuba ilindle elithatyathiweyo lidityaniswa nomthathi nxaxheba ngabantu abasebenza sondeleyo nophando.

Phantse lonke uvavanyo oluyakwenziwa kweli lindle luya kwenziwa e uct.nangona olunye uvavanyo lungenakwenziwa e south africa inxenye yelindle iyakuthunyelwa kwi laboratories zaphesheya.

Emveni kokuba luphelile uphando intwana yelindle iyakugcinwa.kwixa elizayo kusenziwa uphando olutsha lwentsholongwane yokutya namanzi lungenziwa kweli lindle liselugcinweni.uphando lwexesha elizayo kufuneka luqale luphunyezwe yi university of capetown research ethics committee ukuqinisekisa ukhuseleko nama lungelo omthathi nxaxheba ayahlonitshwa.

9.ngubani onelungelo kwi nkcukacha zam nomntwana wam?
Zonke inkcukacha zophando zivalelwa kwi khabhathi ezitshixwayo kwakunye ne computers ezikhuseleke nge nombolo eziyimfihlo.bambalwa abantu abanjenge ngcaphephe no so science kwakunye nabasebenza ngokusondeleyo koluphando abayakukwazi ukujonga ezi nkcukacha.

10.ngubani ovumele oluphando luqhutywe?
I university of capetown research ethics committee iye yajonga ngononophelo lomsebenzi yavumelana ukuba oluphando lubalulekile kwaye luyakwenziwa ngendlela eyiyo kwaye ukhuseleko namalungelo omthathi nxaxheba ahloniphekile.

11.ndingenza ntoni xa ndinemibuzo?
Ungabuza nawuphi na umsebenzi wophando imibuzo nangaliphi na ixesha**.**

Dr.lourens robberts

University of cape town, division of medical microbiology

Umnxeba: 021 406 6727

Mna(mzali okanye isizalwane sika) _____ (igama lomntwana/ isizalwana), ndivile ngengcaciso yophando.ndiyakuqonda konke okufundiweyo nemibuzo yam iphendulekile ngokwaneleyo.

(phendula ngokufanelekileyo)

□ ewe ndiyavuma umntwana wam/isizalwane sam sithathe inxaxheba koluphando

□ ewe ndiyavuma ilindle libeselugcinweni.

□ ewe ndiyavuma ilindle lithunyelwe

Ndiyaqonda ukuba ndingatshintsha isigqibo nangaliphi ixesha kwaye akukho nto izakuchaphazela unyango lwam.

Umzali /isizalwane : igama:_____

(onobumba abakhulu)

Utyikityo:_____umhla _____ ixesha _____

Ndiya qinisekisa ukuba ndiyilandele imigaqo yophando ukuthatha isivumelwano kumthathi nxaxheba/kumzali. Uye wakuqonda ubume nenjongo yophando wavuma ukuthatha inxaxheba(komntana)koluphando.ulifumene ithuba lokubuza imibuzo yaphenduleka ngokwanelisayo.

Igama elipheleleyo lomntu othatha imvume: _____ixesha_____

(onobumba abakhulu)

Utyikityo lomntu othatha imvume _____umhla _____

Xa umzali /isizalwane singakwazi ukufufunda:

Ndiya ngqinisisa ukuba inkcukacha malunga noluphando zicaciswe ngqo kwaye luyaqondwa ngumthathi nxaxheba/umzali kwaye isivumelwano sinikezelwe ngumthathi nxaxheba ngokuzithandela.

Utyikityo lwengqina _____umhla _____

Igama lengqina: _____ixesha _____

*ingqina ngumntu oxhomekeke kuvavanyo okanye kwilungu lomsebenzi elingazanga lizibandakanye ngokuzuza kwisivumelwano**:**

Ubhontsi womzali/ummeli/isizalwane esikhankanyiweyo ngentla ukuba asikwazi kubhala**.**
Umthathi nxaxheba kufuneke enikwe i kopi etyikityiweyo ibekuye.
Amagama okuqala omthathinxaxheba: _____

9.4 Ethics approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492 • **Facsimile** [021] 406 6411
Email: Sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

13 March 2015

HREC/REF: 140/2015

Dr L Roberts
Medical Microbiology
Room 5.06 Level 5
Falmouth Building-FHS

Dear Dr Roberts

Project Title: NYANGA INTEGRATED FOOD- AND WATERBORNE DISEASE SURVEILLANCE (PhD candidate-B Kalule)

Thank you for your response letter dated 10 March 2015, addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has **formally approved** the above mentioned study.

Approval is granted for one year until the 28 March 2016.

Please sent the assent script for the HREC.

Please submit a progress form, using the standardised Annual Report Form, if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approve period.

We acknowledge that the following student:-Bosco Kalule is also involved in this project.

Please note that the on-going ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC REF in all your correspondence.

Yours sincerely

signature removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Hrec/ref:140/2015

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
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Website: www.health.uct.ac.za/research/humanethics/forms

21 January 2014

HREC REF: 015/2014

Dr L Robberts
Medical Microbiology
Room 5.15
Falmouth Building

Dear Dr Robberts

PROTOCOL NO: R&D STEC V1.0
PROJECT TITLE: ENHANCED SURVEILLANCE FOR SHIGA TOXIGENIC ESCHERICHIA COLI IN STOOL SPECIMENS FROM THE WESTERN CAPE

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th January 2015

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/research/humanethics/forms)

We acknowledge that the student Dr Chad Centner is also involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC reference no in all your correspondence.

Yours sincerely

signature removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN ETHICS
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical