GENOTYPIC ANTIBIOTIC RESISTANCE PROPERTIES OF ESCHERICHIA *COLI* AND *CAMPYLOBACTER JEJUNI* ASSOCIATED WITH DIARRHOEA IN YOUNG CHILDREN IN THE VHEMBE DISTRICT

by

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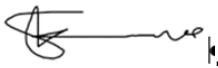
Declaration

I, **Simbarashe Karambwe (Student number 11613034)** hereby declare that this thesis is my original work and design, and all the literature has been duly acknowledged through referencing. This thesis has not been submitted and will not be presented at any other University for a similar or any other degree award.

Student: Signature ……  **…… Date: …28/02/2024……**

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Dedication

I dedicate this thesis to my father Stanley Karambwe, my mother Elesta Mutukura, my siblings Isaac Karambwe and Forget Karambwe.

Preface

This thesis comprises of five chapters.

Chapter 1: This chapter is composed of the introduction, the aim, problem statement, rationale, and objectives.

Chapter 2: This chapter is composed of the literature review.

Chapter 3: This chapter is based on the materials and methods.

Chapter 4: This chapter is based on the results and discussion.

Chapter 5: This chapter is composed of the conclusion, recommendations, and limitations.

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List of abbreviations

3GC	-	Third generation Cephalosporin
AK	-	Amikacin
AMC	-	Amoxicillin–clavulanate
AMP	-	Ampicillin
AMR	-	Antimicrobial resistance
AMX	-	Amoxicillin
ARG	-	antimicrobial resistance genes
AST	-	antibiotic susceptibility testing
bla$_{CTX-M}$	-	β-lactamase CTX-M
C	-	Chloramphenicol
CAZ	-	Ceftazidime
CIP	-	Ciprofloxacin
CL	-	Colistin
CLSI	-	Clinical Laboratory Standard Institute
COT	-	Cotrimoxazole
CTR	-	Ceftriaxone
CTX	-	Cefotaxime
DAEC	-	diffusely adherent E. coli
DEC	-	diarrheagenic *E. coli*
DNA	-	Deoxyribonucleic acid
E	-	Erythromycin
EAEC	-	Enteroaggregative *E. coli*
EHEC	-	Enterohemorrhagic E. coli
EIEC	-	Enteroinvasive E. coli
EMB	-	Eosine methylene blue
EPEC	-	Enteropathogenic *E. coli*
ESBL	-	extended spectrum β-lactamase
ETEC	-	Enterotoxigenic *E. coli*
GEMS	-	Global Enterics Multicenter Study
GEN	-	Gentamicin
*gyr*A	-	gyrase A

IM	-	Imipenem
LE	-	Levofloxacin
MAL-ED	-	Malnutrition and Enteric Disease
MDR	-	Multidrug resistance
MRP	-	Meropenem
NA	-	Nalidixic Acid
ORS	-	Oral Rehydration Solution
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase chain reaction
QRDR	-	Quinolone Resistance Determining Region
rRNA	-	ribosomal ribonuncleic acid
S	-	Streptomycin
ST-ETEC	-	Shiga-toxin enterotoxigenic *E. coli*
WHO	-	World Health Organisation

List of symbols

%	-	Percentage
°C	-	degrees celcius
µl	-	microliters
µM	-	micrometer
β	-	beta
13300 r/min	-	13300 revolutions per minute
ng/µl	-	nanogram per microliter
A260/A280 nm	-	ratio of absorbance at 260 nm and 280 nm
~300bp	-	approximately 300 base pairs

List of outputs

Journal articles in preparation or published.

- Review article:
  - Published on the 15th of January 2024
  - Karambwe, S.; Traoré, A.N.; Potgieter, N. Epidemiology of Cefotaxime-Hydrolysing β-Lactamase-Producing *Escherichia coli* in Children with Diarrhoea Reported Globally between 2012 and 2022. *Microorganisms* 2024, 12, 171. https://doi.org/10.3390/microorganisms12010171 (Appendix C, pp 104).
- Article 1:
  - Faecal Carriage of Multi-drug resistant *Escherichia coli* isolated from Children under 5 years with and without Diarrhoea in the Vhembe District of South Africa
  - In preparation
- Article 2:
  - Carriage of *gyr*A and *bla*$_{CTX-M}$ resistance genes in *E. coli* isolates from children with diarrhoea in Venda, South Africa
  - In preparation

Conference/Workshop Proceedings

- World Antimicrobial Awareness Week (WAAW) Research Symposium 2022
  - Preventing Antimicrobial Resistance Together: A One Health Approach - Oral presentation

  Abstract Title: Carriage of *gyr*A and *bla*$_{CTX-M}$ resistance genes in *E. coli* isolates from children with diarrhoea in Venda, South Africa

DNA Sequences submitted to GeneBank

Accession numbers:

- ***gyr*A**
  - OP132375
  - OP132376

- OP132377
- OP132378
- OP132379
- OP132380
- OP132381
- OP132382

- **mdh**
  - OP132383
  - OP132384
  - OP132385
  - ON783719
- ***bla*CTX-M-15**
  - OP271864
  - OP271862
  - OP271863

Abstract

Background: Diarrhoea continues to threaten the lives of young children in Sub-Saharan Africa. While antibiotic resistance among enteric pathogens such as *Escherichia coli* (*E. coli*) and *Campylobacter* spp. is increasing, surveillance of antimicrobial resistance genes (ARG) is limited in Africa. The need for such studies in Africa was demonstrated by our published review of the literature on surveillance of molecular resistance mechanisms like *bla*$_{CTX-M}$. Therefore, this study sought to investigate the genotypic (*bla*$_{CTX-M}$ and *gyr*A) antibiotic resistance profiles of bacteria causing diarrhoea in young children in the Vhembe district.

Methods: A cross-sectional surveillance was done between August 2020 and August 2021. Diarrhoeal (lose, watery) and non-diarrhoeal (normal, solid) stool samples were collected from children under the age of five at selected hospitals and clinics around the Vhembe District. The Kirby Bauer Disk Diffusion technique was used to screen for antibiotic susceptibility, and PCR and sequencing were used for molecular characterization of antibiotic resistance genes.

Results: Of the *E. coli* positive samples, 39% (18/46; 12 diarrhoeal and 6 non-diarrhoeal) had multi-drug resistance (MDR) to at least three antibiotics, with 33% (6/18) and 11% (2/18) having fluoroquinolone (*gyr*A) and ß-lactam (*bla*CTX-M) resistance mechanisms, respectively. Five percent (1/18) of the samples carried both *gyr*A and *bla*$_{CTX-M}$ genes. The prevalence of *Campylobacter* in diarrhoeal stools was 13.8% and *gyr*A gene was partially detected.

Conclusion: Children under the age of two in the Vhembe District continue to be at risk from diarrhoea due to antibiotic resistant *Escherichia coli* and *Campylobacter*. This study raises awareness of the prevalence of MDR, and aids medical professionals in implementing the appropriate treatment. Future research should consider concurrent studies on clinical and environmental samples to determine the possible role of livestock and river water as carriers of antibiotic resistance genes.

Keywords: *E. coli*, *Campylobacter*, children, diarrhoea, antibiotic resistance, South Africa

Chapter 1: General introduction

This Chapter provides a general introduction of this study and introduce the aim, problem statement, rationale, and objectives of the study.

Chapter 1

GENERAL INTRODUCTION

1.1 Background

In children under five, diarrhoea is a significant cause of death and disability-adjusted life years (DALYs) (Troeger et al., 2018). At least 6 billion episodes of diarrhoea and more than 1 million deaths annually have been reported in nations with low and medium incomes (Garbern et al., 2021; Troeger et al., 2018). In recent years, diarrhoea has remained among the top 3 causes of death in children under 5 years and accounts for 9% of all deaths (UNICEF, 2024; Demissie et al., 2021). The impact of diarrhoea in children is noticeable. In 2019, the Institute for Health Metrics and Evaluation (IHME) report showed that at least half a million children less than 5 years old died from diarrhoeal diseases globally (IHME, 2022).

In South Africa, diarrhoea accounts for at least 20% of deaths in children under the age of five (Nguyen et al., 2021; Chola et al., 2015). The modes of transmission of diarrheagenic pathogens to children include contaminated water, contaminated food, poor hygiene by breastfeeding mothers and caregivers as well as lack of proper environmental hygiene and sanitation practices (Nogueira et al., 2021; GebreSilasie et al., 2018).

Several pathogens such as viruses, parasites, and bacteria are associated with diarrhoea illness in children (Soli et al., 2014). The most reported bacteria causing diarrhoea in children include *Campylobacter* spp, *Escherichia coli [E. coli]* spp, *Salmonella* spp, *Vibrio* spp, *Shigella* spp, and *Aeromonas* spp among others (Kotloff et al., 2019; Amour et al., 2016). While around 30–40% of acute diarrhoea episodes in children under the age of five in underdeveloped nations are caused by diarrheagenic *E. coli* (DEC), (Miliwebsky et al., 2016), *Campylobacter* infections are endemic in Low- and Middle-income countries (LMIC) especially in Asia, Africa, and the Middle East (Behailu et al., 2022). Although diarrhoea caused by *E. coli* strains is usually self-limiting (Huang et al., 2006), the emergence of antibiotic-resistant strains displaying the extended-spectrum beta-lactamase (ESBL) phenotype is becoming a public health concern (Karami et al., 2017; Konate et al., 2017 Swierczewski et al., 2013). The involvement of *E. coli* as a mediator of horizontal transmission of antibiotic resistance plasmids among Enterobacteriaceae species is well documented

(Rapoport et al., 2016). Treatment options are limited since ESBL-producing *E. coli* bacteria frequently show multidrug resistance to other antibiotics, including aminoglycosides and fluoroquinolones (Eltai et al 2020). The World Health Organisation's (WHO) priority list of pathogens includes third-generation cephalosporin-resistant Enterobacteriaceae and fluoroquinolones resistant species namely *Campylobacter* spp, *Salmonella* spp, and *Shigella* spp (WHO, 2017).

Antibiotic resistance in intestinal infections is rising in sub-Saharan Africa (Brander et al., 2017; Tansarli et al., 2014; Woerther et al., 2011). The driving factors of antibiotic resistance have been linked to inappropriate prescription practices of antibiotics in pediatric diarrhoea (Rogawski et al., 2016) and the fact that antibiotics are not used as prescribed (WHO, 2018). The latter can be supported by literature on bystander antibiotic exposure of enteric pathogens in subclinical infections which has been reported in different countries in South Asia, sub-Saharan Africa, and South America (Rogawski et al., 2022). Additionally, there is evidence that children under the age of two who have diarrhoea are consuming antibiotics, which is against WHO recommendations (Qu et al., 2016). According to the findings of prospective Malnutrition and Enteric Disease Study (MAL-ED) cohort research conducted in eight different countries across Africa, Asia, and South America, the first two years of life are marked by a high rate of antibiotic usage in children under the age of two (Rogawski et al., 2016). Although antibiotics used to treat diarrhoea vary between geographical regions (Rogawski et al., 2016), information on antibiotic resistance had been reported to be scarce in Africa (WHO, 2014). The goal of the WHO Global Action Plan on Antimicrobial Resistance was to increase the use of surveillance-based control methods (WHO, 2018). Thus, the purpose of this study was to investigate the genotypic antibiotic resistance profiles of bacteria that cause diarrhoea in young children in the Vhembe district of the Limpopo Province, South Africa, including *E. coli* and *Campylobacter* spp.

1.2 Rationale of Study

Children under the age of five who suffer from diarrhoea are more likely to become ill or die in countries with low and medium incomes (UNICEF, 2024; Kotloff et al., 2019). In South Africa, diarrhoea is one of the prime causes of death in children under 5 years

(van der Westhuizen et al., 2019). The key risk factors associated with the spread of diarrhoea in communities are poor sanitation and hygiene (Mebrahtom et al., 2022). Access to sanitary facilities and clean drinking water is still limited in the Vhembe District of the Limpopo Province in South Africa, where nearly all the settlements are mainly rural (Traore et al., 2016). In these rural communities, open water sources such as rivers are the alternative sources of drinking water, and this poses a serious health risk to young children (Potgieter et al., 2020; Traore et al., 2016). In addition, Brander et al. (2017) discovered a connection between poor sanitation and multi-drug resistance (MDR). Further risk factors for the spread of antibiotic-resistant bacteria to children include unsanitary breastfeeding, poor hygiene practices, contaminated weaning foods, and not enough knowledge of the causes of diarrhoea among caregivers and mothers who breastfeed (Kinkese et al., 2018).

The prevalence of the most common bacteria that cause diarrhoea in children under the age of five has been the subject of several studies conducted in the Vhembe District, Limpopo Province, South Africa. These bacteria include *Campylobacter* spp., Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Shiga toxin-Enterotoxigenic *E. coli* (ST-ETEC), and *Shigella* spp (Potgieter et al., 2023; Samie et al., 2022; Ledwaba et al., 2018; Amour et al., 2016; Platts-Mills et al., 2015). Generally, a high burden of *Campylobacter* infections has been reported among children under 24 months of age (Samie et al., 2022; Amour et al., 2016). Based on the latter reports, it is evident that *Escherichia coli* and *Campylobacter* spp associated with diarrhoea in children under 5 years are endemic in the Vhembe District. However, little has been done on surveillance of associated antibiotic resistance. In addition, extended-spectrum β-lactamase-producing Enterobacteriaceae such as *E. coli* have significantly developed an increase in molecular resistance (Nashwa et al., 2022). Therefore, it is important to understand the various resistance mechanisms which is crucial in the discovery of new antibiotics. Continuous monitoring of antibiotic-resistant clinical isolates is essential in identifying emerging antibiotic resistance patterns which is informative and as well as guiding the prescription of antibiotic therapy. This helps to curb the morbidity and mortality rates due to resistant pathogenic bacteria.

1.3 Problem statement

According to the report by Massyn et al (2015) on the disease profile for the Vhembe Health District, Limpopo Province, diarrhoea was considered among the leading causes of premature mortality in 2011. The case fatality ratio due to diarrhoea in the Vhembe District over the course of four years, from 2010-2013, decreased from 8.9% to 4.3%. Additionally, Sivhaga et al. (2012) noted that the incidence of diarrhoea has decreased by more than 5/1000. The latter findings may be explained by improved management of diarrhoea cases in children (Massyn et al., 2015). However, some sub-districts in the Limpopo province of South Africa, such as the Mutale district, had no data for diarrhoea deaths among children less than 5 years due to the absence of a hospital and this may mask an underlying severe problem (Massyn et al., 2015). On the other hand, diarrhoea is still a challenge in rural communities in the Vhembe District with the costs of treating diarrhoea symptoms in children escalating due to poor management by healthcare workers who unnecessarily prescribe antibiotics (Potgieter et al., 2018), instead of the recommended oral rehydration (ORS), continued feeding and zinc supplements (UNICEF, 2024). Studies in 8 different countries, including South Africa reported high levels of antibiotics prescription and administration for diarrhoea in young children suggesting a high risk for antibiotics resistance in this population (Rogawski et al., 2016). The disadvantage of antibiotic treatment is that microorganisms that are not the target organism (bystander organisms) are exposed which results in acquired resistance. Bacteria such as *Campylobacter*, typical enteropathogenic *E. coli,* and enterotoxigenic *E. coli* are frequently exposed to antibiotics when they are not the causative organism of diarrhoea (Rogawski et al., 2022). Inappropriate treatment is a risk factor that could worsen the course of the disease resulting in high mortality in children with diarrhoea (Lanyero et al 2021).

Despite a noticeable drop in case of fatalities as justified by the reports above (Massyn et al., 2015; Sivhaga et al., 2012), the microorganisms associated with diarrhoea are constantly evolving and resistance to empirical antibiotic treatment due to extended-spectrum β-lactamase (ESBL)-producing *E. coli* has been reported in the Vhembe District (DeFrancesco et al., 2017). Brander et al (2017) study on the correlation of antibiotic resistance in enteric bacteria isolated in children with diarrhoea in Kenya indicated that poor sanitation was associated with resistance to at least three antibiotics. There are still challenges to adequate sanitation and hygiene in the

Vhembe District in South Africa (Murei et al., 2022; Potgieter et al., 2018), which are key risk factors critical for reducing high case fatalities due to diarrhoeal outbreaks (Mebrahtom et al., 2022). All these factors warrant further investigations on antibiotic resistance profiles of diarrheagenic microorganisms in the Vhembe District.

1.4 Research questions

The current study was guided by the following inquiries:

1. Is there evidence of antibiotic or multidrug resistance of diarrhoea-causing bacteria in the Vhembe District?
2. Do the circulating *Campylobacter* species and *E. coli* pathotypes in the Vhembe District exhibit any signs of resistance genes (*bla*$_{CTX-M}$ or *gyr*A)?
3. What is the phylogenetic profile of the resistance genes circulating in the Vhembe District?
4. What is the prevalence of *bla*$_{CTX-M}$ producing *E. coli* in the Vhembe District of South Africa?

1.5 Aim and Objectives

This study aimed to investigate the genotypic antibiotic resistance profiles of *Escherichia coli* and *Campylobacter jejuni* causing diarrhoea in young children in the Vhembe district.

- ✓ To screen diarrhoea stool samples for the presence of specific *E. coli* pathotypes (ETEC, EPEC, EAEC) and *Campylobacter* spp using multiplex PCR, and Allplex™ real-time PCR respectively.
- ✓ To detect specific antibiotic resistance genes associated with *E. coli* pathotypes and *Campylobacter spp.* using conventional PCR and sequencing.
- ✓ To determine the relatedness of the antibiotic resistance genes using phylogenetic analysis

Chapter 2: Literature Review

This chapter provides a summary of the literature and overview of diarrhoea in young children and treatment management, the causative enteric bacteria such as *E. coli* and *Campylobacter*, the antibiogram profiles as well as the antibiotic resistance mechanisms.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

Worldwide, diarrhoeal diseases claim the lives of young children. A study conducted in Beijing on the prevalence of antibiotic resistance in childhood reported that children under the age of two had the highest cases of diarrhoea (Qu et al., 2016). In addition, the prevalence of diarrhoea was shown to go down with increasing age (Rathaur et al., 2014; Jafari et al., 2009). On the other hand, research has demonstrated that as children grow, the human gut microbiota becomes more diverse in terms of antibiotic resistance (Lu et al., 2014). In support of the latter observation, Moore et al (2013) research on pediatric fecal microbiota of healthy infants reported that children and adolescents harboured a diverse array of resistance genes associated with mobilisation elements poised for dissemination.

It is worth noting that diarrhoea is self-limiting in most cases, however, some instances require chemotherapeutic intervention such as antibiotics. According to the World Health Organisation (WHO) guidelines for combating diarrhoea, antimicrobials should be prescribed only for known causative agents. Additionally, findings from prospective MAL-ED cohort research on antibiotic use in young children in eight countries in Africa, Asia, and South America showed that the first two years of life were associated with a higher rate of antibiotic usage (Rogawski et al., 2016).

It is worth noting that antibiotics are only helpful in cases of bloody diarrhoea and suspected cholera with severe dehydration (Bhan, 2005). However, the observations made by Qu et al (2016) in which a similar antibiotic consumption rate was noticed regarding diarrheagenic *E. coli*, *Salmonella* spp, and *Shigella* spp could mean that antibiotics are being used in any case of diarrhoea without confirmation of the etiological agent in question. Similarly, a study done by Rogawski et al (2016) produced empirical evidence regarding the inappropriate use of antibiotics in resource-limited areas. The latter study observed a small difference in the percentages of antibiotic-treated non-bloody diarrhoea (21%) and bloody diarrhoea (33%) in Venda in the Vhembe District, South Africa relative to other countries in the developed world. This could indicate a possible fixed prescription of antibiotics regardless of the type of causative agent in question.

2.2 Antibiotic management of diarrhoea in children

Diarrhoea can be acute (occurs for less than 14 days), or it may be persistent when occurring for more than 14 days (WHO, 2005). According to the WHO Handbook on childhood illness, diarrhoea is common among formula-fed children as well as those using cow's milk (WHO, 2005). Perhaps contamination from the hands of mothers or caregivers and contaminated cow's milk could account for the latter observations. There is evidence of a lack of proper education among caregivers which negatively impacts their knowledge, attitudes, and practices in preventing diarrhoea in children (Ndou et al., 2021). Deaths of children suffering from diarrhoea is known to occur due to dehydration and malnutrition. Therefore, the standard primary management of diarrhoea is oral rehydration solution (ORS), continued feeding, and zinc supplements (UNICEF, 2024). Rational antibiotic use is recommended by the World Health Organisation (WHO) in which antibiotics are preferably prescribed for bloody diarrhoea (WHO, 2005). However, a study by Pernica et al (2016) in Botswana found that treatable bloody diarrhoea due to *Shigella* was not associated with poor outcomes such as death. On the contrary, a considerable number of children with non-bloody diarrhoea attributable to *Campylobacter* and ETEC accounted for most deaths (Pernica et al., 2016).

2.3 Overview of diarrhoeal-causing bacteria and Antibiotic Resistance in Africa

Due to the emergence of antibiotic resistance patterns against routinely given medications, pediatric diarrhoea in Africa is becoming increasingly difficult to treat (WHO, 2018; WHO, 2017). Children's diarrhoea can be caused by bacteria like *Campylobacter*, diarrheagenic *E. coli* (DEC) pathotypes, and Noroviruses (Samie et al., 2022; Ledwaba et al., 2018; DeFrancesco et al., 2017; Kabue et al., 2016; Samie et al., 2009). Diarrheagenic *E. coli* (DEC) consists of six strains; enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) and diffusely adherent *E. coli* (DAEC) and is reported as a major cause of diarrhoea in pediatric population (GebreSilasie et al., 2018; Miliwebsky et al., 2016; Croxen et al., 2013).

The most often reported DEC strains that cause diarrhoea in young children are EAEC, EPEC, and ETEC (Kotloff et al., 2019; Saka et al., 2019; Platts-Mills et al., 2018; Shah et al., 2016; Langendorf et al., 2015; Mosquito et al., 2015; Lanata et al., 2013). Several studies including the Global Enterics Multicenter Study (GEMS) have identified EPEC, ETEC, rotavirus, calicivirus, and *Cryptosporidium* as the prominent causes of diarrhoea (Black et al., 2024., Kotloff, 2017., Lanata et al., 2013). Studies in South Africa have reported that EAEC, EPEC, and ETEC are the dominant *E. coli* pathotypes involved in pediatric diarrhoea in communities living in rural areas (Potgieter et al., 2023; Ledwaba et al., 2018; Potgieter et al., 2018).

The EAEC strains have been the frequent cause of acute and persistent diarrhoea in all age groups and are also responsible for malnutrition in children (Guerrieri et al., 2019; Gomes et al., 2016; Huang et al., 2006). It has also been reported that the prevalence of EAEC increases with age within the first two years of life (Rogawski et al., 2017). The symptoms of EAEC infection include watery diarrhoea with or without blood and mucus, nausea, abdominal pain, fever, and vomiting (Huang et al., 2006). Enteropathogenic *E. coli* (EPEC) is linked with diarrhoea outbreaks and is considered a high-risk pathogen causing fatal cases of diarrhoea in infants (Langendorf et al., 2015; Lanata et al., 2013). In addition, EPEC has also been strongly linked with community-acquired diarrhoea (Thakur et al., 2018; Estrada-Garcia et al., 2009). According to data from the MAL-ED cohort study (Platts-Mills et al., 2018) and the Global Enterics Multicenter Study (GEMS) (Vidal et al., 2019; Zimmermann et al., 2019), enterotoxigenic *E. coli* (ETEC) is one of the top four consistently occurring causes of moderate-to-severe diarrhoea in children under five.

According to the WHO Global Antimicrobial Resistance Surveillance System (GLASS), *E. coli* is one of the specific bacteria that infect people (Pholwat et al., 2019; WHO, 2018). While diarrhoea due to *E. coli* typically resolves on its own (Huang et al., 2006), the rise of antibiotic-resistant strains that exhibit co-resistance to other antibiotic classes and the extended-spectrum beta-lactamase (ESBL) phenotype is a growing public health concern (Karami et al., 2017; Konate et al., 2017b; Swierczewski et al.,2013). The WHO priority list of pathogens includes third-generation cephalosporin (3GC) resistant Enterobacteriaceae and fluoroquinolone-resistant species; *Campylobacter* spp, *Salmonella* spp and *Shigella* spp (WHO, 2017).

In sub-Saharan Africa, antibiotic resistance in enteropathogens is increasing (Brander et al., 2017; Tansarli et al., 2014; Woerther et al., 2011). The driving factors of antibiotic resistance has been linked with prescription practices of antibiotics in pediatric diarrhoea (Rogawski et al., 2016) and the fact that antibiotics are used inappropriately (WHO, 2018). Although antibiotics used to treat diarrhoea vary between geographical regions (Rogawski et al., 2016), information on antibiotic resistance was reported to be scarce in Africa (WHO, 2014). The WHO Global Action Plan on Antimicrobial Resistance sought to scale up efforts on control strategies through surveillance (WHO, 2015).

2.3.1 Review of studies in Africa between 2010-2020: An update on antibiotic resistance

A mini-review of studies published between 2010 and 2020 was conducted using PubMed, Google Scholar, and Science Direct as search engines. Key words included the following: diarrhoea AND antibiotic resistance AND Africa; *E. coli* AND pediatric diarrhoea AND Africa; pediatric diarrhoea AND Africa; diarrheagenic *E. coli* AND antibiotic resistance AND Africa; diarrheagenic *E. coli*. In addition, the bibliographies of relevant studies were scanned through for additional eligible studies. Articles were selected if (i) the study was done in Africa, (ii) the study group included children under 5 years, (iii) data on diarrheagenic *E. coli* (DEC) pathotypes were available, (iv) the study reported on selected *E. coli* pathotypes (EPEC, ETEC, and EAEC) and (v) the study was published between 2010 and 2020. Studies on DEC including all age groups were not included in the assessment.

The name of the author, year of publication, study period, country, region of the African continent, age group, type of diarrhoea, sample size, study site (rural or urban), study setting (hospital, community, outpatient), etiology (*E. coli* only or mixed bacterial pathogens), the percentage of *E. coli* isolates detected, and antibiotic resistance were extracted.

The search identified 72 studies published between 2010 and 2020. After screening, 34 studies from 15 countries on children under five years of age met the criteria and were included for further assessment (Table 2.1). The 15 countries included Kenya, Tanzania, Ethiopia, Sudan, Niger, Burkina Faso, Guinea, Nigeria, Egypt, Tunisia,

Lybia, Mozambique, Malawi, Zambia, and South Africa. Of these 15 countries, 80% (12/15) fall under the sub-Saharan Africa region.

A total of 52,9% (18/34) of the studies found, reported on phenotypic resistance profiles of diarrheagenic *E. coli* (DEC). Only one study (5.6%; 1/18) in South Africa reported on the molecular aspects of antibiotic resistance (DeFrancesco et al., 2017). A total of five studies (5/18; 27.8%) investigated the resistance profiles of EPEC, ETEC, and EAEC to first-line antibiotics such as ampicillin, tetracycline, cotrimoxazole (or trimethoprim-sulfamethoxazole) and chloramphenicol which are commonly used for the treatment of diarrhoea (Table 2.2). In total, only 3 studies (3/18; 16.7%) reported on the MDR profiles of the EAEC, EPEC, and ETEC strains (Saka et al., 2019; Seidmann et al., 2016; Ali et al., 2014).

The proportions of EAEC, EPEC, and ETEC isolates resistant to ampicillin and cotrimoxazole ranged between 75%-100% (Saka et al., 2019; Seidman et al., 2016; Odetoyin et al., 2016; Langerndorf et al., 2015; Ali et al., 2014). Multidrug resistance (MDR) in *E. coli* isolates was reported in 55.5% (10/18) of the studies (Dabo et al., 2019; GebreSilase et al., 2019; Saka et al., 2019; Konate et al., 2017; Seidman et al., 2016; Shah et al., 2016; Odetoyin et al., 2016; Langendorf et al., 2015; Ali et al., 2014; Moyo et al.,2011). However, only 3 studies reported MDR data for EAEC, EPEC, and ETEC pathotypes (Table 2.2). The EAEC strains were commonly linked with MDR phenotype (Saka et al., 2019; Seidman et al., 2016; Ali et al., 2014) (Table 2.2).

Out of the 18 studies that surveyed phenotypic resistance, only 22.2% (4/18) studies screened extended-spectrum beta-lactamase (ESBL) phenotype using the double disk synergy assay (Konate et al., 2017; Dembele et al., 2015; Langendorf et al., 2015; Ali et al., 2014). Whereas high proportions of the EAEC strains (91.3%) resistant to third-generation cephalosporins (3GCs) were reported (Ali et al., 2014), the proportion of the EPEC strains associated with resistance to 3GCs was approximately 14% (Behiry et al., 2011). According to a South African study, the primary mechanism of beta-lactamase resistance in diarrheagenic *E. coli* is TEM-1. Additionally, 4.9% (2/41) of ESBL such as CTX-M-14 was reported. (DeFrancesco et al., 2017).

Table 2.1: Studies published between 2010 and 2020 on paediatric diarrhoea caused by ETEC/EPEC/EAEC strains in African Countries

Africa Region	Country	Setting	Health Facility	DEC Pathotypes (% Detection rate)			Period of Study	Reference
				EPEC	ETEC	EAEC		
Eastern	Ethiopia	Urban	Hospital	NC	NC	NC	2015	GebreSilasie et al 2018
	Kenya	Sub-urban	Hospital	11.6	24.3	50	2009-2013	Shah et al 2016
	Kenya	Urban	Hospital	6.8	9.1	12.3	2012	Karambu et al 2013
	Kenya	Urban	Hospital	4.5	10.5	21.2	NS	Nyanga et al 2017
	Kenya	Urban	Hospital	16.5	16.3	45.1	2007-2009	Iijima et al 2017
	Kenya	NS	Hospital	NC	1.2	8,9	2007-2008	Sang et al 2012
	Kenya	Urban	Hospital	19.3	7.25	3.86	2005-2008	Makobe et al 2012
	Kenya	NS	Hospital	1.6	4.3	1.1	2013-2015	Mbuthia et al 2018
	Sudan	Urban	Hospital	29	18	43	2013	Saeed et al 2015
	Tanzania	Urban	Hospital	20.3	15.6	64.1	2005-2006	Moyo et al 2011
	Tanzania	Rural	Community	5.9	14	12.9	2009	Seidman et al 2016
Northern	Egypt	NS	Hospital	3..2	3.2	30.7	2007	Ali et al 2014
	Egypt	Urban	Hospital	5.2	NC	NC	2009	Behiry et al 2011
	Libya	Urban	Hospital	4.6	0	5.4	2008	Rahouma et al 2011
	Niger	NS	Hospital	11	NC	NC	2010-2012	Langendorf et al 2015
	Tunisia	NS	Clinic	13.7	21	23.4	2008-2009	Nejma et al 2014
Southern	Malawi	Urban	Hospital	NC	10.6	NC	1997-2007	Trainor et al 2015
	Mozambique	Urban	Hospital	19	13	15	2012	Sumbana et al 2015
	South Africa	Rural	Clinics	17.9	27.9	26.8	2014-2015	Ledwaba et al 2018
	South Africa	Rural	Community	NC	NC	6.2		Tanih et al 2019
	South Africa	NS	Hospital	31.4	9.3	20	2016-2017	Chukwu et al 2020
	South Africa	Rural	Community	NC	NC	NC	NS	DeFrancesco et al 2017
	Zambia	Urban	Hospital	13.3	40	20	2016	Chiyangi et al 2017
Western	Burkina Faso	Urban	Hospital	25.8	3.2	48.4	2013-2015	Konate et al 2017b
	Burkina Faso	Urban/ Rural	Hospital	16	13	26	2009-2010	Bonkoungou et al 2012
	Burkina Faso	Rural	Hospital	19.3	NC	NC	2009-2010	Dembele et al 2015
	Burkina Faso	Urban	Hospital	9.7	NC	NC	2009-2010	Nitiema et al 2011
	Burkina Faso	Urban	Hospital	25.8	3.2	48,4	2013-2015	Konate et al 2017a
	Guinea	Urban	Hospital	8.8	11.1	11.1	2009-2010	Soli et al 2014
	Nigeria	Urban	Hospital	5.6	15.9	8.7	2008-2011	Odetoyin et al 2017
	Nigeria	Urban	Hospital	6	18.14	36.75	2017	Saka et al 2019
	Nigeria	Urban	Hospital	15	18	34.4	2008-2009	Onanuga et al 2014
	Nigeria	Urban	Hospital	4.5	4	2	2011	Ifeanyi et al 2015
	Nigeria	Urban	Hospital	NC	NC	NC	NS	Dabo et al 2019

NC= not characterised *E. coli* pathotypes, NS= not specified

Table 2.2: Antibiotic resistance profile of EPEC, ETEC, and EAEC isolates towards first line antibiotics.

First line Antibiotic	Resistance ranges of *E. coli* pathotypes (%)			Reference
	EAEC	EPEC	ETEC	
Ampicillin	100	66.7	100	Ali et al., 2014
	82.9	79	76	Seidman et al., 2016
	100	92.9	97.5	Odetoyin et al., 2016
Cotrimoxazole	95.7	-	100	Ali et al., 2014
	81	61.5	74.4	Saka et al., 2016
	87.5	79	76.6	Seidman et al., 2016
	-	90	-	Langerndorf et al., 2015
	100	78.6	90	Odetoyin et al., 2016
Tetracycline	78.3	66.7	100	Ali et al., 2014
	100	100	92.5	Odetoyin et al., 2016
Chloramphenicol	73.9	-	-	Ali et al., 2014
	26.7	9	10.1	Seidman et al., 2016
	57.2	42.9	32.5	Odetoyin et al., 2016
MDR	>91	-	-	Ali et al., 2014
	6.3	-	5.1	Saka et al., 2016
	61	42	16.8	Seidman et al., 2016

MDR= Multidrug resistance

2.4 Evolutionary trends of antibiotic resistance in diarrheagenic bacteria

The World Health Organisation (WHO) considers diarrhoea to be among the frequent ailments that pose a hazard to public health because antibiotic resistance is making it increasingly difficult to cure (WHO, 2017). The bacterial pathogens making headlines on the WHO priority list of pathogens include third-generation cephalosporin-resistant Enterobacteriaceae, fluoroquinolone-resistant species especially *Campylobacter* spp, *Salmonella* spp and *Shigella* spp (WHO, 2017). Pourmand et al (2017) posit that the identification of emerging antibiotic resistance patterns brings awareness and guides providers to tailor antibiotic therapy.

Campylobacter spp is a major cause of acute diarrhoea (WHO, 2017). In a study done by Samie et al (2007) in Venda in the Vhembe District on human diarrheal stools, about a third of the children under the age of 2 years were infected with *Campylobacter*. Normally, diarrhoea due to *Campylobacter* is self-limiting (Abbasi et al., 2019); however, serious cases require chemotherapeutic intervention with antibiotics such as macrolides and fluoroquinolones (Tian et al., 2016; Samie et al;

2007). It is highly recommended to take note of resistance when antibiotic prescription is prescribed in pediatric diarrhoea (Abbasi et al., 2020). The results of the studies done in Venda in the Vhembe District so far regarding *Campylobacter* have shown an increasing trend in resistance to macrolides and quinolones (Samie et al., 2007; Obi et al., 2004; Obi and Bessong, 2002).

In addition, observations made by Samie et al (2007) on carbapenems which are β-lactam antibiotics reserved for multi-drug-resistant bacteria, little resistance was observed against meropenem and imipenem. This prompts further investigations to monitor any evolutionary developments in carbapenem resistance in *Campylobacter* species. The question remains to be answered on whether carbapenems are often prescribed in diarrhoeal cases in South Africa, specifically in Venda. Reports regarding the resistance mechanisms of *Campylobacter* spp circulating in Venda are scarce. Mutations in the 23S rRNA gene were linked to macrolide resistance in a study on the molecular processes of antibiotic-resistant *Campylobacter* spp. conducted in Beijing, China by Zhou et al. (2016).

Acquisition of quinolone resistance by *Campylobacter jejuni* has been attracting increasing attention for decades (Gibreel et al., 1998). Quinolone drugs are known to interfere with DNA replication. The mechanism of bacterial resistance to quinolones has to do with altered genes that codes for special enzymes, DNA gyrase and topoisomerase, which are critical during DNA replication (Drlica and Zhao, 1997; Maxwell, 1992). Whether bacterial pathogens currently circulating in the Venda region, South Africa, are resistant to quinolones requires an update. However, reports from a MAL-ED cohort study on antibiotic use indicated that fluoroquinolones were rarely used in treating diarrhoea in most areas including Venda (Rogawski et al., 2016).

More information on *E. coli* than any other bacteria has been uncovered by numerous research on antibiotic resistance conducted worldwide. When it comes to children under five, one of the harmful bacteria that cause diarrhoea is *E. coli.* (Zhou et al., 2018; DeFrancesco et al., 2017). There are few studies that have been done in Venda in the Vhembe District regarding antibiotic resistance mechanisms. It is still unclear how *E. coli* responds to antibiotics such as ciprofloxacin, imipenem, and aminoglycosides due to a study by DeFrancesco et al. (2017) on children under five in rural Venda, South Africa, which did not find any evidence of resistance to these

drugs. However, the β-lactam resistance mechanism was studied since penicillins were seen as the most used antibiotics in the Venda region (Rogawski et al., 2016).

When treating extreme antibiotic-resistant bacteria, colistin, carbapenems, and tigecycline are regarded as the last resort antibiotics in South Africa (Sekyere, 2016). However, the increasing prevalence of ESBLs and carbapenemases among Gram-negative bacteria such as *E. coli* is worrisome and this leaves colistin as the only option in alleviating the burden due to such resistant bacteria (Tangden and Gisken, 2015). In a study conducted by Bi et al. (2017) in China, resistance to colistin was noted in β-lactamase-producing *E. coli* isolated from human fecal samples, while resistance to carbapenems and tigecycline was not observed. In this case, the *mcr*-1 gene was identified as the predominant colistin-resistant mechanism in *E. coli* (Bi et al., 2017). According to Zhang et al (2014), the production of ESBLs was inferred to be associated with carbapenem resistance which however contradicts Bi et al (2017) and Mandal et al (2017) observations.

A study by Ni et al (2016) on ESBLs *E. coli* in China also observed insignificant resistance of ESBLs *E. coli* to carbapenems and based on the results of the very same study, amikacin, cefoxitin, and imipenem could be seen as potential drugs of choice against ESBLs *E. coli* since very little if not insignificant resistance was reported.

2.5 Extended spectrum beta-lactamase (ESBL) resistance mechanisms

Gram-negative genera in most cases exhibit naturally occurring, chromosomally mediated β-lactamases (Bradford, 2001). Conversely, ESBLs can be readily transmitted across isolates since they are typically encoded on mobile genetic components like plasmids (Bradford, 2001). The emergence of ESBLs has been linked with the overuse of extended spectrum cephalosporins in hospital settings (Bradford, 2001).

The first detected β-lactamase is the TEM-1 which was discovered in the 1960s in *E. coli* and in a space of few years afterward, it spread among distinct species worldwide (Bradford, 2001; Datta and Kontomichalou, 1965). The functionality of ESBLs rests on

the composition of the active site and the majority of ESBLs have amino acid serine on the active site and belong to Ambler's molecular Class A enzymes which include mainly the TEM-1 and SHV-1 (Bradford, 2001; Ambler, 1980). Up to date, there are at least 100 ESBLs which are derivatives of TEM and SHV enzymes (Bradford, 2001; Bush et al., 1995). In addition, TEM and SHV ESBLs are most often found in *E. coli* (Bradford, 2001). However, TEM has also been reported in *Salmonella* spp (Tessier et al., 1998). As a result of evolution, a new family of plasmid-mediated ESBLs, that is, CTX-M emerged and is mainly found in *Salmonella* enterica serovar *Typhimurium* and *E. coli*. A review by Rocha et al (2015) on the spread of β-lactamase CTX-M (*bla*$_{CTX-M}$) in Brazil confirmed the association of Gram-negative bacteria such as *Klebsiella pneumoniae* and *E. coli* with the presence of *bla*$_{CTX-M}$.

CTX-M is a broad-spectrum ESBL whilst TEM and SHV are narrow-spectrum ESBLs (DeFrancesco et al., 2017). According to Tzouvelekis et al. (2000), the most identified β-lactamases, TEM and SHV, are not closely related to CTX-M. In terms of phylogenetics, molecular Class A ESBLs such as TEM and SHV families are closely related to each other and are in no way any closer to the molecular Class D OXA-type enzymes (Bradford, 2001). The OXA-type enzymes are characterised by their ability to hydrolyse oxacillin and cloxacillin (Bush et al., 1995). Of the ESBLs, the *bla*$_{CTX-M}$ is the most widely spread and has greater impact relative to that of TEM and SHV ESBLs (Rossolini et al., 2008).

2.5.1 The CTX-M family

There are about six sub-lineages that houses all the CTX-M variants, and each group is named after the first member (Figure 2.1). CTX-M variants were first described in clinical practice in the late 1980s and CTX-M-1 pioneered the history of CTX-M types (Rossolini et al., 2008). These CTX-M variants differ from each other by at least one amino acid residue.

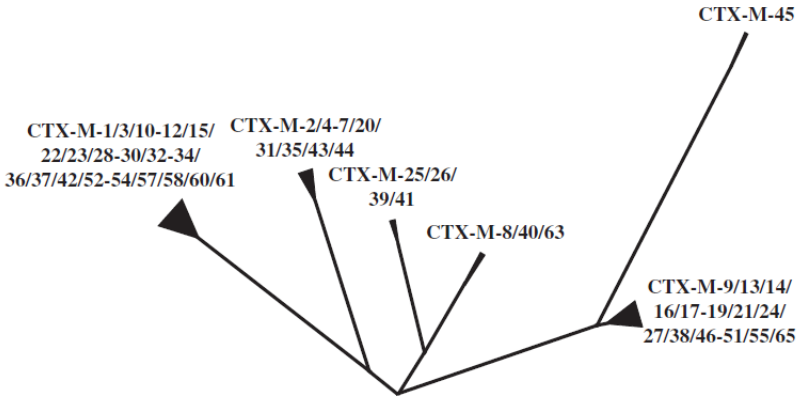


Figure 2.1: Phylogenetic tree showing the six sub-lineages of CTX-M type beta-lactamases (Rossolini et al., 2008).

2.5.1.1 Molecular details of blaCTX-M type genes

The spread of CTX-M genes among different species of bacteria has been associated with plasmid mediation. Various plasmids play a role in the transfer of genes from one bacterium to the next. According to Partridge et al. (2011), *bla*$_{\text{CTX-M-15}}$ is linked to FII plasmids. Despite their narrow host range, FII plasmids are so-called *'epidemic resistance plasmids'* because of their capacity to develop resistance and mediate the transfer between bacteria. The FII plasmids are mostly found in Enterobacteriaceae (Mathers et al., 2015).

On the other hand, insertion sequences (IS) dictate the state of genetic plasticity in prokaryotes, and this promotes the dissemination of genes (Poirel et al., 2003). Accordingly, the upstream region of the CTX-M genes contains the insertion sequence *ISEcp1*, which may be a major factor in the translocation and dissemination of the CTX-M genes (Awosile et al., 2021; Chanawong et al., 2002).

The primary phylogenetic groupings A, B1, B2, and D contain most of the CTX-M beta-lactamases. The epidemic-associated beta-lactamases in *E. coli,* that is, *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$ are members of the B2 phylogenetic group (Araque and Labrador, 2018). The majority of commensal pathogens are linked to phylogroups A and B1 (Habeeb et al., 2014).

2.5.1.2 Epidemiology of the CTX-M gene

Global reports assert that *E. coli* harbouring CTX-M genes is the most common among ESBL producers and this has become a cause of concern in public health (Karambwe et al., 2024; Peirano and Pitout, 2019). According to Rossolini et al. (2008), bacteria that produce CTX-M have been linked to nosocomial infections as well as community-acquired infections. There are several CTX-M variants that are categorized into sub-families, including CTX-M groups 1, 2, 8, 9, 25, and 45 (Rossolini et al., 2008). The most widespread variants globally are the Group1 CTX-M and they are more common in Africa (Rossolini et al., 2008). The most common CTX-M Group 1 variant at the moment, *bla*$_{\text{CTX-M-15}}$, is found throughout Europe, North and South America, and Asia, and is a source of concern in clinical practice (Osawa et al., 2015). Table 2.3 shows that there is a significant incidence of CTX-M-producing *E. coli* in Asian nations, which is linked to instances of diarrhoea in young children. Even though studies (Dembele et al., 2020; Khairy et al., 2020; De Francesco et al., 2017; Tellevik et al., 2016; Woerther et al., 2011) have looked into CTX-M-producing *E. coli* in cases of diarrhea in Africa (Table 2.3), little is known about beta-lactamase resistance (*bla*$_{\text{CTX-M}}$) in *E. coli* linked to diarrhoea in children.

In South-East Iran, a study done by Alizade et al (2015) on diarrheagenic *E. coli* isolated from diarrhoeal samples linked the CTX-M-15 gene with *E. coli* resistance to beta-lactam antibiotics. Mandal et al (2017) study on molecular epidemiology of ESBL-producing *E. coli* pathotypes isolated from hospitalised children with diarrhoea in India, reported CTX-M as the most common resistance gene. Production of plasmid encoded-ESBL has been considered as the predominant mechanism of resistance of diarrheagenic *E. coli* (DEC) to beta-lactams (Mandal et al., 2017). Among ESBL (CTX-M) producers, particular DEC pathotypes have been linked, including enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) (Kim et al., 2014). There is little information available about particular *E. coli* pathotypes linked to CTX-M genes (Kim et al., 2014).

Table 2.3: Incidence rates of CTX-M-producing *E. coli* isolated from diarrhoea cases, surveillance reports from studies.

Country (District/Region)	Study Group	Scope of study & samples	CTX-M Detected in isolates (%)	Reference
South Africa (Venda)	Children < 5 years	Diarrhoea stools	CTX M-14 (4.9%)	DeFrancesco et al., 2017
Indonesia (Surabaya)	0-3 years	Diarrhoea stools	CTX M-15 (84%)	Wasito et al., 2017
Tanzania (Dar es Saalam)	0-2 years		CTX-M (94%)	Tellevik et al., 2016
Niger	0.5-5 years	Stools -Malnourished children	CTX-M (44%)	Woerther et al., 2011
Iran (Zanjan)	0-5years	Diarrhoea & non-diarrhoea stools	CTX-M (63.1%)	Khoshvaght et al.,2014
India (Bahir)	0-5 years	Diarrhoea stools	CTX-M (86.1%)	Mandal et al., 2017
Qatar	0-10	Diarrhoea	CTX-M (88.23%)	Eltai et al 2020
Egypt	0-5 years	Diarrhoea stools	CTX-M (22.7%)	Khairy et al. 2020
Iran	0-10	Diarrhoea stools	CTX-M (94.4%)	Abbasi et al 2020
Burkina Faso	Young children	Diarrhoea	CTX-M (7.14%)	Dembele et al 2020

Nevertheless, Memarian et al. (2015) emphasized the growing significance of enteropathogenic *E. coli* (EPEC) that produces CTX-M-15 in pediatric diarrhoea. Africa has seen limited research on the diarrheagenic *E. coli* strain that produce CTX-M. (Tellevik et al., 2016, Woerther et al., 2011; DeFrancesco et al., 2017) (Table 2.3). Studies on intestinal *E. coli* that produce CTX-M are uncommon in South Africa; however, Peirano et al. (2011) reported on uropathogenic *E. coli* that produceCTX-M. It is known that environmental bacteria, such as those in the genus *Kluyvera*, may be the source of the CTX-M genes that facilitate the horizontal transfer of antibiotic resistance genes (Chong et al., 2018; Rossolini et al., 2008). Self-conjugative plasmids which carry additional determinants for resistance are implicated in the transfer of antibiotic resistance genes either between bacterial members of the same species or varied species (Rossolini et al., 2008).

There is a growing concern over the spread of *E. coli* that produces CTX-M-15. Sequence type 131 species of *E. coli* that produce CTX-M-15 is attracting attention as a major problem (Osawa et al., 2015).

2.6 Mechanism of quinolone resistance in *E. coli*

Fluoroquinolone-resistant bacterial species are among the WHO pathogen list attracting attention and the need for surveillance (WHO, 2017). The enzymes topoisomerase IV and DNA gyrase, which are essential for DNA replication, are major targets of fluoroquinolones. The primary cause of fluoroquinolone resistance in bacterial species is changes in the genes encoding topoisomerase IV (*parC*) and DNA gyrase (*gryA* and *gryB*). In *E. coli*, the *gryA* is the most common target of fluoroquinolones (Jaktaji and Mohiti, 2010). Generally, *gyr*A mutations are found within the quinolone resistance-determining region (QRDR) spanned by codons 67 to 106 (Johnning et al., 2015). Sequence analysis by comparison with a reference sequence such as *E. coli* K-12 is a key approach to determining any nucleotide substitutions in *gyr*A (Johnning et al., 2015; Jaktaji and Mohiti, 2010).

Virulence factors and antibiotic resistance are correlated, and reports indicate that pathogenic strains are more liable to antibiotic selection pressures and genetic mechanisms leading to the development of antibiotic resistance (Zhang et al., 2015). Of the *E. coli* pathotypes, the EAEC strain is known to possess a solid resistance to fluoroquinolones (Kim et al., 2012). In addition, a combination of mutations in the target enzyme and increased expression of efflux pumps is common in EAEC. Studies on fluoroquinolone resistance mechanisms in diarrheagenic *E. coli* are scarce in Africa. For example, phenotypic resistance to fluoroquinolone (ciprofloxacin) was found in a study conducted in the Eastern Cape Province of South Africa on *E. coli* isolates from children who had acute diarrhoea; however, the resistance mechanism for fluoroquinolone resistance was not investigated (Omolajaiye et al., 2020). In addition, there is a dearth of literature on fluoroquinolone resistance (*gyr*A) producing *E. coli* isolated from diarrhoea cases in young children. Similarly, as observed with CTX-M, studies on fluoroquinolone-resistant *E. coli* isolated from diarrhoea cases are prominent in Iran and India (Table 2.4). On the other hand, there is evidence of fluoroquinolone-resistant *E. coli* in healthy children. According to a study by Zhao et al. (2021), 98.2% of the isolates had the *gyr*A resistance mechanism.

Table 2.4: Incidence rates of fluoroquinolone-resistant *E. coli* isolated from diarrhoea cases, surveillance reports from studies.

Country	Study Group	Scope of study & samples	*gyrA* Detected in isolates (%)	Reference
Iran (central Iran)	0-10 years	Diarrhoea	*gyrA 6* (68.7)	Abbasi et al., 2019
India	0-5 years	Diarrhoea	*gyrA* 20(24.4%)	Moharana et al., 2019

2.7 Colistin resistance mechanism

Colistin among other polymyxin is considered the last resort antibiotic for the treatment of aggressive infections with carbapenemase-producing *Enterobacteriaceae.* However, the emergence of colistin resistance has raised concerns recently in many parts of the world. Numerous research has reported on the emergence of the phosphoethanolamine transferase enzyme-coding gene, *m-cr1* (Newton-Foot et al., 2017). The transferase enzyme confers resistance to colistin by mediating the translocation of phosphoethanolamine to Lipid A of the lipopolysaccharide membrane. The mechanism of colistin is governed by its polycationic nature which permits interaction with the bacterial outer membrane resulting in cell lysis.

2.8 *Campylobacter* mutations

Although *Campylobacter* infections are self-limiting, one in five patients may experience chronic or severe illness requiring antibiotic therapy (Kovac et al., 2015). Fluoroquinolone-resistant *Campylobacter* spp is increasing and that is a public health concern that requires several studies to unravel the epidemiology of such *Campylobacter spp.* Assumptions are that this escalation in fluoroquinolone-resistant *Campylobacter* spp is associated with the spreading of certain resistant genotypes. The Thr86Ile point mutation in the *gyrA* gene of DNA gyrase confers resistance in *Campylobacter spp* (Wieczoreck and Osek, 2013). DNA gyrase is an enzyme that plays a role in DNA replication and is primarily targeted by fluoroquinolone antibiotics such as ciprofloxacin. The Thr86Ile point mutation arises from changes in the nucleotide sequence of the *gyrA* gene at position 257 (codon 86). The change is a nucleotide substitution from ACA to ATA which results in threonine (Thr) to isoleucine (Ile) substitution in the *gyrA* protein. Several studies have confirmed and validated the

importance of the Thr86Ile point mutation in *Campylobacter* resistance against fluoroquinolones (Aksomaitiene et al., 2018).

2.9 Phenotypic and genotypic correlates of antimicrobial-resistant isolates

Assessment of antimicrobial resistance follows two dimensions, that is, phenotypic which is based on antibiotic susceptibility tests such as Kirby Bauer disc diffusion test, E-test, and broth microdilution while genotypic is based on molecular approaches such as PCR and sequencing. Based on the assumptions that phenotypic profiles derive from the status of the genetic makeup of microorganisms, is interesting to always assess the correlation of observations made on either phenotypic tests or genotypic tests. A study done in Peru on *Campylobacter* isolates from diarrhoeal patients showed a 100% correlation between phenotypic observations (ciprofloxacin resistance) and genotypic results in which it was observed that all ciprofloxacin-resistant isolates possessed the *gyr*A-point mutation Thr86Ile while ciprofloxacin sensitive isolates did not possess such point mutation (Espinoza et al., 2020).

In Northwest Province, South Africa, empirical results from a study by Chukwu et al. (2019) showed that all isolates of *Campylobacter* spp. resistant to ciprofloxacin expressed the *gyr*A gene. Another study done in Lithuania on *Campylobacter jejuni* reported a match between phenotypic and genotypic observations (Aksomaitiene et al., 2018). Although the hypothesis that phenotypic observations are highly likely to match with genotypic determinants of resistance, as confirmed in several studies, it is worthwhile to caution that it is not always the case in minor scenarios. For instance, Jesse et al (2005) reported an anomaly from the expected phenotypic and genotypic correlates among *Campylobacter* isolates. In the latter study, no *gyr*A mutations were observed in eight isolates that had shown resistance to ciprofloxacin.

Similarly, studies on antibiotic resistance in Enterobacteriaceae spp such as *E. coli* also reported on the association between phenotypic observations and molecular resistance mechanisms. This study focuses on the CTX-M resistance mechanism, hence the selection of studies included in this review. In a study done by Paul et al (2020) in India using sewage samples, cefotaxime-resistant *E. coli* was found to

possess *bla*$_{CTX-M}$ and *bla*$_{CMY-42}$ resistance genes. Both CTX-M and CMY are variants of extended-spectrum beta-lactamase (ESBL) which have broad spectrum activities against beta-lactam drugs and third-generation cephalosporins such as cefotaxime. Empirical observations in the same study by Paul et al (2020) suggest that, of the *bla*$_{CTX-M}$, the variant *bla*$_{CTX-M-15}$ is the most common gene conferring resistance to cefotaxime resistant *E. coli*. Antibiotic-resistant isolates are not only being reported in symptomatic cases but in asymptomatic cases as well.

In a study on healthy children from a rural area in Venezuela, phenotypic assays were used to screen for potential ESBL producers, and the study revealed that all the ESBL-producing *E. coli* harboured *bla*$_{CTX-M-15}$ resistance mechanism (Araque and Labrador, 2018). In a longitudinal study done in Korea on characterisation of *E. coli* isolates from diarrheic patients, all phenotypic ESBL-producers were confirmed to possess resistance genes in member groups such as TEM-1, CTX-M-1, and CTX-M-9 with CTX-M-14 being the most common variant of the CTX-M-1 group (Kim et al., 2019)

2.10 Summary of the Literature Review

The incidence of diarrhoea is high in countries with low and medium incomes, and it is thought to be a primary reason why children need antibiotic treatment (Brennhofer et al., 2022). In the Vhembe District, South Africa, *Campylobacter* and *E. coli* are frequent causes of endemic diarrhoea (Potgieter et al., 2023; Samie et al., 2022). In addition, *E. coli* and *Campylobacter* have acquired antibiotic resistance mechanisms over the years (Behailu et al., 2022; Ramatla et al., 2022; Rogawski et al., 2022). The global spread of CTX-M-type ESBLs especially *bla*$_{CTX-M-15}$ is a threat to public health due to limited therapeutic options available (Awosile et al., 2021; Mandal et al., 2017; Karanika et al., 2016; Storberg, 2014). Surveillance studies on *bla*$_{CTX-M-15}$-positive *E. coli* linked to pediatric diarrhoea in Africa and the Vhembe District are extremely rare (Karambwe et al., 2024). Furthermore, the World Health Organization (WHO) has stated that surveillance on fluoroquinolone resistance in Enterobacteriaceae, including *E. coli* and fluoroquinolone-resistant *Campylobacter*, is warranted (WHO, 2017). However, there is limited literature on fluoroquinolone resistance (*gyr*A) producing *E. coli* isolated from diarrhoea cases in young children. Although much is known about *Campylobacter* in the Vhembe District (Samie et al 2022; Samie et al., 2009; Samie

et al., 2007), there is no latest information on antibiotic resistance profiles of *Campylobacter*. Thus, the purpose of this study was to investigate the molecular resistance profiles of the bacteria in the Vhembe District that cause diarrhoea in young children. To the best of the author's knowledge, this is the first study evaluating the presence of *gyr*A and *bla*$_{\text{CTX-M-15}}$ in pediatric diarrhoea.

Chapter 3: Materials and Methods

The chapter describes the approach taken from sample collection to laboratory analysis. In summary, the methods cover the detection of target bacteria (*E. coli* pathotypes and *Campylobacter* spp) from diarrhoeal and non-diarrheal stool samples, the susceptibility of *E. coli* isolates to antimicrobial agents, detection of resistance genes, and phylogenetic analysis.

Chapter 3

MATERIALS AND METHODS

3.1 Ethical clearance/ consent

The approval to conduct this study was obtained from the Research Ethics Committee of the University of Venda (*SMNS/19/MBY/06/0207*), the Department of Health, Limpopo Province (*LP-2020-03-004*), and the Department of Health, Vhembe District (*S5/6*). Permission to conduct research was sought from the study sites, Donald Frazer Hospital, Tshilidzini, Siloam, Makhado, and Elim hospitals as well as from primary health care clinics serving these hospitals within the Limpopo province of South Africa. The study was explained to parents and/or guardians of the children and consent was obtained from parents and/or guardians on behalf of their children (subjects).

3.2 Study site

The current investigation was conducted in South Africa's Vhembe District, in the province of Limpopo. The Vhembe district is largely rural with limited infrastructure for water, sanitation, and electricity which negatively impacts community health in the district (Murei et al., 2022). The district has four local municipalities, namely, Makhado, Musina, Mutale, and Thulamela (Figure 3.1). Vhembe District has nine hospitals offering various health care services namely Donald Frazer, Elim, Evuxaken, Hayani, Louis Trichadt (Makhado), Malamulele, Messina, Siloam and Tshilidzini (Potgieter et al., 2021). In addition, the Vhembe District is also served by several Primary Health Care Centres and/or clinics providing healthcare services in remote areas (Potgieter et al., 2021). According to Statistics South Africa (2016), 33,2% (1.9 million) of the people living in the province of Limpopo are under 15 years old.

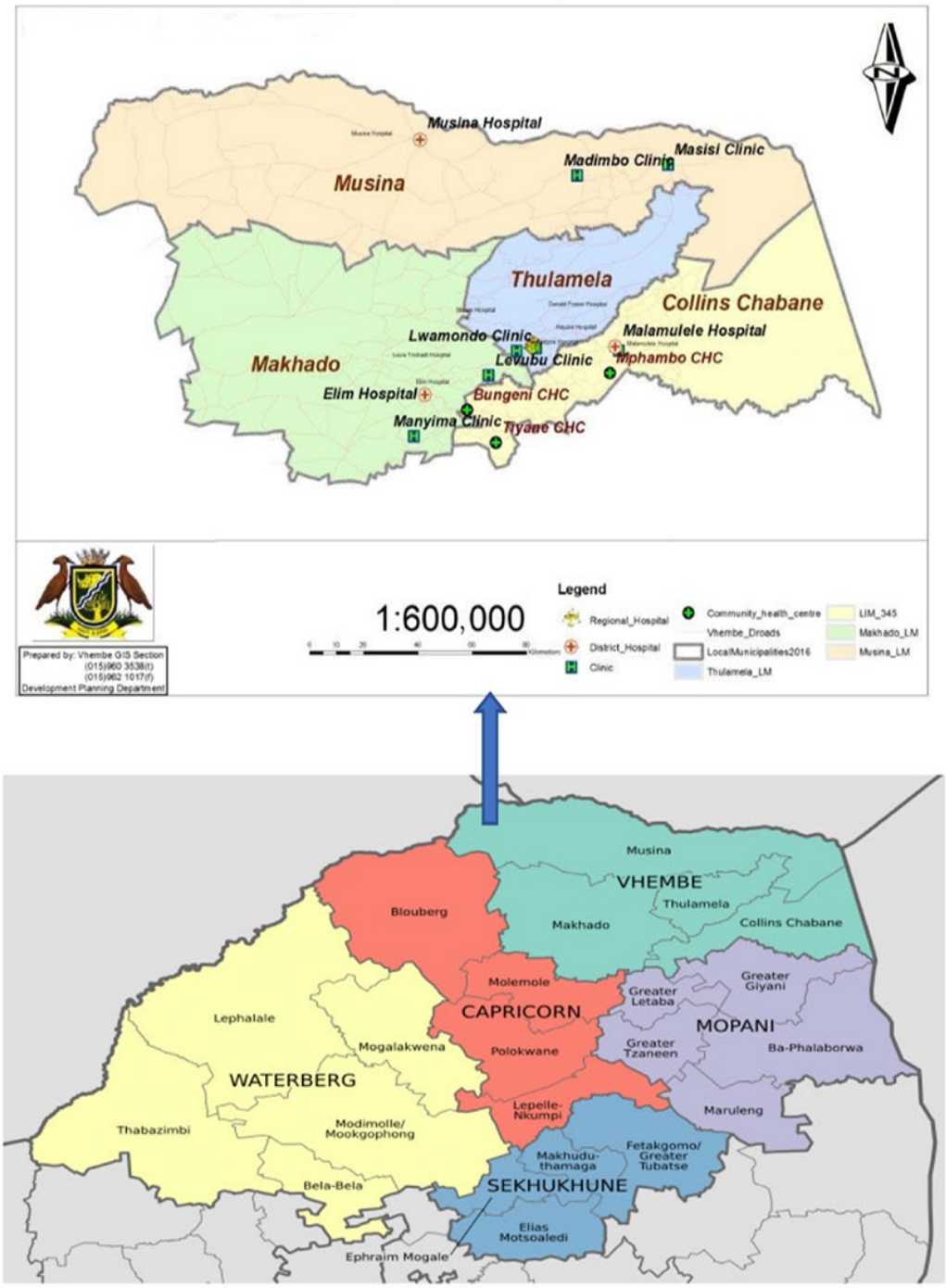


Figure 3.1: Map of the Vhembe District and some health care facilities (Olaniyi et al., 2019).

3.3 Sample collection

In 2021, a cross-sectional surveillance was carried out in April and August. Children under the age of five who reported having acute diarrhoea at five hospitals (Donald Frazer, Siloam, Elim, Makhado, and Tshilidzini) and one clinic (Mpambo) in the Vhembe District had their stool samples taken for both symptomatic and non-symptomatic diarrhoea. According to Kotloff (2017), diarrhoea was defined as the

frequent passing of loosely formed or watery feces three or more times per day. Inclusion criteria focused on children with diarrhoea that have not taken antibiotics in the past 1 month. In addition, the inclusion criteria also considered patients hospitalised within 24 hrs of admission. The zero-age group was incorporated as a comparison group on assumption that no antibiotics were prescribed to this age group. Non-diarrhoeal stool samples were collected to serve as a reference group for the purposes of comparison control samples. Demographic information regarding age, socio-economic background of participants, and symptoms associated with diarrhoea were collected using a standard capture form. Samples were excluded based on the following reasons:

- If admission time at the time of sample collection was more than 24 hrs
- If culture results did not yield any *E. coli*-positive data
- If no growth of bacteria was observed on the agar plates

All collected samples were transported on ice to the lab at the University of Venda for analysis. Some of the samples were stored at -20°C prior to examination.

3.4 Screening for *E. coli* pathotypes and *Campylobacter* spp

3.4.1 Cultivation of *E. coli*

A swab was used to mix the stool sample and a small amount of the stool sample was transferred into a sterile tube with 500 µl Phosphate Buffered Saline (PBS) (pH 6.8-7.4, Davies Diagnostics Pty, Limited). The isolation and screening for *E. coli* from stool samples were done using Eosin Methylene Blue (EMB) agar as published by DeFrancesco et al (2017). *E. coli* ATCC 25922, *E. coli* NCTC 13846, *E. coli* NCTC 13353, and *Klebsiella pneumoniae* ATCC 700603 (supplied by Anatech Pty, Limited) were used as control strains (Table 3.1). A sterile prepared EMB agar (pH 7.2, Condalab, SA) plate was inoculated with the emulsified PBS diluted stool sample using the streaking technique. For enrichment, 20 µl of the PBS stool suspension was inoculated into Nutrient Broth (pH 7.5+/0.2; Sigma-Aldrich). The EMB agar plates and the nutrient broth tubes were incubated at 37°C for 18 to 24 hours. When no growth was observed on direct culturing on EMB agar plates, the enriched samples (20 µl) were then cultured on EMB agar.

Table 3.1: Observed outcomes of control strains.

Control strain	Culture results on EMB Agar
E. coli ATCC 25922	Small green metallic shining colonies + purple colonies with dark centres
E. coli NCTC 13846	Green metallic shining colonies + big purple colonies with dark centres
E. coli NCTC 13353	Small green metallic shining colonies + purple colonies with dark centres
Klebsiella pneumoniae ATCC 700603	Confluent growth watery-like colonies

Isolates were considered presumptive positive *E. coli* if they matched the descriptions in Table 3.1 and shown in Figure 3.2. Presumptive positive isolates were sub-cultured on Nutrient agar (pH 7.5, Condalab, SA) in duplicate for preservation and further testing. The *E. coli* isolates were chosen at random to undergo additional PCR characterization, and the amplicons were forwarded to Inqaba Biotec (Inqaba Biotechnical Industries (Pty) Ltd) for sequencing.

The isolation and screening for *Campylobacter* from stool samples were done using Campylobacter Agar Base (HIMEDIA M944) supplemented with *Campylobacter* Supplement III (Skirrow). The addition of the recommended blood supplement (5-7% v/v sterile lysed horse blood or 10% sterile defibrinated sheep blood) was not possible due to a lack of supply at the time this study was completed. *Campylobacter jejuni* ATCC 33560 (supplied by Anatech Pty, Limited) were used as control strains. The agar plates were placed into anaerobic containers and incubated at 42 °C for 48 hrs.

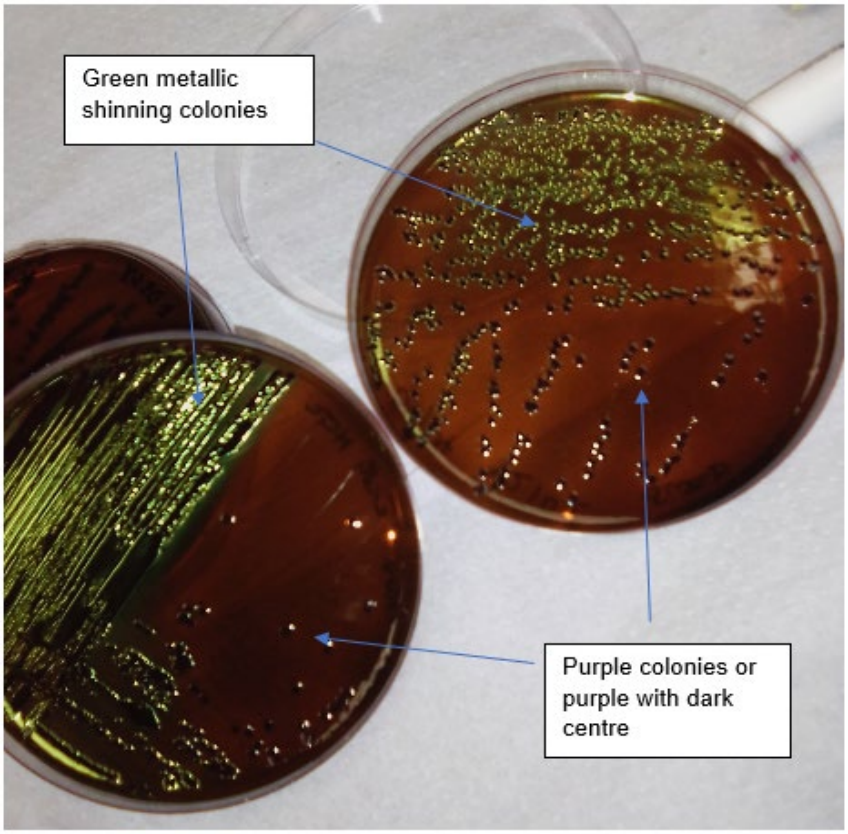


Figure 3.2: Presumptive isolates of *E. coli* on EMB agar (colonies with Green metallic shining, purple or purple with dark centre).

3.4.2 Molecular identification of *E. coli* isolates

DNA was extracted from presumptive *E. coli* isolates using the boiling method as described by Iwu et al (2017), using PBS as an extraction medium (Scaletsky et al., 2010). Depending on the sizes of colonies, 1-3 colonies were suspended in 500µl of PBS solution. The suspension was heated in a heating block at 100°C for 15 minutes. The tubes were allowed to cool and thereafter centrifuged for 10 minutes at 13 300 r/min. The supernatant was collected and stored at -20°C for further analysis. The quantity (ng/µl) and quality of DNA extracted was analysed using a nano-spectrophotometer (IMPLEM, Labotec). The A260/A280 nm reading determines the protein contamination of the nucleic acid sample. Pure dsDNA has an A260/280 ratio of 1.85-1.88 (Koetsier & Cantor, 2019). A lower ratio indicates high protein contamination.

Diarrheagenic *E. coli* (DEC) was further characterised by multiplex-PCR following the QIAGEN ® Multiplex PCR kit. A 10x primer mix was prepared following the QIAGEN ® Multiplex PCR kit. A total volume of 50µl was used for each PCR reaction. The final

concentration of each primer was 0.2µM (Table 3.2). The primer targets included the *mdh*, *Gapdh, eagg, lt, st, eaeA*, and *bfp* genes. A total of 5µl of template DNA was used for the PCR reactions. A BioRad T-100 Thermocycler was used, and the cycling condition was adopted from Omar and Barnard (2014). The gel electrophoresis was run at 80V for 1hr 15 minutes. The amplicons were visualised on 1.5% agarose gel (Figures 3 and 4). *E. coli* ATCC 25922 and *E. coli* NCTC 13846 were used as positive controls for *E. coli* (*mdh*). The negative control was DNAse-free water. The *Gapdh* gene serves as an internal control to keep track of PCR inhibition and false positives. Detection of the *Gapdh* gene in all samples may signify the absence of false positives as well as no PCR inhibition (Omar and Barnard, 2014). The *mdh* positive amplicons were sent to Inqaba Biotec for sequencing. The sequences were edited using Finch TV and analysed by the NCBI nucleotide BLAST algorithm.

Table 3.2: Multiplex PCR primers for *E. coli* and *E. coli* pathotypes

***E. coli* strain**	**Target gene**	**Primer (5'->3')**	**Size (bp)**	**Final Conc. (µM)**	**Reference**
E. coli	*Mdh*	F) GGT ATG GAT CGT TCC GAC CT R) GGC AGA ATG GTA ACA CCA GAG T	304	0.2	Tarr et al. (2002)
EHEC/ atyp EPEC	*eae*A	F) CTG AAC GGC GAT TAC GCG AA R) CCA GAC GAT ACG ATC CAG	917	0.2	Aranda et al. (2004)
Typ EPEC	Bfp	F) AAT GGT GCT TGC GCT TGC TGC R) TAT TAA CAC CGT AGC CTT TCG CTG AAG TAC CT	410	0.2	Aranda et al (2004)
EAEC	Eagg	(F) AGA CTC TGG CGA AAG ACT GTA TC (R) ATG GCT GTC TGT AAT AGA TGA GAA C	194	0.2	Pass et al. (2000)
ETEC	Lt	(F) GGC GAC AGA TTA TAC CGT GC (R) CGG TCT CTA TAT TCC CTG TT	360	0.2	Pass et al (2000)
ETEC	St	(F) TTT CCC CTC TTT TAG TCA GTC AAC TG (R) GGC AGG ATT ACA ACA AAG TTC ACA	160	0.2	Pass et al (2000)
Internal control	Gapdh	(F) GAG TCA ACG GAT TTG GTC GT (R) TTG ATT TTG GAG GGA TCT CG	238	0.2	Mbene et al. (2009)

F = Forward primer; R = reverse primer

3.4.3 Molecular identification of *Campylobacter* spp

Campylobacter was identified directly from the stool samples, bypassing the culturing challenges due to the fastidious behaviours of *Campylobacter*. The QIAmp Fast DNA Stool Mini kit was used to extract DNA from all stool samples by the manufacturer's instructions. The extracted DNA was sent to Inqaba Biotec for analysis of

Campylobacter using Allplex™ GI-Bacteria(I) Assay (Inqaba Biotechnical Industries (Pty) Ltd). *Campylobacter* spp. was further characterised using the NCBI BLAST algorithm.

3.4.4 Data analysis

Data was captured on Microsoft Office 365 Excel spreadsheet and analysed using Python version 3.9.7. Microsoft Excel was also used for drawing graphs. The association between variables was tested using the Chi-square test. The association was concluded significant if the p-value < 0.05 and the Cramer's V statistic criteria were used to evaluate the association's strength (Kim et al., 2019).

- >0.25 = Very strong
- >0.16 = strong
- >0.10 = Moderate
- >0.05 = Weak
- >0 = No or very weak

3.5 Detecting specific antibiotic resistance genes associated with *E. coli* pathotypes and *Campylobacter spp.*

The flow chart below (Figure 3.3) shows the criteria used for selecting isolates tested for genotypic antibiotic resistance.

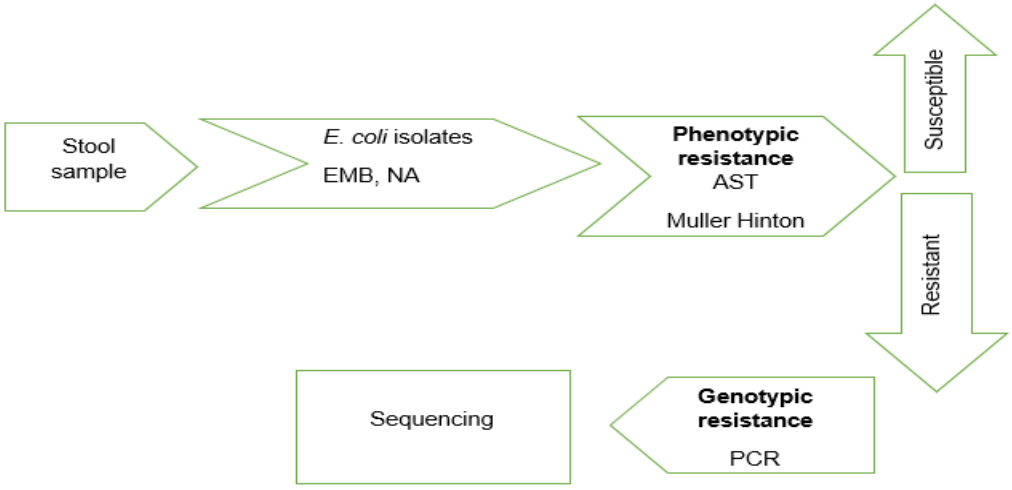


Figure 3.3: Flow chart showing the methodology followed (*E. coli*) to achieve objective no. 2 of the study as shown in Chapter 1

3.5.1 Antibiotic Susceptibility of *E. coli*

The Kirby Bauer disc diffusion method was used to determine the susceptibilities of 63 presumed *E. coli* isolates to a panel of eighteen antibiotics. The antibiotics included ciprofloxacin (CIP), ampicillin (AMP), amoxicillin (AMX), amoxicillin–clavulanate (AMC), chloramphenicol (C), cotrimoxazole (COT), nalidixic acid (NA), ceftriaxone (CTR), gentamicin (GEN), erythromycin (E), streptomycin (S), cefotaxime (CTX), ceftazidime (CAZ), levofloxacin (LE), imipenem (IM), meropenem (MRP), colistin (CL) and amikacin (AK) (supplied by HiMedia Laboratories Pvt Limited). To ensure quality control for the antibiotic susceptibility tests, *Escherichia coli* ATCC 25922 was employed. Ten distinct types of antibiotics were employed in this investigation (Table 3.3). The CLSI recommendations (CLSI, 2020) were followed in the interpretation of the zone of inhibition's diameter, which was measured in millimetres.

Table 3.3: Panel of antibiotics, class, and disc content

Antibiotic & Disc content (µg)	Antibiotic class
GE (10), AK (30), S (25)	Aminoglycoside
LE (50), CIP (5)	Fluoroquinolone
• CL (50)	• Polymixins
C (30)	Amphenicol
CTX (30), CAZ (30), CTR (30)	Cephalosporin
IPM (10), MRP (10)	Carbapenems
COT (25)	Sulfonamides
AMP (10), AMC (30), AMX (25)	Penicillin
NAL (30)	Quinolone
E (5)	Macrolides

3.5.2 Molecular characterisation of resistant phenotypes

The isolates that showed phenotypic resistance to cefotaxime were characterised for beta-lactamase gene (blaCTX-M-15) using Group 1 & 2 primers (Table 3.4). To detect other antibiotic resistance genes such as blaCTX-M-15) for cefotaxime resistance and gyrA for quinolone resistance, conventional PCR was done following the AccurisTM Taq DNA Polymerase Master Mix PR1001 PCR protocol (https://accuris-usa.com/Products/accuris-taq/). The primers used were previously published (Table 3.4). A total PCR volume of 50 µl was used while 5 µl was used for the template DNA. E. coli NCTC 13846 and E. coli ATCC 13353 were used as positive controls for the gyrA and blaCTX-M-15 target genes respectively. The negative control was DNAse-free water. A BioRad T-100 Thermocycler was used, and the cycling conditions were adopted from the Accuris PCR protocol, that is, an initial denaturation step at 95 ºC for 1 minute, followed by 40 cycles of denaturation at 95ºC for 15 s, annealing at 55ºC for 15 s, extension at 72ºC for 30s and a final elongation step at 72 ºC for 2 min. The gel electrophoresis was run at 80V for 1hr 15mins. The amplicons were visualised on 1.5% agarose gel stained with ethidium bromide and photographed under Omega Fluor™ UV light after. The *gyr*A and *bla*$_{\text{CTX-M-15}}$ positive amplicons were sent to Inqaba Biotec for sequencing. The sequences were edited using Finch TV and analysed by the NCBI nucleotide BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Table 3.4: CTX-M Group 1 &2 primers and *gyrA* primers

Antibiotic	Resistance gene	Primer	Size (bp)	Reference
Beta-lactams e.g penicillins, cephalosporins	*bla*$_{CTX-M}$	MultiCTXMGp1_for TTAGGAARTGTGCCGCTGYA MultiCTXMGp1-2_rev CGATATCGTTGGTGGTRCCAT	688	(Dallene et al.,2010; DeFrancesco et al., 2017)
Quinolones e.g nalidixic acid, ciprofloxacin	*gyrA* Quinolone resistance determining region (QRDR) in ***E. coli***	F: 5'-GCT GCC AGA TGT CCG AGA T-3' R: 5'-TCC GTG CCG TCA TAG TTA TCA-3'	360	Shenagari et al 2018

3.5.3 Statistical analysis

A Microsoft Excel spreadsheet was used to enter all of the data. Analysis was done using both Microsoft Excel and Python. Resistance to three or more antibiotics was referred to as multidrug resistance (MDR). The association between diarrhoeal and non-diarrhoeal as well as resistance to antibiotics was assessed using Chi-square.

3.6 Determining the relatedness of the antibiotic resistance genes

3.6.1 Sequencing of *E. coli* samples

The PCR products for resistant genes (*bla*$_{CTX-M-15}$ and *gyr*A) were verified by sequencing, which was done by Inqaba Biotech, South Africa. The sequences were compared with the GenBank database & β-lactamase classification system. MEGA 11 was used for alignment and constructing a phylogenetic tree to present the relationships based on the neighbour-joining method. Phylogenetic relations can be drawn from sequences to help trace links between sources such as clinical, community, and environmental. Molecular Evolutionary Genetics Analysis (MEGA) is a computer software for constructing phylogenetic trees in addition to statistical analysis of molecular evolution. Neighbour-joining and maximum likelihood are some of the methods used for constructing phylogenetic trees using the MEGA Software. The sequences are first aligned using muscle and the aligned sequences are then used to construct a phylogenetic tree.

3.6.2 Prediction of Antibiotic Resistance

ResFinder for predicting the resistance genes (https://cge.cbs.dtu.dk/services/

ResFinder-4.0/) was used. ResFinder analyzes the whole or partial DNA sequence of bacteria to identify acquired genes and/or chromosomal changes causing antimicrobial resistance. A ResFinder can generate a prediction for both the genotypic and phenotypic profile from the sequence of a microorganism under investigation.

3.6.3 Sequence analysis of *Campylobacter*-positive samples

The *Campylobacter*-positive samples were further analysed by Sanger sequencing at Inqaba Biotec (Pretoria, South Africa). Amplification was done using primers that target the conserved region of the 16S rRNA, the basis for the identification of the *Campylobacter* genus. Furthermore, the Cj-GyrA primers Cj-gyrA-759r (5'-TCGCTTTCTGAACCATCA-3') and Cj-gyrA-393 (5'-CTTTGCCTGACGCAAGAG-3') (Hakanen et al., 2002) were used to amplify the antibiotic resistance genes of *Campylobacter.* The sequences were edited using Finch TV software. The editing involved the replacement of ambiguous nucleotides based on the NCBI bioinformatics standards(https://www.bioinformatics.org/sms/iupac.html). The processed sequences were then analysed using the BLAST algorithm for nucleotides (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to align the present sequences with the reference sequences in the NCBI GeneBank. ResFinder was employed to predict the antibiotic resistance of *Campylobacter.*

Chapter 4: Results and Discussion

The chapter provides an overview of the results of the investigations conducted and the discussion of these results. The chapter compares the findings of this study against previous studies done in different geographical locations.

Chapter 4

RESULTS AND DISCUSSION

4.1 Prevalence of *E. coli* pathotypes and *Campylobacter* spp

4.1.1 Demographic data of the study population

Following the screening of 69 samples from six different healthcare institutions, 46 positive samples were taken into consideration for further analysis; the majority of these samples came from Elim Hospital (35%; 16/46), Tshilidzini Hospital (20%; 9/46), and Mpambo Clinic (20%; 9/46) (Table 4.1). About 74% of samples were collected from children between the ages 0-2 years while 26% were from children between 2-5 years (Table 4.2). The age range observed in this study was 1- 47 months and the mean age was 18 months. With regards to gender, about 65% (30/46) of the samples were collected from males while 35% (16/46) came from females (Table 4.2).

Table 4.1: Demography of samples per Healthcare Facility

Healthcare Facility	Age group (yrs)	Diarrhoea		Non-diarrhoea		Total
		Male	Female	Male	Female	
TLDH	0-1	2 (4%)	1 (2%)	-	-	3 (7%)
	1-2	5 (11%)	-	-	-	5 (11%)
	2-3	1 (2%)	-	-	-	1 (2%)
	3-4	-	-	-	-	0
	4-5	-	-	-	-	0
DFH	0-1	2 (4%)	-	1 (2%)	1 (2%)	4 (8%)
	1-2	-	-	-	-	0
	2-3	-	-	-	-	0
	3-4	-	-	-	1 (2%)	1 (2%)
	4-5	-	-	-	-	0
ELH	0-1	1 (2%)	1 (2%)	1 (2%)	-	3 (7%)
	1-2	1 (2%)	3 (7%)	3 (7%)	2 (4%)	9 (20%)
	2-3	-	-	1 (2%)	-	1 (2%)
	3-4	1 (2%)	-	2 (4%)	-	3 (7%)
	4-5	-	-	-	-	0
SLMH	0-1	-	1 (2%)	-	-	1 (2%)
	1-2	-	-	-	1 (2%)	1 (2%)
	2-3	-	-	-	1 (2%)	1 (2%)
	3-4	-	-	-	1 (2%)	1 (2%)
	4-5	-	-	-	-	0

MPC	0-1	2 (4%)	1 (2%)	-	-	3 (7%)
	1-2	2 (4%)	-	-	-	2 (4%)
	2-3	1 (2%)	2 (4%)	1 (2%)	-	4 (8%)
	3-4	-	-	-	-	0
	4-5	-	-	-	-	0
MAK	0-1	1 (2%)	-	2 (4%)	-	3 (7%)
	1-2	-	-	-	-	0
	2-3	-	-	-	-	0
	3-4	-	-	-	-	0
	4-5	-	-	-	-	0
TOTAL		**19 (41%)**	**9 (20%)**	**11 (24%)**	**7 (15%)**	**46 (100%)**

MAK = Makhado Hospital, MPC = Mpambo Clinic, SLMH = Siloam Hospital, ELH = Elim Hospital, DFH = Donald Fraser Hospital, TLDH = Tshilidzini Hospital

Table 4.2: Demographic profile of children positive for *E. coli* in the Vhembe District

Age group (years)	Male	Female	Total
0-1	12 (26%)	5 (11%)	17 (37%)
1-2	11 (24%)	6 (13%)	17 (37%)
2-3	4 (9%)	3 (7%)	7 (15%)
3-4	3 (7%)	2 (4%)	5 (11%)
4-5	0	0	0
TOTAL	**30 (65%)**	**16 (35%)**	**46 (100%)**

p-value = 0.5951, Cramer's phi = 0.0784

The ratio of females to males was approximately 1:2 (Table 4.2). Gender and age group did not significantly correlate (p-value = 0.5951). The participants considered in this study were children suffering from acute diarrhoea which is based on the duration of less than 14 days. The results showed that diarrhoea lasted for at most 5-6 days (only 2 children) while the shortest duration was 1 day. The diarrhoea case of 5- and 6-day duration was noted on a female and a male child under 1 year of age who were hospitalised due to acute diarrhoea.

Table 4.3: Distribution of samples as per gender

Sample	Males	Females	Total
Diarrhoea	19 (41%)	9 (20%)	28 (61%)
Non-diarrhoea	11 (24%)	7 (15%)	18 (39%)
Total	**30 (65%)**	**16 (35%)**	**46 (100%)**

Cramer's V correlation statistic = 0.0

Of the total samples, about 61% were diarrhoea cases while 39% were non-diarrhoea samples from healthy children. Gender and sample state (diarrhoea or not) did not significantly correlate; the Cramer V correlation statistic was 0.0 (Table 4.3). However, there was a noteworthy link (Cramer V correlation statistic =0.17; Table 4.4) between age group and sample status.

Table 4.4: Distribution of samples as per age group

Sample	0-2 years	2-5 years	Total
Diarrhoea	23 (50%)	5 (11%)	28 (61%)
Non-diarrhoea	11 (24%)	7 (15%)	18 (39%)
Total	**33 (71.7%)**	**13 (28.3%)**	**46 (100%)**

Cramer's V correlation statistic = 0.17

Children under the age of two years with diarrhoea made up about half of the samples. (Table 4.4). Most of the children experienced diarrhoea that lasted for 1 day followed by 2 days and 3 days. Only two children experienced diarrhoea that lasted for 4 days. Of the diarrhoea cases, about 57% of children experienced 3 episodes of diarrhoea within 24 hours. The maximum number of episodes experienced was 4.

Children who experienced more episodes were below the age of 12 months and were more likely to be hospitalised (Table 4.5). In addition, there was a very strong association between the number of episodes and the duration of diarrhoea (p=0.0000, Cramer's V = 0.87).

Table 4.5: Profile of children with the maximum number of episodes

Lab ID	Age (months)	No. of Episodes within 24hrs	Duration (days)	Admission
113	6	4	3	Hospitalised
169	2	4	2	Outpatient
158	27	4	1	Outpatient
166	3	4	1	Hospitalised

p=0.0000, Cramer's V = 0.87

In addition, the association between breastfeeding and the number of diarrhoea episodes was not significant (p-value = 0.4311, Cramer's V = 0.2021) (Table 4.6). The observation that breastfeeding was not strongly associated with episodes of diarrhoea agree with previous studies that found no association between breastfeeding and diarrhoea (Dagnew et al., 2019; Demissie et al., 2021). However, other studies have reported on the association between partial breastfeeding (Acharya et al., 2018), spoiled breastmilk (McMahon et al., 2013), and diarrhoea in children. Moreover, the association of breastfeeding and diarrhoea episodes has been reported in the a previous study on diarrhoea and associated factors among young children in Ethiopia which found that the most vulnerable children to contracting diarrhoea were those whose mothers used water only to wash their hands while the risk was kept at a minimum when water and soap were used for handwashing (Dagnew et al., 2019). Among the known risk factors for diarrhoea are the conditions of sanitation and hygiene at their individual homesteads, as well as the hygiene habits of breastfeeding mothers (Nguyen et al., 2021; George et al., 2014).

Table 4.6: Association of Breastfeeding and number of episodes

Number of Episodes	Breastfeeding	
	Yes	No
0	6 (13%)	12 (26%)
1	0	1 (2.2%)
2	2 (4.3%)	6 (13%)
3	9 (19.6%)	6 (13%)
4	3 (6.5%)	1(2.2%)
Total	**20 (43.5%)**	**26 (56.5%)**

p-value = 0.4311, Cramer's V = 0.2021

The presence of livestock at home was not associated with diarrhoea episodes, p-value = 0.1523, Cramer's V = 0.000 (Table 4.7).

Table 4.7: Association of the number of episodes and having livestock at home

Number of Episodes	Livestock	
	Yes	No
0	9 (19.6%)	9 (19.6%)
1	0	1 (2.2%)
2	7 (15.6%)	1 (2.2%)
3	10 (21.7%)	5(10.9%)
4	2 (4.3%)	2 (4.3%)
Total	**28 (60.9%)**	**18 (39.1%)**

p-value = 0.1523, Cramer's V = 0.000

The association of water source and the number of diarrhoea episodes was not significant, p-value = 0.1819, Cramer's V = 0.0000 (Table 4.8).

Table 4.8: Association of number of episodes and water source

Diarrhoea Episodes	Water source					
	Borehole	Tap	Tap/ Borehole	Tap/Borehole/ River	Tap/ Spring	Tap/Spring/ River
0	2 (4.3%)	6 (13%)	9 (19.6%)	1 (2.3%)	0	0
1	0	1 (2.3%)	0	0	0	0
2	0	8 (17.4%)	0	0	0	0
3	2 (4.3%)	3 (6.5%)	4 (8.7%)	4 (8.7%)	1 (2.3%)	1 (2.3%)
4	0	3 (6.5%)	1 (2.3%)	0	0	0
Total	**4** (8.7%)	**21 (45.6%)**	**14 (30.3%)**	**5 (10.9%)**	**1 (2.2%)**	**1 (2.2%)**

p-value = 0.1819, Cramer's V = 0.0000

4.1.2 Prevalence of *E. coli*

A total of 69 samples were screened for *E. coli* infection and consisted of diarrhoeal (42) and non-diarrhoeal (27) samples. *E. coli* was detected in 71 % (46/69) samples. Twenty-three (23) samples were excluded: 13 samples tested negative for *E. coli*; 7 samples were from patients hospitalised for more than 24 hrs while 3 samples did not produce any growth on EMB agar plates. Overall, 46 samples were considered for further characterisation of *E. coli* (Table 4.9). From the positive samples, 98 presumptive *E. coli* isolates were collected, and 63 representative isolates were selected for antibiotic susceptibility testing (AST) and molecular characterisation.

Table 4.9: Breakdown of samples examined in this study.

E. coli+	*E. coli*-	>24hrs hospitalisation	No growth on EMB agar	Total
46	13	7	3	69

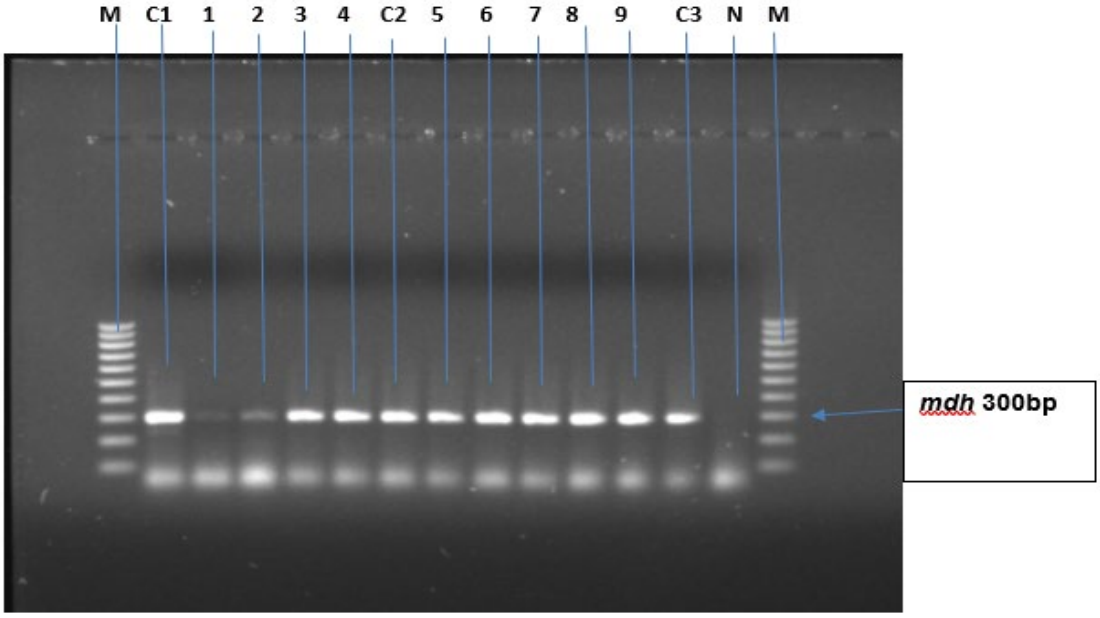
E. coli+: samples that tested positive for E. coli on EMB agar.

E. coli-: Samples that tested negative for E. coli (identified as either *Klebsiella* spp or *Enterobacter* spp)

>24hrs hospitalisation: Samples excluded based on more than 24hrs of hospitalisation.

No growth on EMB agar: Samples that did not yield any growth of bacteria on EMB agar.

For quality control (QC), the *mdh* gene was successfully amplified (Figure 4.1) and the results of the amplicons are shown in Figures 4.1, 4.2 and 4.3.



M= 100bp-1000bp DNA ladder,

C1= *E.coli* ATCC 25922,

C2 = *Klebsiella pneumonia* ATCC 700603,

C3 = *E.coli* NCTC 13846,

Lanes 1-9 = presumptive *E. coli* isolates,

N= negative control

Figure 4.1: Gel electrophoresis results for surveillance of *mdh* (~300bp) gene for characterization of presumptive *E. coli* isolates

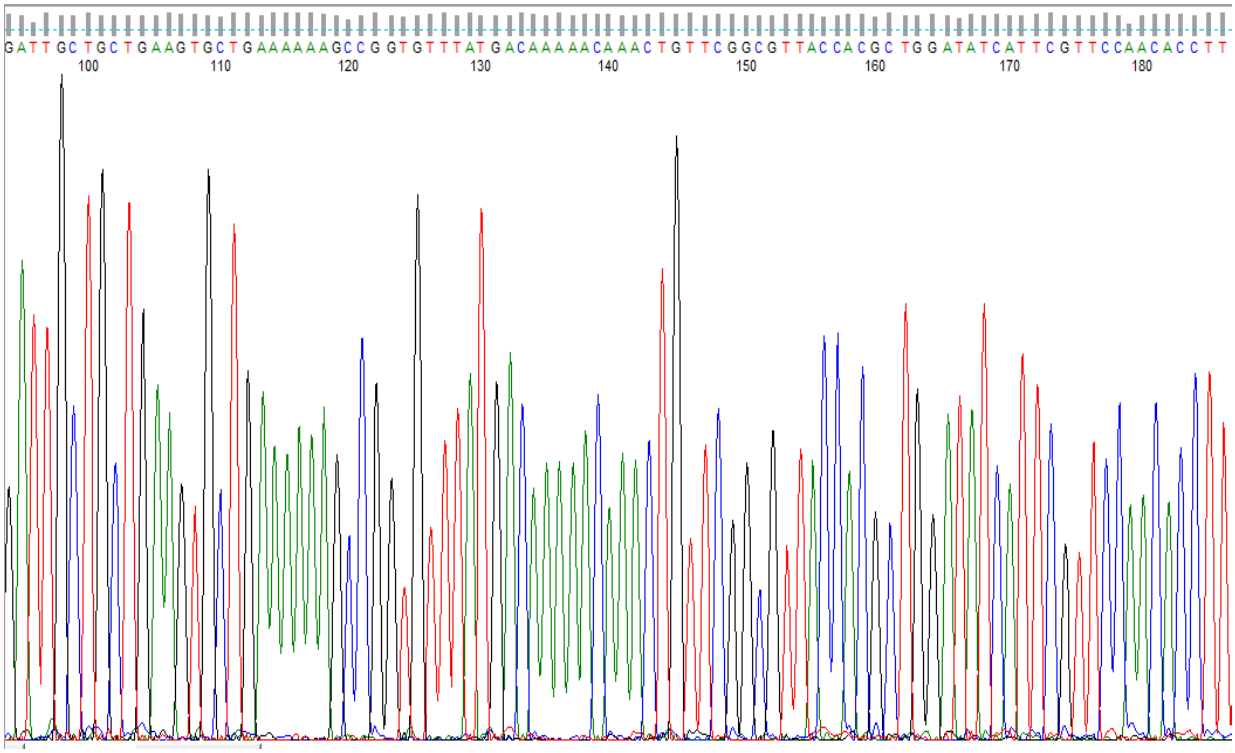


Figure 4.2: Chromatogram for positive *mdh* positive amplicons

Escherichia coli strain WCHEC025970 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	4794992	CP036177.1
Escherichia coli strain NCTC11105 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	4692802	LR134157.1
Escherichia coli strain NCTC9104 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	4692996	LR134152.1
Escherichia coli strain NCTC10444 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	5295029	LR134092.1
Escherichia coli strain NCTC12655 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	4757324	LR134083.1
Escherichia coli strain NCTC9038 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	4682556	LR134082.1
Escherichia coli strain NCTC9033 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	5249174	LR134081.1
Escherichia coli strain NCTC9112 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	5468700	LR134079.1
Escherichia coli strain NCTC9084 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	4601921	LR134075.1
Escherichia coli strain NCTC9066 genome assembly, chromosome: 1	Escherichia coli	426	426	99%	5e-115	100.00%	4644134	LR134000.1
Escherichia coli strain 214-4 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	5138709	CP025840.1
Escherichia coli strain 504838 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	5104962	CP025856.1
Escherichia coli strain 300709 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	4876488	CP025903.1
Escherichia coli strain 203740 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	4922099	CP025913.1
Escherichia coli strain WPB121 chromosome	Escherichia coli	426	426	99%	5e-115	100.00%	4637028	CP034426.1
Escherichia coli strain WPB102 chromosome	Escherichia coli	426	426	99%	5e-115	100.00%	4595459	CP034428.1
Escherichia coli strain 4/2-1 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	4735721	CP023834.1
Escherichia coli strain ER1709 chromosome, complete genome	Escherichia coli	426	426	99%	5e-115	100.00%	4582842	CP030240.1

Figure 4.3: BLAST results showing 100% percent identity for *E. coli mdh* sequence analysis.

Samples from children younger than two years old makeup more than twice as many as samples from older children. The immune system of children under 2 years of age is not yet fully adapted and hence children under 2 years of age are vulnerable to infection by various pathogens (Hugho et al., 2023).

Studies in the Vhembe District, South Africa previously reported on the vulnerability of children under 2 years of age to infection by *E. coli* pathotypes such as ETEC and EAEC (Potgieter et al., 2023; Ledwaba et al., 2018). In a study in Burkina Faso in children under 5 years with diarrhoea, at least 67% of DEC were recovered from children less than 1 year (Konaté et al., 2017). The results of these investigations are consistent with the current investigation, which found that a considerable number of children under the age of two had *E. coli* infections.

4.1.3 Occurrence of *Campylobacter* spp in children under five years

Sixteen out of 48 (16/48) stool samples tested positive for either *Campylobacter spp, Shigella spp./Enteroinvasive E. coli (EIEC), Clostridium difficile toxin B* and/or *Aeromonas spp.* (Table 4.10). Although it was not the main pathogen being examined in this study, *Clostridium difficile* was found in a few samples (6/48) in addition to *Campylobacter*, the principal pathogen of concern. Overall, in both diarrhoeal and non-diarrhoeal stool samples, *Campylobacter spp* were detected in 10.4% (5/48) samples. Of these 5 positive samples, only one sample was asymptomatic (non-diarrhoeal stool samples), and the rest were symptomatic (diarrhoeal stool samples). One of the symptomatic samples showed a co-infection by both *Campylobacter spp, Shigella spp./Enteroinvasive E. coli (EIEC).* The NCBI BLAST results showed that *Campylobacter* spp detected in this study aligned well with *Campylobacter jejuni* strain from chicken detected in Egypt (Accession number: MG773488.1).

Table 4.10: Enteric bacterial infection profile of diarrhoeal and non-diarrhoeal stool samples from children under 5 years in the Vhembe District

Pathogen	Symptomatic (n=10)	Asymptomatic (n=6)	Total (N=16)
Shigella spp/EI	3	2	5 (31%)
Campylobacter spp	4	1	5 (31%)
Yersinia enterocolitica	0	0	0
Vibrio spp	0	0	0
Clostridium difficile toxin B	3	3	6 (37.5%)
Aeromonas spp	1	0	1 (6%)
Salmonella spp	0	0	0
Total	**11**	**6**	**17**

EI: Enteroinvasive E. coli, Total = 16, extra one is due to co-infection hence 17

The prevalence of *Campylobacter* in diarrhoeal stools was 13.8% (Table 4.11). The current observations are lower than the 20.4% prevalence reported by Samie et al (2022) in the same study area, Vhembe District in South Africa. However, the findings from this study are in line with a systematic review on studies done in South Africa which reported a pooled prevalence of 16.4% of *Campylobacter* isolated from human samples (Ramatla et al., 2022). In addition, the current observations are within the prevalence range reported in a systematic review of case-control studies done in South Asia which showed that *Campylobacter* spp. were detected in diarrhoeal cases at a prevalence range of 3.2-17.4% (Murugesan et al., 2022). Studies elsewhere in Africa have reported lower prevalence relative to the current findings. For instance, a previous study in Africa reported a 9.7% prevalence of *Campylobacter* in children under five with diarrhoea in Tanzania (Deogratias et al., 2014) which is slightly lower than the present study. Furthermore, the current results within the range of a 9% pooled prevalence that was reported in a Hlashwayo et al. (2021) comprehensive review research on antibiotic resistance of *Campylobacter* spp. in Sub-Saharan Africa.

Table 4.11: Prevalence of *Campylobacter* in diarrhoeal and non-diarrhoeal stools

Category	Diarrheal N=29	Non-diarrheal N=19	Total N=48
Campy^{+}	4 (13.8%)	1 (5.3%)	5 (10.4%)
Campy^{-}	25 (86.2%)	18 (94.7%)	43 (89.6%)

Campy^{+} = *Campylobacter* positive, Campy^{-} = *Campylobacter* negative

In addition, the present study observed that male children were more likely to be infected by *Campylobacter* than their female counterparts (Table 4.12). These observations do not agree with the findings by Samie et al (2022) study on the epidemiology of *Campylobacter* in children under 2 years in South Africa which reported a higher frequency of *Campylobacter* in non-diarrheal stools as well as in female children. The reason could be the disproportionate number of samples used in the latter study. On the other hand, children under the age of 2 years (24 months) were more likely to suffer from acute diarrhoea due to *Campylobacter*. This observation agrees with a previous study that reported a high susceptibility to *Campylobacter* infection among children less than 2 years (Murugesan et al., 2022). However, the present findings on *Campylobacter* are limited and future research is needed to confirm these observations.

Table 4.12: Profile of the *Campylobacter*-positive samples in terms of age, gender, and admission status

Sample ID	Admissions Status	Diarrhoea status	Symptoms	Age (months)	Gender
169	Outpatient	Acute	Symptomatic	2	Male
H055	Outpatient	-	Asymptomatic	29	Female
134	Hospitalised	Acute	Symptomatic	21	Male
106	Outpatient	Acute	Symptomatic	12	Male
166	-		Symptomatic	-	-

Given that the sample came from a child aged 12 months, there are probably poor sanitation and hygiene practices by the caregivers of the child. An important observation in this study is that, of the four children that experienced the maximum number of diarrhoea episodes (Table 4.5), half (2/4) had been co-infected by both *E. coli* and *Campylobacter,* causing watery diarrhoea and were both children under the age of 2 years. The prevalence of *Campylobacter* in children under 2 years has been reported (Samie et al., 2022; Amour et al 2016). Sample ID 106 showed co-infection between *Campylobacter* and *Shigella*/Enteroinvasive *E. coli*. In a parallel analytical examination, the five samples that tested positive for *Campylobacter* also tested positive for *E. coli*. It seems like environmental factors such as water source, livestock, and sanitation possibly were involved. This study did not observe any co-infection between *Campylobacter* and *Clostridium difficile* toxin B. Contrary, a previous study

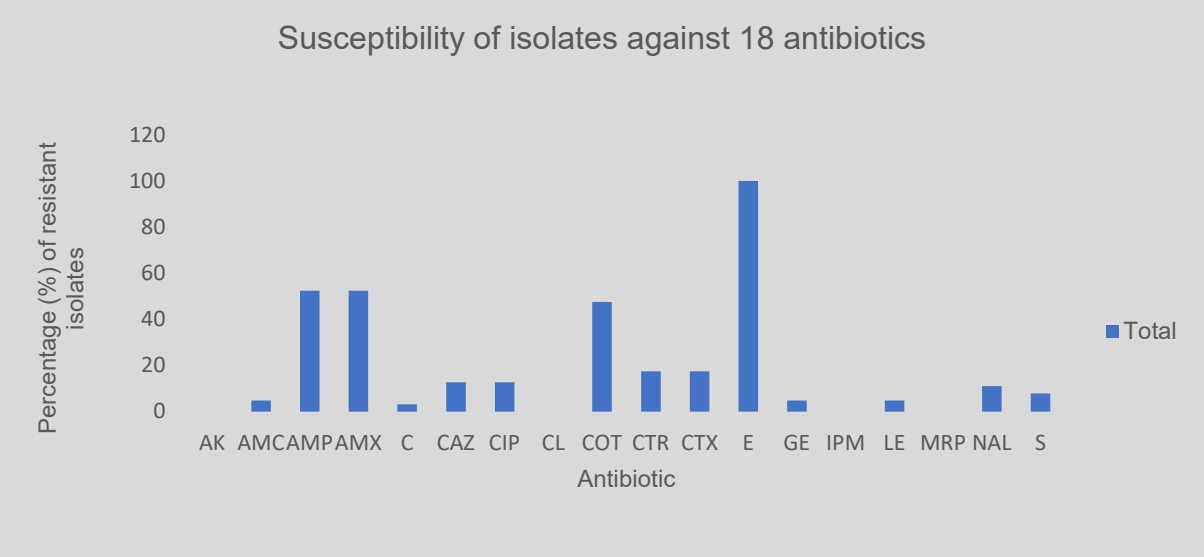
done in the Vhembe District on children with diarrhoea reported on co-infection of *Campylobacter* and *Clostridium difficile* toxin B (Potgieter et al., 2023).

Although *Clostridium difficile* was not a principal pathogen in this study, its noticeable detection informs future studies to have a comprehensive approach in investigating enteric bacteria associated with diarrhoea in children. Nevertheless, there is still limited literature on the role of *Clostridium difficile* in paediatric diarrhoea as children are often colonised without showing symptoms (Perumalsamy and Riley, 2021; Santiagoa et al., 2015).

4.2 Antibiotic resistance profiles associated with *E. coli* and *Campylobacter spp.*

4.2.1 Susceptibility of *E. coli* isolates and the level of resistance in *E. coli* isolates

Of the 18 antibiotics, only 4 antibiotics (AK, CL, MRP, and IMP) were effective against *E. coli* isolates. About 52% and 47.6% of *E. coli* isolates showed resistance to AMP and COT respectively. The isolates showed 100% resistance to erythromycin (E) (Figure 4.4). The most common resistance pattern was GEN-CTX-CAZ-CTR-AMP-AMX-E. The highest resistance pattern had a combination of 11 antibiotics, GEN-S-CTX-CIP-COT-CAZ-CTR-AMP-AMC-AMX-E (Tables 4.13 and 4.14).



GE=Gentamicin, AK=Amikacin, S=Streptomycin, LE=Levofloxacin, CIP=Ciprofloxacin, CL=Colistin, C=Chloramphenicol, CTX=Cefotaxime, CAZ=Ceftazidime, CTR=Ceftriaxone, IMP=Imipenem, MRP=Meropenem, COT=Cotrimoxazole, AMP=Ampicillin, AMC=Amoxicillin/Clavulanic acid, AMX=Amoxicillin, NAL=Nalidixic acid, Erythromycin

Figure 4.4: Susceptibility of *E. coli* isolates isolated from stools of children under 5 years with and without diarrhoea.

At least 58% of the isolates exhibited resistance to 3 or more antibiotics (MDR) (Table 4.12).

Table 4.13: Distribution of MDR profiles by source sample

Source	Resistance profile	
	≤ 2 antibiotics	**≥ 3 antibiotics (MDR)**
Diarrhoeal **(n = 42)**	17 (40.5%)	25 (59.5%)
Non- diarrhoeal **(n = 21)**	9 (42.9%)	12 (57.1%)
Total (n= 63)	**26 (41.3%)**	**37 (58.7%)**

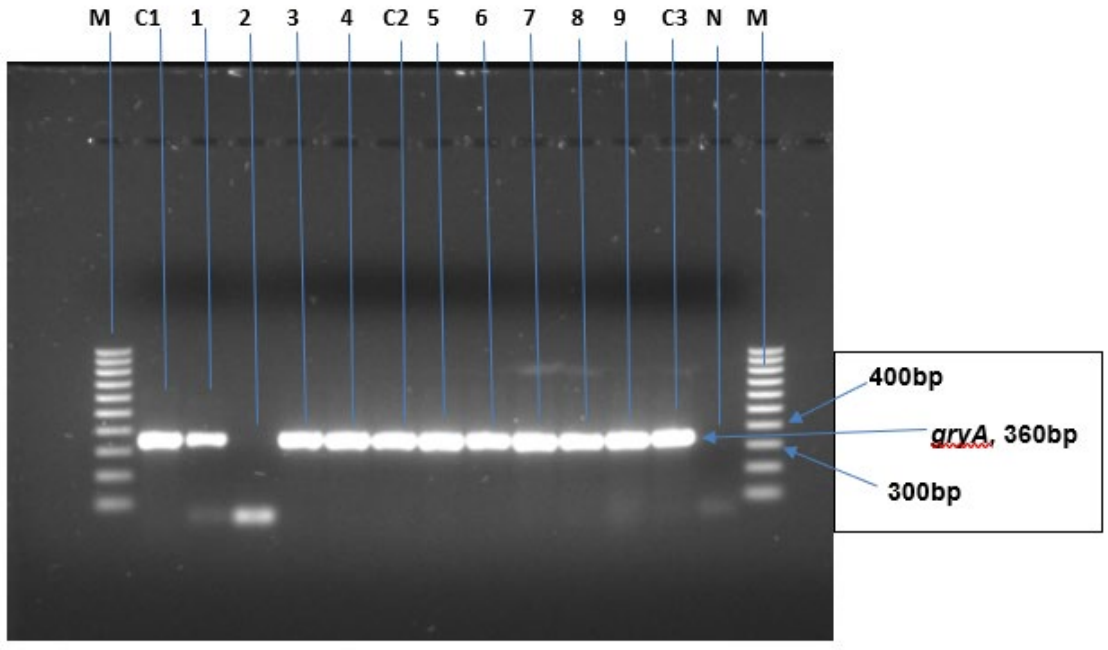
Table 4.14: Resistance patterns as per antibiotics

Resistance Pattern	Frequency	MDR Type
GEN-COT-E	3	
AMP-AMX-E	2	3
COT-AMP-AMX-E	4	
GEN-AMP-AMX-E	4	4
CIP-COT-NA-E	1	
LE-CIP-NA-E	1	
GEN-COT-AMP-AMX-E	4	
GEN-CIP-COT-NA-E	1	5
GEN-S-AMP-AMX-E	1	
GEN-S-COT-AMP-AMX-E	2	
GEN-CIP-COT-AMP-AMX-E	1	
GEN-C-COT-AMP-AMX-E	1	6
GEN-COT-AMP-AMC-AMX-E	1	
GEN-CTX-CAZ-CTR-AMX-E	1	
GEN-CTX-CAZ-CTR-AMP-AMX-E	6	
LE-CIP-COT-AMP-AMX-NA-E	1	
GEN-LE-C-CIP-COT-NA-E	1	7
GEN-CIP-COT-CAZ-AMP-AMX-NA-E 1	1	
GEN-CTX-COT-CAZ-CTR-AMP-AMX-E	1	8
GEN-S-C-COT-CTR-AMP-AMX-NA-E	1	
GEN-CTX-COT-CAZ-CTR-AMP-AMC-AMX-E	1	
GEN-CTX-COT-CAZ-CTR-AMP-AMX-NA-E	1	9
GEN-S-CTX-CIP-COT-CAZ-CTR-AMP-AMC-AMX-E	1	11

4.2.2 Resistance mechanisms in *E. coli* isolates

A total of 18 isolates showed phenotypic resistance to quinolones (9; 50%) (Table 4.15) such as NA, CIP, and LE broad-spectrum cephalosporins, cefotaxime (11;61%). The distribution of phenotypic resistance was as follows; CIP (8/9, 88.9%), LE (3/9, 33.3%), and NA (7/9, 77.8%). Two of the 18 isolates exhibited a mixed phenotypic resistance pattern, that is, CIP-CTX and NA-CTX. However, only one of these isolates tested positive for both *gyr*A and *bla*$_{CTX-M}$ resistance genes. Overall, 50% (9/18) isolates tested positive for the *gyr*A gene while 22% (4/18) isolates tested positive for the CTX-M Gp1 gene (Table 4.15). In summary, *gyr*A and *bla*$_{CTX-M}$ resistance genes were detected in 17% (8/46) of the *E. coli*-positive samples. While 17% (8/46) samples (3 diarrhoeal and 5 non-diarrhoeal) were positive for *gyr*A gene, only 7% (3/46) samples (2 diarrhoeal and 1 non-diarrhoeal) were positive for *bla*$_{CTX-M}$ resistance gene. Carriage of both *gyr*A and *bla*$_{CTX-M}$ genes was observed in 7% (3/46) of the samples (Table 4.15).

The *gyr*A gene was detected at 360bp (Figure 4.5) as expected while the *bla*$_{CTX-M}$ was detected at 688bp using Gp1&2 primers (Figures 4.6 & 4.7).



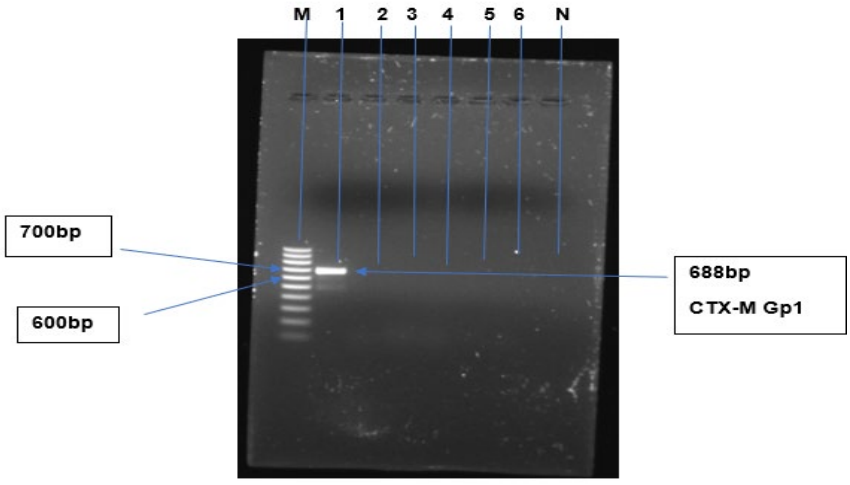
M = 100bp-1000bp DNA ladder, C1 = *E.coli* ATCC 25922, C2 = *Klebsiella pneumonia* ATCC 700603, C3 = *E.coli* NCTC 13846, Lanes 1,3-9 = *gyr*A positive *E.coli* isolates, Lane 2 = *gyr*A negative *E.coli* isolates, N= negative control

Figure 4.5: Gel electrophoresis results for surveillance of *gyrA* gene for quinolone resistance.

Table 4.15: Phenotypic and Genotypic Profiles of resistant *E. coli* isolates from diarrhoea and non-diarrhoea cases.

Isolate ID	Sample ID	Source	Phenotypic Resistance (N=18)				Genotypic profiles	
			CIP	NA	LE	CTX	bla-ESBL (CTX-M)	QRDR (gyrA)
44	TLD07	D	+	+	-	-	-	+
93	MP31	D	+	+	+	-	-	+
MP31		D	+	+	+	-	-	+
92		D	+	+	-	-	-	+
48	EL43	ND	+	+	-	-	-	+
47	TLD010	D	+	+	+	-	-	+
31	EL23	ND	+	-	-	+	-	+
94		D	-	-	-	+	+	N/A
8		ND	-	-	-	+	-	N/A
12		ND	-	-	-	+	-	N/A
30		ND	-	-	-	+	-	N/A
98	MP30	D	+	-	-	-	-	+
34	TLD09	D	-	+	-	+	+	+
36		D	-	-	-	+	+	N/A
51	V204	ND	-	-	-	+	+	N/A
9	EL22	D	-	-	-	+	-	N/A
28		D	-	-	-	+	-	N/A
32		D	-	-	-	+	-	N/A
Percentage + (%)			**8(44.4%)**	**7(38.9%)**	**3(16.7%)**	**11(61.1%)**	**4(22.2%)**	**9(50%)**

Key: ND=non-diarrhoeal, D=diarrhoeal stools, N/A= not tested for *gyr*A based on criteria

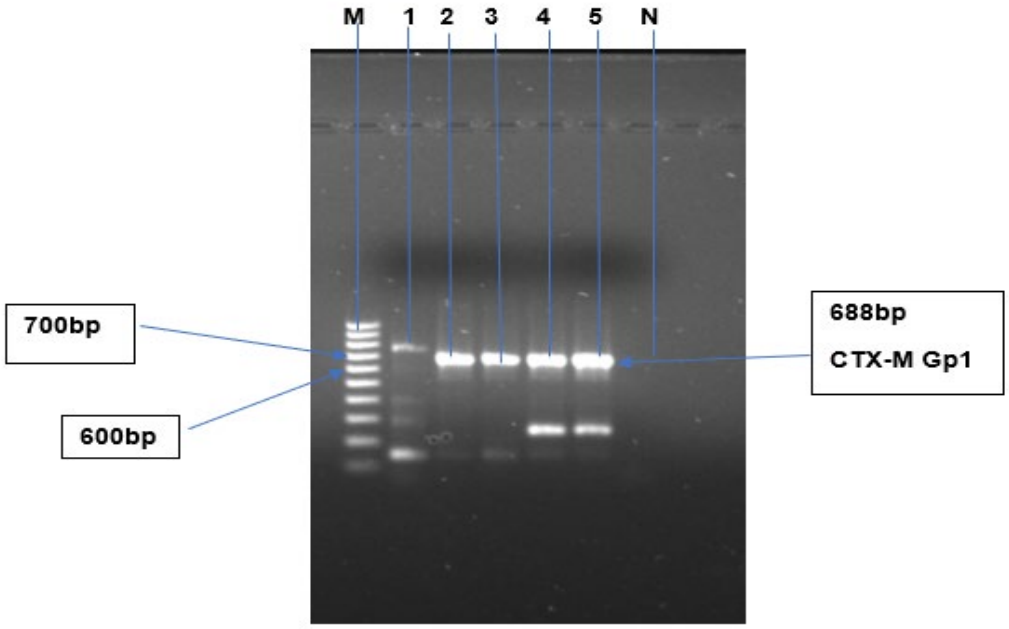


M = 100bp-1000bp marker,

Lane 1= *E. coli* ATCC13353 CTX-M positive control,

Lanes 2-6 = CTX-M negative isolates

Figure 4.6: CTX-M Group1 & 2: Positive control showing positive for CTX-M Group 1



M= 100bp-1000bp marker,

Lane 1= *E. coli* ATCC13846 CTX-M control,

Lanes 2-5 = CTX-M positive isolates,

N= negative control

Figure 4.7: CTX-M Group1 & 2: Positive control showing positive for CTX-M Group 1

Table 4.16: Point mutation profiles of fluoroquinolone-resistant *E. coli* isolates from diarrhoea and non-diarrhoea cases.

Isolates ID	Nucleotide positions & Amino acid (aa)					
	248 aa83	255	259 aa87	273	300	333
51	-	GTC->GTT		CGC->CGT	TAT->TAC	TCT->TCC
31		GTC->GTT		CGC->CGT	TAT->TAC	TCT->TCC
98		GTC->GTT		CGC->CGT	TAT->TAC	TCT->TCC
93	TCG->TTG S83L	GTC->GTT	GAC->AAC D87N	CGC->CGT	TAT->TAC	TCT->TCC
48	TCG->TTG S83C	GTC->GTT		CGC->CGT	TAT->TAC	TCT->TCC
44	TCG->TTG S83L	GTC->GTT		CGC->CGT	TAT->TAC	TCT->TCC
34		GTC->GTT		CGC->CGT	TAT->TAC	TCT->TCC

aa83 = amino acid 83; aa87 = amino acid 87

Table 4.17: Summary of Phenotypic and Genotypic Profiles of resistant *E. coli* isolates

Isolate ID	Source	Phenotypic Resistance			Genotypic resistance (gyrA)	Point mutation (Nucleotide position 248 & 259)	Amino acid codon
		CIP	NA	LE			
44	D	+	+	-	+	248	83
48	ND	+	+	-	+	248	83
93	D	+	+	+	+	248 & 259	83 & 87
31	ND	+	-	-	+	None	None
98	D	+	-	-	+	None	None
34	D	-	+	-	+	None	None
51	ND	-	-	-	+	255,273,300, 333	

4.2.3 Susceptibility and resistance of *Campylobacter* isolates

The microbiological culture method was not successful in isolating *Campylobacter* in this investigation. Molecular techniques were then employed to determine the prevalence of *Campylobacter*. Therefore, the findings of *Campylobacter* resistance are based on prediction which was done using the resistance finder software (ResFinder 4.1).

4.2.4 Prediction of Antibiotic resistance of *Campylobacter* spp

The selected nucleotide sequences of the *gyr*A gene fragments from the *Campylobacter* positive samples detected in Venda were analysed using the bioinformatics software ResFinder 4.1. The BLAST analysis for *Campylobacter* sequence **134_Cy-gyrA-393_G10_3730XL** matched *Campylobacter jejuni* strain MRC-09/00033 DNA gyrase subunit A (gyrA) gene, partial cds (Accession number: KP794749.1) with 94.14% identity. The latter strain is associated with ciprofloxacin-resistant *C. jejuni* in European countries. Although this study did not assess the phenotypic resistance of *Campylobacter* spp due to unsuccessful cultivation, the current prediction using bioinformatics highlights the potential of *Campylobacter* spp to harbour resistance genes that expresses resistance against fluoroquinolones such as ciprofloxacin. However, ResFinder 4.1 confirmed the partial presence of antibiotic-resistant *gyr*A gene far below the threshold (Tables 4.18 and 4.19). Despite the challenges associated with the cultivation of *Campylobacter*, future studies should

consider antibiotic susceptibility testing to ascertain the true nature of *Campylobacter* spp.

Table 4.18 ResFinder results for *Campylobacter* sequence 134_Cy-gyrA-393_G10_3730XL

Detection of PointFinder Genes	
rpsL	No gene found
23S	No gene found
cmeR	No gene found
gyrA_2	No gene found
gyrA	Gene found with coverage, 0.125000, below minimum coverage threshold: 0.6

Table 4.19 ResFinder results for *Campylobacter* sequence 134_Cy-gyrA-759r_B11_3730XL

Detection of PointFinder Genes	
rpsL	No gene found
23S	No gene found
cmeR	No gene found
gyrA_2	No gene found
gyrA	Gene found with coverage, 0.121914, below minimum coverage threshold: 0.6

4.2.5 Antibiotic Resistance of *E. coli*

The activity of ampicillin (AMP), cotrimoxazole (COT), and erythromycin (E) against *E. coli* isolates was quite low (Figure 4.4). This shows that these antibiotics are no longer effective against *E. coli* due to acquired resistance. Ampicillin (AMP), cotrimoxazole (COT), and chloramphenicol (C) have been widely reported in the literature as among the first-line antibiotics prescribed for treating diarrhoea in Africa (Seidman et al., 2016; Odetoyin et al., 2015; Ali et al., 2014). Given the findings from this study, it might be time to reevaluate AMP's inclusion in first-line defensive regimens for the treatment of children's diarrhoea. Several studies have made similar observations about *E. coli* developing resistance to AMP and COT (Ali et al., 2014; Odetoyin et al., 2015; Seidman et al., 2016).

The activities of CTX (3rd generation cephalosporin), IMP & MRP (carbapenems) in this study were quite high. Like polymixins such as colistin, carbapenems are also last resort antibiotics which are only prescribed against multi-drug resistant bacteria (Sekyere, 2016). Thus, the isolates did not show any resistance to the last line of defense such as colistin (CL) and carbapenems (IMP and MRP), resistance to these last-resort antibiotics is a cause of concern. Since cefotaxime (CTX) is a third-generation cephalosporin antibiotic with a broad spectrum of action, it is concerning when organisms develop resistance to such drugs. A high level of multi-drug resistance (MDR) to at least 2 antibiotics was observed in this study.

4.2.6 Surveillance of Quinolone resistance gene (*gyr*A)

Most isolates were resistant to NA (Table 4.15) . In this study, the majority of isolates showed resistance to CIP, and this is a cause of concern. The nine isolates that were resistant to quinolones (NA) and fluoroquinolones (CIP & LE) were evaluated for quinolone resistance mechanism (*gyrA*) which is the most common target of fluoroquinolones (Jaktaji and Mohiti, 2010). Eight out of nine isolates (88.9%) were positive for the *gyrA* gene (Figure 4.5). The literature that is now available indicates that the genes that confer resistance to nalidixic acid are the plasmid-mediated quinolone resistance determinants (*qnr*) (Nordmann and Poirel, 2005) and only the chromosomal QRDR (*gyr*A) gene was examined in this investigation. Published research indicates that DNA gyrase is the main target of quinolones in Gram-negative bacteria (Nordmann and Poirel, 2005). The fact that all nine isolates had positive *gyr*A gene tests was a noteworthy finding. It is important to remember, nevertheless, that quinolone resistance originates from mutations in the *gyr*A gene.

Multiple mutations in the *gyr*A gene's quinolone resistance determining region (QRDR) have been related to resistance to fluoroquinolones including ciprofloxacin and levofloxacin (Moharana et al., 2019). In addition, in quinolone-resistant *E. coli*, the most frequent mutation of *gyr*A is observed at codon 83 followed by codon 87 (Mahmud et al., 2021).

In this study, only isolate 93 showed a double transition mutation at positions 248 (codon 83) and 259 (codon 87) which were changes C → T and G → A respectively

(Table 4.16). From Table 4.17, mutations at positions 248 and 259 are linked with phenotypic resistance to both ciprofloxacin (CIP) and levofloxacin (LE). The results of a study conducted by Fu et al. (2013) on clinical samples from pediatric patients in China are in line with the current observations. The latter study observed that a double mutation at positions 248 and 259 was correlated with resistance to both ciprofloxacin and levofloxacin. Also, a study by (Moharana et al., 2019) reported that resistance to fluoroquinolones was linked with a double mutation at amino acid position 83 (serine → leucine) and position 87 (aspartic acid (D) → asparagine (N)). The same study also reported that resistance to narrow-spectrum quinolone such as Nalidixic acid (NA) was associated with a single mutation in the *gyr*A gene at amino acid 83 (serine → leucine). However, this study did not find any conserved *gyr*A mutation (S83L & D87N) in NA resistant (NA^{R+}) isolates (Table 4.17). Resistance to CIP alone had no link with mutation at positions 248 and 259. Mutation at position 248 corresponded with resistance to both NA and CIP while a double mutation at position 248 and 259 corresponded to resistance to all quinolones, NA, CIP, and LE

This is the first study in the Vhembe District, Limpopo Province to explore quinolone resistance in diarrheagenic *E. coli.* Fluoroquinolone-resistant Enterobacteriaceae such as *E. coli* are considered high-priority pathogens by the Word Health Organization (WHO) and thus continual surveillance was warranted. Surveillance of antimicrobial resistance genes (ARG) is scarce in Africa. A review of the distribution of ARG in South Africa revealed that ESBLs and carbapenemase resistance mechanisms are common in Limpopo (Ekwanzala et al., 2018). The latter study reported quinolone resistance genes in Northwest Province, however, this study observed quinolone resistance genes (*gyr*A) circulating in clinical isolates in rural areas in the Vhembe District, Limpopo Province, South Africa.

4.2.7 Surveillance of CTX-M resistance genes from *E. coli* isolates

Group 1 CTX-M includes the most prevalent $bla_{CTX\text{-}M\text{-}15,}$ and its global spread is a public health concern. A similar study done on children in rural Limpopo Province, South Africa observed two *E. coli* isolates which belonged to CTX-M group 9 (includes CTX-M-9 and CTX-M-14) (DeFrancesco et al., 2017). In this study, the CTX-M group1 was

observed in four *E. coli* isolates from young children in the same region of Limpopo Province (Table 4.15). This observation could indicate the evolution of *E. coli* due to antibiotic selection pressures. Elsewhere in Africa and other parts of the world, CTX-M Group 1 (*bla*$_{\text{CTX-M-15}}$) was detected in *E. coli* isolated from young children in Egypt (Khairy et al., 2020), Tanzania (Tellevik et al., 2016), Indonesia (Wasito *et al.*, 2017), Iran (Abbasi et al., 2020).

Apart from resistance to cefotaxime (CTX), the four isolates also showed resistance to other 3rd generation cephalosporins (3GC) such as CAZ, and CTR. Thus these *E. coli* isolates exhibited 3GC MDR phenotype. In addition, the same isolates showed resistance to other non-β-lactam antibiotics such as COT, AMP, and AMX, which are primarily used for treating diarrhoea in children. The phenotypic resistance pattern of these four isolates ranged between 7-9 antibiotics (Table 4.14). The rationale for this observation can be accounted for at the molecular level. It is known that the same conjugative plasmids often harbour ESBLs encoding genes and those genes conferring resistance to other non- β-lactam antibiotics (Amin et al., 2018; Franiczek et al., 2012).

4.3 Phylogenetic analysis of antibiotic resistance genes (CTX-M and *gyr*A)

4.3.1 Neighbour-joining phylogenetic tree for *E. coli* DNA gyrase (*gyr*A) and CTX-M genes

The nucleotide sequences of the *gyr*A gene fragments from the *E. coli* isolates detected in Venda were submitted the GeneBank and the accession numbers are as follows; OP132375, OP132376, OP132377, OP132378, OP132379, OP132380, OP132381, and OP132382 (Figure 4.8). Furthermore, as shown in Figure 4.9, the CTX-M-15 gene fragments were also added to the GeneBank under the accession numbers OP271864, OP271862, and OP271863.

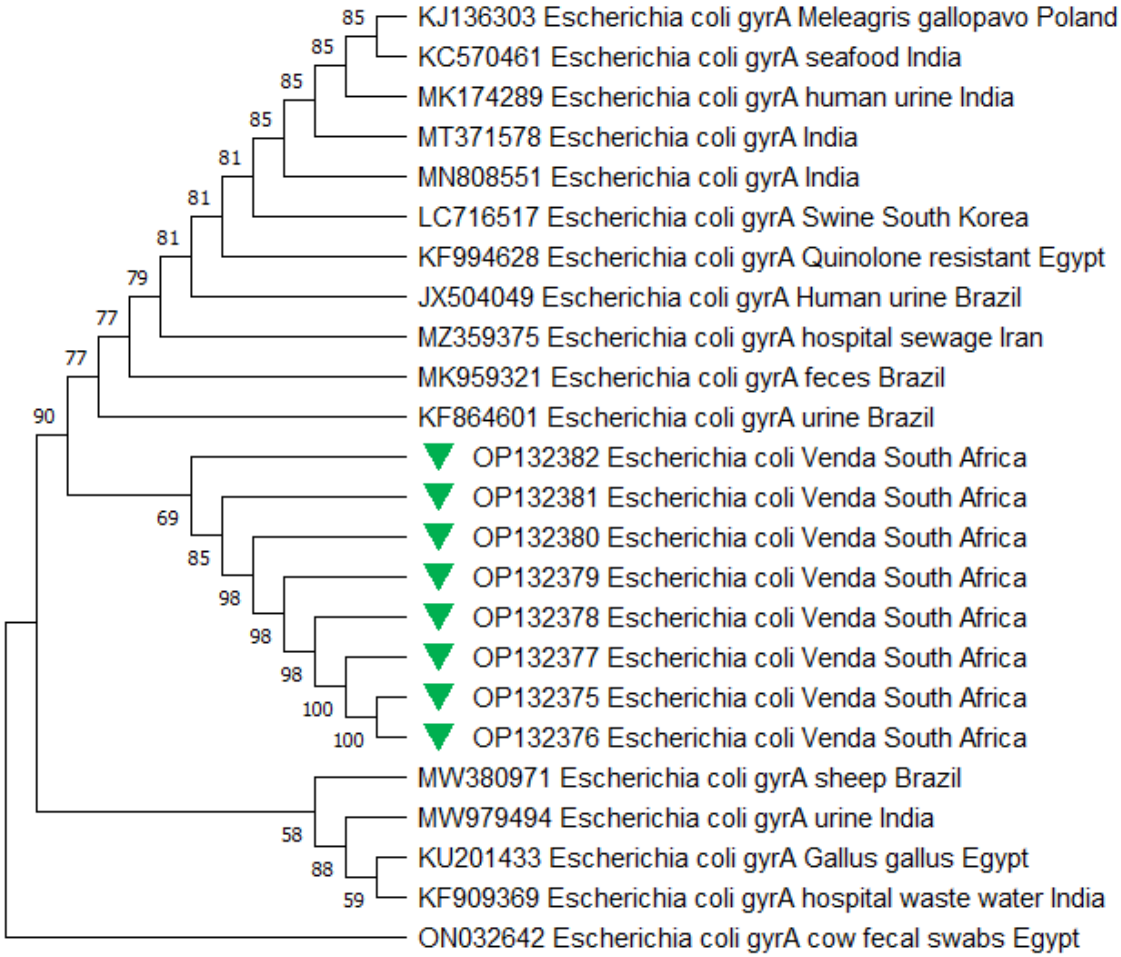


Figure 4.8: Neighbour-joining phylogenetic tree based on the 360-nucleotide sequence of *E. coli* DNA gyrase gene (*gyr*A) fragment was constructed using MEGA 11. Quinolone-resistant *E. coli* strains circulating in rural communities in the Vhembe District between 2020 and 2021. The green triangles show the *gyr*A genes from this study. Sixteen reference strains were selected from the GeneBank, and their respective accession numbers were noted.

Generally, the *E. coli* strains in this study were not diverse. According to the phylogenetic tree, the *E. coli* strains that were prevalent in the rural areas of Venda clustered under the same clade. This may indicate that these strains are clonal isolates of the same strain of *E. coli* that is common in Venda. The possibility of isolates from the same area clustering together under the same clade was the subject of a study done in Mozambique on antibiotic-resistant *E. coli* from bovine stool (Taviani et al., 2021). In addition, the *E. coli* strains from Venda shared a common ancestor with other *E. coli* strains circulating across the continents such as Africa (Egypt), Asia (India, Iran, South Korea), South America (Brazil) and Europe (Poland) (Figure 4.8). One striking observation is that the *E. coli* strains circulating in the Vhembe District which are

phenotypically resistant to quinolones and fluoroquinolones shared a common ancestor with quinolone-resistant *E. coli* strain detected in Egypt (Accession number: KF994628). Moreover, it is worth noting that *E. coli* species undergo horizontal transfer of genes with other members of the Enterobacteriaceae. The phylogenetic relationship between local strains and other strains isolated from human urine in India as depicted in Figure 4.8 could hint at the potential transfer of genes from uropathogenic *E. coli* to diarrheagenic *E. coli.* A previous study has reported on the possible existence of hybrid *E. coli* species co-habouring uropathogenic and diarrheagenic genes (Tanabe et al., 2022). Future studies in Venda should consider exploring on the epidemiology of hybrid *E. coli* strains circulating in the Vhembe District.

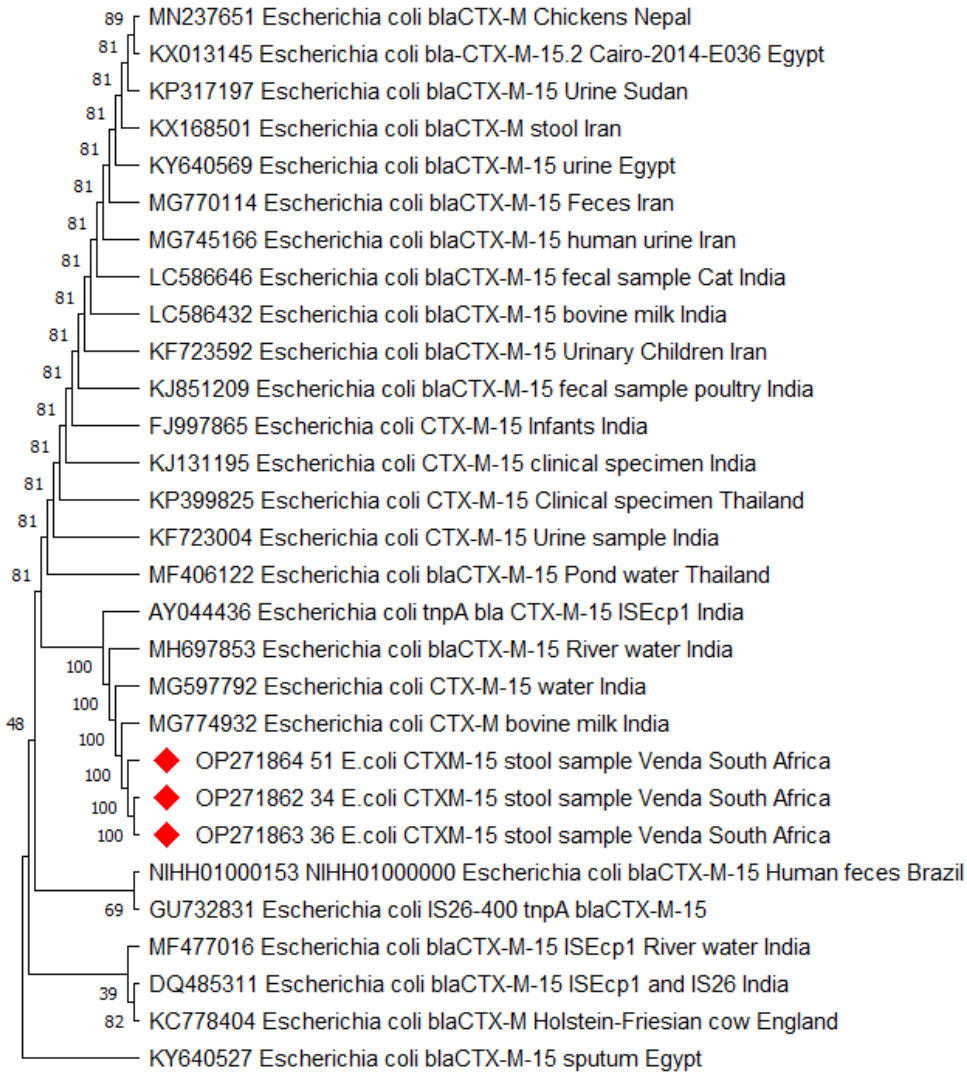


Figure 4.9 Beta-lactamase CTX-M Neighbour-joining phylogenetic tree based on the 688-nucleotide sequence of *E. coli* beta-lactamase gene fragment was constructed using MEGA 11. CTX-M-resistant *E. coli* strains circulating in rural communities in the Vhembe District between 2020 and 2021. The CTX-M genes from this study are highlighted in red. Twenty-six reference strains were selected from the GenBank, and their respective accession numbers were noted.

The three *bla*$_{\text{CTX-M-15}}$ resistance genes, OP271864 (51), OP271862 (34), and OP271863 (36) from this study, clustered together (Fig 4.9). While OP271864 (51) was found from a sample collected from a different hospital, OP271862 (34) and OP271863 (36) were recovered from samples taken from the same hospital. According to the phylogenetic tree, it is evident that the *bla*$_{\text{CTX-M}}$ variant sought in this study is indeed *bla*$_{\text{CTX-M-15}}$. This extended-spectrum beta-lactamase is known to be strongly associated with the insertion sequence *ISEcp1* which is responsible for the dissemination of *bla*$_{\text{CTX-M-15}}$ (Zhao and Hu, 2013). From the phylogenetic tree, the *bla*$_{\text{CTX-M-15}}$ detected in this study, OP271862 (34) and OP271863 (36) are more closely related to the insertion sequence *ISEcp1* reported in India (accession no. AY044436) compared to OP271864 (51) (Fig 4.9). Another observation is that the *bla*$_{\text{CTX-M-15}}$ detected in this study were closely related to *bla*$_{\text{CTX-M-15}}$ reported in India which were recovered from bovine milk (accession no. MG774932) and river water (accession number MG597792 & MH697853). This could suggest that livestock such as cattle and water sources such as rivers are potential sources of transmission of CTX-M-producing *E. coli* to humans in the Vhembe District. However, further studies are needed to validate the latter hypothesis.

Chapter5

CONCLUDING REMARKS

5.1 Conclusion and Recommendations

The findings from this study revealed a prominent level of multi-drug resistance (MDR) to at least two antibiotics including ampicillin, amoxycillin, cotrimoxazole, ciprofloxacin and cefotaxime. Empiric antibiotic treatment against bacteria such as *E. coli* is no longer effective. This study confirms the circulation of CTX-M-15-producing *E. coli* variants among children under five years in rural communities in the Vhembe District. The phylogenetic relationships revealed a close association of $bla_{\text{CTX-M}}$ with insertion sequence *ISEcp1* (Fig 4.9) which is responsible for the dissemination of $bla_{\text{CTX-M-15}}$. Future studies could investigate the role of *ISEcp1* in the dissemination of $bla_{\text{CTX-M-15}}$ among pediatric population in the Vhembe District. The phylogenetic relationship observed between the CTX-M-producing isolates in this study and other CTX-M-producing isolates from livestock and river water warrants further studies to consider parallel investigations on both clinical and environmental samples to ascertain the potential roles of livestock and river water as vehicles of transmission of CTX-M-producing *E. coli* to humans in the Vhembe District. Given that, few studies in Africa have reported on CTX-M-producing *E. coli* in pediatric diarrhoea cases, this study contributes to the body of knowledge about genotypic antibiotic resistance.

In addition, this study explored the circulation of fluoroquinolone-resistant *E. coli* in diarrhoea cases in young children in the Vhembe District. The findings from this study revealed that resistance to fluoroquinolones such as ciprofloxacin and levofloxacin among other antibiotics is attributed to mutations in the *gyrA* gene. In this study, mutation at codon 83 was more common a cause of resistance relative to double mutation at positions 83 and 87 (Table 4.16). The phylogenetic relationship revealed the association between diarrheagenic *E. coli* strains in this study, and uropathogenic *E. coli* strains detected in urine samples from various countries as shown in Figure 4.8. Future studies could consider investigations on both stool samples and urine samples collected from the same participant to characterise hybrid isolates of uropathogenic and diarrheagenic *E. coli*.

Although the small number of samples in this study is inadequate to establish appropriate statistical significance, our results showed a clear trend, and emphasizes the need for future research on the state of molecular resistance mechanisms circulating in the Vhembe District.

5.2 LIMITATIONS OF THE STUDY

The current study has several limitations, among which is the limited number of samples included in this study. The collection of stool samples was a challenge during the COVID-19 period hence the small number of samples included in this study. Despite attempts to inform them of the study's scope, not all mothers and/or caregivers who were consulting for diarrhoea on behalf of their children were willing to take part. Some stool samples were stored in a freezer upon arrival at the laboratory before analysis could be done. This most likely explains why the culture approach was unable to isolate *Campylobacter*. The lack of resources at the time to satisfy *Campylobacter's* stringent fastidious requirements, however, may have been the most likely factor in the failure to isolate the bacteria. Nevertheless, the researcher was able to alternatively assess the prevalence of *Campylobacter* directly from the stools using molecular techniques. This is the reason antibiotic resistance of *Campylobacter* was only done based on predictions from bioinformatic tools such as the ResFinder. Furthermore, the current study did not manage to characterise *E. coli* into pathotypes which limit the findings of this study from being reported as ESBL-producing diarrheagenic *E. coli* but rather as fecal carriage of ESBL-producing *E. coli*.

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APPENDIX

APPENDIX A : Presentation at a Conference

WORLD ANTIBIOTIC AWARENESS WEEK SYMPOSIUM 2022

Preventing Antimicrobial Resistance Together: A One Health Approach

Virtual and In-person Symposium

Venue: Stellenbosch Institute for Advanced Study (STIAS; Address - 10 Marais Road, Stellenbosch, 7600)

PROGRAMME MONDAY 21 NOVEMBER 2022

07:45 – 08:25 **On-site Registration**

SESSION 1: ENVIRONENTAL RESEARCH (IN-PERSON PRESENTATIONS)
CHAIRPERSON: Dr Nonhlanhla Kalebaila (WRC)

08:30 – 09:00	**Welcome and Plenary Address: Dr Jennifer Molwantwa (CEO, WRC)**	
09:00 – 09:30	**Keynote: Nature's solutions to the antibiotic resistance Armageddon**	**Prof Marina Rautenbach (SUN)**
09:30 – 09:45	Analysis of resistance profiles within domestic greywater systems	Bronwyn Kirby-McCullough (UWC
09:45 – 10:00	Genome mining of South African marine actinobacteria strains for the discovery of novel antimicrobial compounds	Jo-Marie Vreulink (CPUT)
10:00 – 10:15	Evaluation of antibiotic resistance in mixed bacterial populations isolated from ostrich chicks.	Ruan Kitshoff (SUN)
10:15 – 10:30	Distribution of *Campylobacter* antibiotic resistance genes (ARGs) in selected source waters and potential human exposure	Mary Chibwe (RU)
10:30 - 10:45	Comparative genome analysis of the novel phages infecting multi-drug resistant *Escherichia coli* O177 strain	Peter Kotsoana Montso (NWU)

10:45 – 11:15 **Coffee/Tea Break**

SESSION 2: MEDICAL RESEARCH (IN-PERSON PRESENTATIONS)
CHAIRPERSON: Dr Mae Newton-Foot (SUN)

11:15 – 11:45	**Keynote: The Role of a One Health Approach in Antimicrobial Resistance Surveillance (ONLINE)**	**Dr Yogandree Ramsamy (JDJ Diagnostics, UKZN)**
11:45 – 12:00	Comparative genomic analysis of colistin resistant *Acinetobacter baumannii* clinical isolates circulating in hospitals in South Africa	Chane Buys (UP)
12:00 – 12:15	Antibiotic-resistant staphylococci other than *Staphylococcus aureus* in South Africa: A One Health Approach	Remous Ocloo (SUN)
12:15 – 12:30	Fosfomycin susceptibility in Enterobacterales: Antibiotic susceptibility testing and molecular mechanisms of resistance	Jessica Hurwitz (SUN)
12:30 – 12:45	Modulation of oligomerization and antimicrobial activity in cyclic decapeptide-metal nanoformulations	Carmen de Villiers (SUN)
12:45 – 13:00	Formulation of natural cyclodecapeptides for surface treatment applications	Christopher Borrageiro (SUN)
13:00 – 13:15	Activity and structural analysis of Arg- and Trp-rich peptide candidates for antifungal drug development	Gamuchirai Mamhende (SUN)

13:15 – 14:00 **Lunch Break and On-site Poster Presentations**

SESSION 3: ENVIRONMENTAL and MEDICAL RESEARCH (ONLINE PRESENTATIONS)
CHAIRPERSON: Dr Eunice Ubomba-Jaswa (WRC)

14:00 – 14:15	Evidence of virulent multi-drug resistant and biofilm-forming *Listeria* species isolated from various sources in South Africa	Christ Kaptchouang Tchatchouang (NWU)
14:15 – 14:30	An audit of antimicrobial use in surgery at a public hospital: A stepping stone towards antimicrobial stewardship	Kwena Mathobela (UL)
14:30 – 14:45	Carriage of *gyrA* and $bla_{CTX\text{-}M}$ resistance genes in *E. coli* isolates from children with diarrhoea in Venda, South Africa	Simbarashe Karambwe (UV)
14:45 – 15:00	Prevalence of multidrug resistant diarrhoeagenic bacteria isolated from cattle faecal samples: Possible source of diarrhoea outbreak	Tesleem Abolarinwa (NWU)
15:00 – 15:15	Assessment of commercial and wild birds as potential vectors of antibiotic-resistant *Escherichia coli* and *Enterococcus* species	Nerisa Chetty (UKZN)
15:15 – 15:30	Probiotics/effective microbes as an alternative for antimicrobials in chicken growth promotion: The need for smart regulations for resistance prevention	Norman Nyazema (UL)
15:30 – 16:00	**End of Day 1 - Closing Remarks**	**Prof Carlos Bezuidenhout (NWU)**
16:00 –16:30	**Coffee/Tea Break**	
16:15-17:00	**National Organising Committee Meeting**	

NWU® Stellenbosch University iYunivesithi University of Johannesburg saMRC WATER RESEARCH COMMISSION

APPENDIX B: Project Registration and Approval from the University of Venda, Limpopo Provincial Department of Health, and Vhembe District Department of Health

UNIVERSITY OF VENDA

OFFICE OF THE DEPUTY VICE-CHANCELLOR: ACADEMIC

TO : MR/MS S. KARAMBWE

SCHOOL OF MATHEMATICAL AND NATURAL SCIENCES

FROM: SENIOR PROF L.B KHOZA

ACTING DEPUTY VICE-CHANCELLOR: ACADEMIC

DATE : 27 NOVEMBER 2018

DECISIONS TAKEN BY UHDC OF 27TH NOVEMBER 2018

Application for approval of Thesis research proposal in Mathematical and Natural Sciences: **S. Karambwe (11613034)**

Topic: "Phenotype and Genotypic antibiotic resistance properties of Enteric Bacteria associated with diarrhoea in young children in Vhembe District."

Promoter	UNIVEN	Prof. N. Potgieter
Co-promoters	UNIVEN	Prof. A.N Traore
	UNIVEN	Prof. P.O Bessong

UHDC approved Thesis proposal

SENIOR PROF L.B KHOZA
ACTING DEPUTY VICE-CHANCELLOR: ACADEMIC

RESEARCH AND INNOVATION
OFFICE OF THE DIRECTOR

NAME OF RESEARCHER/INVESTIGATOR:

Mr S Karambwe

Student No:
11613034

PROJECT TITLE: **Phenotype and Genotypic antibiotic resistance properties of enteric bacteria associated with diarrhoea in young children in Vhembe District.**

PROJECT NO: **SMNS/19/MBY/06/0207**

SUPERVISORS/ CO-RESEARCHERS/ CO-INVESTIGATORS

NAME	INSTITUTION & DEPARTMENT	ROLE
Prof N Potgieter	University of Venda	Promoter
Prof AN Traore	University of Venda	Co - Promoter
Prof PO Bessong	University of Venda	Co - Promoter
Mr S Karambwe	University of Venda	Investigator – Student

ISSUED BY:
UNIVERSITY OF VENDA, RESEARCH ETHICS COMMITTEE

Date Considered: July 2019
Decision by Ethical Clearance Committee Granted
Signature of Chairperson of the Committee: .....
Name of the Chairperson of the Committee: Senior Prof. G.E. Ekosse

UNIVERSITY OF VENDA
DIRECTOR
RESEARCH AND INNOVATION
2019 -07- 04
Private Bag X5050
Thohoyandou 0950

University of Venda
PRIVATE BAG X5050, THOHOYANDOU, 0950, LIMPOPO PROVINCE, SOUTH AFRICA
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REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH

Ref : LP -2020-03-004
Enquires : K. Letseparela
Tel : 015-293 6028
Email : Kurhula.Hlomane@dhsd.limpopo.gov.za

S. Karambe

PERMISSION TO CONDUCT RESEARCH IN DEPARTMENTAL FACILITIES

Your Study Topic as indicated below;

Phenotype and Genotype Antibiotic resistance properties of enteric bacteria associated with Diarrhoea in young Children in Vhembe District

1. Permission to conduct research study as per your research proposal is hereby Granted.
2. Kindly note the following:
   a. Present this letter of permission to the institution supervisor/s a week before the study is conducted.
   b. In the course of your study, there should be no action that disrupts the routine services, or incur any cost on the Department.
   c. After completion of study, it is mandatory that the findings should be submitted to the Department to serve as a resource.
   d. The researcher should be prepared to assist in the interpretation and implementation of the study recommendation where possible.
   e. The approval is only valid for a 1-year period.
   f. If the proposal has been amended, a new approval should be sought from the Department of Health
   g. Kindly note that, the Department can withdraw the approval at any time.

Your cooperation will be highly appreciated

pp _____
Head of Department

05/06/2020
Date

Private Bag X9302 Polokwane
Fidel Castro Ruz House, 18 College Street. Polokwane 0700. Tel: 015 293 6000/12. Fax: 015 293 6211.
Website: http/www.limpopo.gov.za

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LIMPOPO
PROVINCIAL GOVERNMENT
REPUBLIC OF SOUTH AFRICA

DEPARTMENT OFHEALTH
VHEMBE DISTRICT

Ref: S5/6
Enq: Muvari MME
Date: 23-10-2020.....

Dear Sir/Madam..Karambwe S.....

Permission to conduct a research on the
"Phenotypic and genotypic antibiotic resistance profiles".

1. The above matter refers.

2. Your letter received on the 23-10-2020.....requesting for permission to conduct a research is hereby acknowledged.

3. The District has no objection to your request.

4. Permission is therefore granted for the study to be conducted within Vhembe District. You are expected to submit the results to the District.

5. You are however advised to make the necessary arrangements with the facilities concerned.

Wishing you success in your endeavors.

..... 2020-10-28.
CHIEF DIRECTOR: DISTRICT HEALTH DATE

Private Bag X5009 THOHOYANDOU 0950
OLD parliamentary Building Tel (015) 962 1000 (Health) (015) 962 4958 (Social Dev) Fax (015) 962 2274/4623
Old Parliamentary Building Tel: (015) 962 1848, (015) 962 1852, (015) 962 1754, (015) 962 1001/2/3/4/5/6 Fax (015) 962 2373, (015) 962 227

The heartland of Southern Africa – development is about people!

RESEARCH ETHICS COMMITTEE

UNIVEN Informed Consent

Appendix B

LETTER OF INFORMATION

Title of the Research Study	:Phenotypic and genotypic antibiotic resistance properties of enteric bacteria associated with diarrhea in young children in Vhembe District
Principal Investigator/s/ researcher	: (*Karambwe Simbarashe, MSc*)
Co-Investigator/s/supervisor/s	: (*Prof N. Potgieter, PhD MBY; Prof AN Traore, PhD BCM*)

Brief Introduction and Purpose of the Study:

- The study will include stools from young children under 5 years and older participants less than 14 years old with diarrhoea. At least 50 children will take part in this project.
- The project(s) aimed to investigate the phenotypic and genotypic antibiotic resistance properties of bacteria causing diarrhea in the Vhembe district.
- This information will help outline an update on evolutionary trends regarding antibiotic resistance and also crucial in developing proper intervention strategies in order to curb the debilitating effects of paediatric diarrhea
- General information will be taken from you (participant), including contact details, age, gender, use of toilet and type of toilet, type of water used for drinking, date of onset of diarrhoea, episodes of diarrhea per day, any use of antibiotics prior to consulting about diarrhea, HIV status and other illnesses, etc.
- A total of 10g of stools will be collected from the participant and will be transported to the laboratory for analysis.

Outline of the Procedures: The collection of samples will be random, diarrheic stool samples will be collected from children under the age of 14 years reporting at two hospitals hospitals (Donald Frazer and Tshilidzini) around the Vhembe District during the period July 2019-November 2019. Participant will be requested to provide their stool sample and also answer some few questions on the capture form/ questionnaire. Inclusion criteria would be children with diarrhea that have not taken antibiotics in the past 1 month. However, the zero age group would be incorporated as a control group since by this time no antibiotics are generally prescribed to this age group. Demographic information regarding age, socio-economic background of participants and symptoms associated with diarrhea will be collected using a structured questionnaire/ capture form.

Bacteriological analysis will be done at the University of Venda Microbiology laboratory and the results will be analysed using statistical methods.

Risks or Discomforts to the Participant: There are no risks involved in participating. Collection of stools will be done after or when the participant is eliminating waste during diarrhea episodes

Benefits: This research is a cross-sectional study, so there are no individual benefits attached. However, the results of the study will be made available through publication.

Reason/s why the Participant May Be Withdrawn from the Study: This study is a cross-sectional study, only interact with participant once.

Remuneration	: No monetary compensation is offered for your participation
Costs of the Study	: The participant will not be required to pay any money towards the study
Confidentiality	: Only the medical doctor/nurse and the research team will have access to your medical information. The participant's identity will not be made public and if the results are published or presented, a participant will only be referred to by a code number. The participant's identity will be strictly kept confidential
Research-related Injury	: There are no risks associated with this study.

Persons to Contact in the Event of Any Problems or Queries:

(Prof N. Potgieter, Dean, School of Mathematical and Natural Sciences, University of Venda; Private Bag X5050; 0950 Thohoyandou, South Africa) Please contact the researcher (0766624752), my supervisor (+27 15 962 8107) or the University Research Ethics Committee Secretariat on 015 962 9058. Complaints can be reported to the Director: Research and Innovation, Prof GE Ekosse on 015 962 8313 or Georges Ivo.Ekosse@univen.ac.za

General:
Potential participants must be assured that participation is voluntary and the approximate number of participants to be included should be disclosed. A copy of the information letter should be issued to participants. The information letter and consent form must be translated and provided in the primary spoken language of the research population

CONSENT

Statement of Agreement to Participate in the Research Study:

- I hereby confirm that I have been informed by the researcher, (*name of researcher*), about the nature, conduct, benefits and risks of this study - Research Ethics Clearance Number: _,
- I have also received, read and understood the above written information (*Participant Letter of Information*) regarding the study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

Full Name of Participant Date Time Signature

I, ...

(*Name of researcher*) herewith confirm that the above participant has been fully

Informed about the nature, conduct and risks of the above study.

Full Name of Researcher

..... Date..... Signature.....

Full Name of Witness (If applicable)

..... Date ..... Signature.....

Full Name of Legal Guardian (If applicable)

..... Date..... Signature.....

Please note the following:

Research details must be provided in a clear, simple and culturally appropriate manner and prospective participants should be helped to arrive at an informed decision by use of appropriate language (grade 10 level- use Flesch Reading Ease Scores on Microsoft Word), selecting of a non-threatening environment for interaction and the availability of peer counseling (Department of Health, 2004)

If the potential participant is unable to read/illiterate, then a right thumb print is required and an impartial witness, who is literate and knows the participant e.g. parent, sibling, friend, pastor, etc. should verify in writing, duly signed that informed verbal consent was obtained (Department of Health, 2004).

If anyone makes a mistake completing this document e.g. a wrong date or spelling mistake, a new document has to be completed. The incomplete original document has to be kept in the participant's file and not thrown away, and copies thereof must be issued to the participant.

References:

Department of Health: 2004. *Ethics in Health Research: Principles, Structures and Processes*

http://www.doh.gov.za/docs/factsheets/guidelines/ethnics/

Department of Health. 2006. *South African Good Clinical Practice Guidelines*. 2nd Ed. Available at:

http://www.nhrec.org.za/?page_id=14

Department of Microbiology, School of Mathematical and Natural Sciences University of Venda

RESEARCH PROJECT DATA CAPTURE FORM

Subject Number .....

Consultation Details

Date.....Visit Number.....Site/Clinic name.....

Patient Information:

Name.....D.O.B.....Gender.....

Contact Details.....Parent status.....

Family living conditions: Water source/Tap ☐ Spring ☐ River ☐ Boreholes ☐

Use of toilet ☐ Type of toilet ☐

Medical history

Date of onset.....Diarrheal episodes.....

Source of contamination: Community ☐ Outbreak ☐ Hospital ☐

Symptoms: Diarrheal ☐ Vomiting ☐ Fever ☐ Abdominal pains ☐

Dehydration ☐ Reported clinical immunodeficiency ☐

Any other infections:.....

Sample Collection:

Date of collection.....

Type of stools: Watery ☐ Sausage ☐ Mushy ☐

Treatment:

Current:.....

Previous:.....

Laboratory Results:

Etiological Agent(s).....

.....

Antibiotic Susceptibility Profile.....

.....

Resistance mechanism (s).....

.....

APPENDIX C: Publications

microorganisms

MDPI

Review

Epidemiology of Cefotaxime-Hydrolysing β-Lactamase-Producing *Escherichia coli* in Children with Diarrhoea Reported Globally between 2012 and 2022

Simbarashe Karambwe, Afsatou Ndama Traoré and Natasha Potgieter*

Department of Biochemistry and Microbiology, University of Venda, Private Bag X5050, Thohoyandou 0950, South Africa; karambwesim@gmail.com (S.K.); afsatou.traore@univen.ac.za (A.N.T.)
* Correspondence: natasha.potgieter@univen.ac.za

Abstract: The global spread of cefotaxime-hydrolysing β-lactamase (CTX-M)-producing *Escherichia coli* (*E. coli*) and its associated impact on paediatric diarrhoeal treatment and management has become a public health concern. This review assessed surveillance studies on CTX-M-producing *E. coli* associated with diarrhoea in children published between 2012 and 2022 globally. A total of thirty-eight studies were included for data analysis, categorised into continental regions, and tabulated. The majority (68%) of studies were conducted in Asian countries while few studies were conducted in Europe (11%) and Africa (18%), respectively. On the African continent, the majority (11%) of studies were conducted in Northern Africa while no studies were reported in East Africa. On the American continent, 3% of the studies were reported from South America. The studies included were classified into diarrheagenic *E. coli* (74%; 28/38) and faecal carriage (26%; 10/38). Of all the *E. coli* pathotypes associated with CTX-M production, EPEC was frequently reported. The prevalence of CTX-M-producing *E. coli* including the CTX-M-15-producing variants ranged between 1% and 94%. About 37% of the studies generalised the report as bla_{CTX-M}-positive *E. coli*. The use of sequencing in characterising the CTX-M-producing *E. coli* was reported in only 32% of all the studies. This review provides information on the epidemiology of CTX-M-15-producing *E. coli* in paediatric diarrhoea and the extent to which surveillance is being performed. This is relevant in informing clinical practice for the management of diarrhoea as well as the design of future surveillance studies.

Keywords: *Escherichia coli*; diarrhoea; children; CTX-M-producing *E. coli*

Citation: Karambwe, S.; Traoré, A.N.; Potgieter, N. Epidemiology of Cefotaxime-Hydrolysing β-Lactamase-Producing *Escherichia coli* in Children with Diarrhoea Reported Globally between 2012 and 2022. *Microorganisms* 2024, *12*, 171. https://doi.org/10.3390/microorganisms12010171

Academic Editors: Dobroslava Bujňáková and Ivana Čirković

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1. Introduction

The Global Burden of Disease, Injuries, and Risk Factors Study (GBD) ranked diarrhoea as one of the prime causes of death and disability-adjusted life years (DALYs) for children younger than 5 years. In 2016 alone, close to half a million deaths in children under 5 years were due to diarrhoea [1]. Asia, Africa, and America are among the continents that have reported high rates of deaths of children under two years of life due to diarrhoea [2].

Pathogenic strains of *Escherichia coli* (*E. coli*) are one of the causes of diarrhoea in children in developing countries [3]. These *E. coli* strains with diarrhoea-causing properties are known as diarrheagenic *E. coli* (DEC). There are six DEC pathotypes namely, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC) also known as Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [3,4]. Of the six pathotypes, EAEC, EPEC, and ETEC are the most common causes of diarrhoea episodes in children under five years in developing countries [5]. Diarrheagenic *E. coli* has also been characterised into phylogroups such as A, B1, B2 and D. Phylogroups A and D are mostly associated with diarrhoea [6,7] and human faecal matter is a possible source of DEC in these two phylogroups [8]. However, the association of DEC and phylogroups varies geographically [8].

According to the World Health Organisation (WHO), paediatric diarrheal infection in low-income countries is not only a risk to public health, but it is becoming increasingly untreatable due to emerging antibiotic-resistant patterns against commonly prescribed antibiotics [9]. Antibiotic resistance among diarrheagenic *E. coli*, which has spread across developing countries, has been associated with the overuse of antibiotics [4]. Travelling has also been implicated as another key driving factor facilitating the global spread of antibiotic resistance [10,11].

Cefotaxime (CTX) is a broad-spectrum cephalosporin antibiotic normally used in treating infections caused by bacteria resistant to first-line antibiotics. Cefotaxime-hydrolysing β-lactamases (CTX-M) which together with some variants of Temoneira (TEM) and sulphydryl variable (SHV) enzymes, are considered the most clinically significant beta-lactamases with extended-spectrum activity (ESBLs) [12,13]. Unlike TEM and SHV genes, which also have variants that exhibit non-ESBL characteristics, all CTX-M types are exclusively ESBL genes [14]. The CTX-M gene variants are not closely related to the most isolated β-lactamases, TEM and SHV genes [13]. Of the ESBLs, the CTX-M variants are leading in terms of spread and their impact is either comparable or even greater to that of Temoneira (TEM) and sulphydryl variable (SHV) ESBLs [15]. There are several CTX-M variants grouped into sub-families, CTX-M group 1, 2, 8,9, 25 and 45 among others [15]. Group 1 CTX-M variants are the most widespread globally compared to other variants and in Africa and Asia, reports indicated that CTX-M group 1 are more common [10,15]. Of the CTX-M Group 1, CTX-M-15 is currently the dominant variant and a cause of concern in clinical practice [16]. The literature suggests that the CTX-M-15 gene variants are widely distributed in countries in Europe, North and South America as well as Asia [17]. It is important to note that the prevalence of CTX-M-producing *E. coli* varies between regions [10,18]. The prevalence rates of CTX-M-producing *E. coli* are at least 60% in Asia [10] while a lower rate of 34% has been reported in West Africa [18].

Specific DEC pathotypes such as EPEC, ETEC and EAEC have been implicated among extended-spectrum beta-lactamases (ESBL) CTX-M (cefotaxime resistant) producers [4,19]. *E. coli* strains producing ESBLs such as CTX-M are a threat to public health and can exhibit co-resistance to other classes of antibiotics such as aminoglycosides and fluoroquinolones [20–22]. Information regarding specific *E. coli* pathotypes associated with CTX-M genes is scarce [19]. While CTX-M are the predominant ESBL genes encountered, two other genes, namely TEM, and SHV, which encode enzymes that confer beta-lactam resistance are also encountered in the *Enterobacteriaceae* group such as *E. coli* [16,18].

Diarrhoea in children under 5 years is implicated among the risk factors for acquiring ESBL-producing *E. coli* [23]. CTX-M-producing *E. coli* associated with diarrhoea cases in young children has been mostly reported in Asian countries [10] while antimicrobial resistance (AMR) surveillance in other regions such as Africa is slow or rather underreported due to limited resources and infrastructure [18]. Despite studies that investigated CTX-M-producing *E. coli* in diarrhoea cases in Africa [12,23–26], there is a dearth of information on beta-lactamase (CTX-M) resistance in *E. coli* associated with diarrhoea in young children. In addition, detailed genomic studies using sequencing techniques to uncover the epidemiology of high-risk clones such as sequence type 131 (ST131), which are associated with the dissemination of CTX-M genes are limited in Africa [27]. Previous studies that have been conducted in children investigated CTX-M-producing *E. coli* recovered from urinary infections [28]. This narrative review aimed to give an update on the reported prevalence of CTX-M-producing *E. coli* recovered from children less than 5 years of age with diarrhoea, especially tracing the epidemiology of the CTX-M-15 gene variant in the literature published between 2012 and 2022. This is relevant in understanding the local and regional epidemiology of CTX-M-producing *E. coli*, which is essential in guiding interventions and antimicrobial stewardship.

2. Methodology

2.1. Search Strategy and Selection Criteria

A literature search was conducted for studies published between 1 January 2012 and 31 September 2022 using PubMed, Web of Science, Google Scholar and Science Direct databases. The following keywords were used: "*Escherichia coli*" OR "*E. coli*" and "CTX-M beta-lactamase" OR "CTX-M β-lactamase" OR "*bla*CTX-M" OR "CTX-M" AND "diarrhoea" OR "diarrhea". The literature search was restricted to the following: last decade 2012–2022, studies on humans, age group 5 years and under and studies published in English language. In addition, supplementary literature search was carried out using the bibliographies of studies relevant to the objective of this study (Figure 1). The studies were thoroughly screened based on the title and the abstracts reporting on CTX-M-producing *E. coli* (Figure 1). For studies to be included in this review, both phenotypic and genotypic resistance must have been reported.

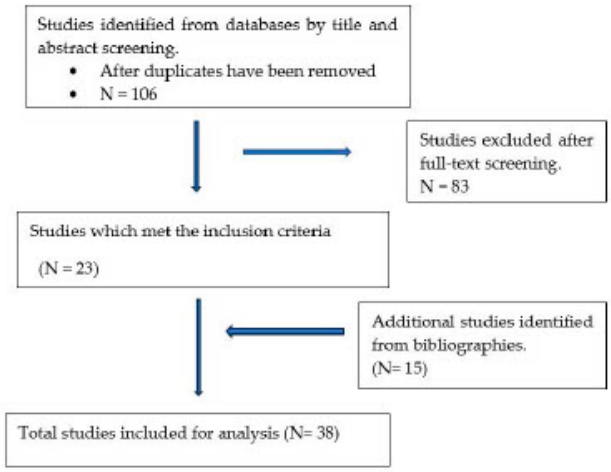

Figure 1. Flow diagram showing the filtering process followed on selection of studies.

2.2. Data Categorisation

Data on the author name, publication year, study period, country, continent, identified gap, age group, study design (prospective or retrospective), sample size, study setting (hospital, community), method of detection ([1]*E.coli* and [2]ESBL genes), causative organism (*E. coli* or *E. coli* pathotypes or *E. coli* phylogroups), the percentage of CTX-M genes reported, other ESBLs genes such as TEM, SHV and OXA among others, and most common ESBLs were extracted and entered into an excel spreadsheet (Supplementary File S1).

2.3. Data Analysis

Python programming language (Version 3.8.8) was used for data analysis. Python Libraries used included Pandas, a package used for storing and manipulating data and data visualisation libraries such as Matplotlib and Seaborn [29]. Analysis was limited to descriptive statistics.

3. Results

3.1. Causative Organism and Study Setting

A total of 38 studies were included in the analysis. The studies were grouped into two, diarrheagenic *E. coli* (28/38) (Table 1) and faecal carriage (10/38) (Table 2). Generally, *E. coli* isolates recovered from stool samples were characterised into pathotypes or phylogroups

by PCR and/or a combination of PCR and serotyping. ESBL genes were also characterised using PCR and sequencing (Tables 1 and 2). Of the studies that reported on the specific *E. coli* pathotypes, the distribution of pathotypes was as follows: EPEC (82%; 23/28), EAEC (53.6%; 15/28), ETEC (35.7%; 10/28), EIEC (21%; 8/28), EHEC (10.7%; 3/28), STEC (10.7%; 3/28) and none of the studies reported on DAEC pathotype (Table 1). Of the 23 studies that reported on EPEC pathotype, 3/23 studies provided details of typical (tEPEC) and atypical EPEC (aEPEC) [24,30,31].

Most studies on faecal carriage (6/10), generalised the causative organism as *E. coli* while 4/10 studies characterised *E. coli* based on the four phylogroups A, B1, B2 and D (Table 2). Phylogroups A, B1 and D were the most prominent [7,28,32,33] (Table 2).

Most of the studies were conducted at hospitals (25/38), followed by primary health-care centres (6/38) and community settings (2/38). Only a few studies have specified the geographical settings as either urban (13%; 5/38 studies) or rural (5%; 2/38 studies) (Tables 1 and 2).

3.2. Distribution of Studies on CTX-M-Producing *E. coli* by Region

Most of the studies on CTX-M-producing *E. coli* were conducted in countries in Asia (68%; 26/38) compared to studies found in European countries (11%; 4/38) and in countries on the African continent (18%; 7/38). Only one (3%) study was conducted in countries in South America (Figure 2). On the African continent, 11% (4/38) of the studies were conducted in North Africa, 5% of the studies were conducted in West Africa (2/38) and 3% (1/38) of the studies were conducted in Sub-Saharan Africa. In Asia, eight studies were reported from Iran, four studies were reported in India and three studies were reported in China (Tables 1 and 2). Overall, faecal carriage studies were mostly reported in Europe and Africa, while most of the studies in Asia were mainly based on diarrheagenic *E. coli*.

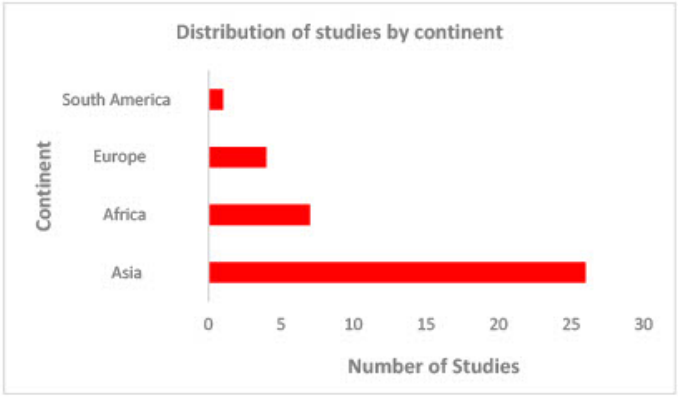

Figure 2. Distribution of studies conducted on CTX-M-producing E. coli in paediatric diarrhoea cases across continents.

Table 1. Summary of studies on *bla*CTX-M-15-producing diarrheagenic *E. coli* recovered from children with diarrhoea across different continents.

Country and Continent	Setting	Design	Age Group	Sample Size	Detection Methods ([1]*E. coli* and/or Pathotype, [2]ESBL Genes)	Causative Organism	% bla_{CTX-M} Reported	CTX-M Genes Detected	Other ESBLs Genes Detected	Study Period	Reference
Brazil, South America	ND	Case–control	0–5	162	[1]PCR, [2]PCR	EPEC, EAEC	15	CTX-M	TEM		[34]
Egypt, North Africa	Hospital	Prospective	0–5	113	[1]PCR, [2]Sequencing	EAEC	4.0	CTX-M	TEM	2016	[35]
Egypt, North Africa	Hospital	Prospective	0–5	320	[1]m-PCR, [1]phylogrouping, [2]PCR	EAEC, tEPEC, aEPEC	37.5	CTX-M-15	TEM	2018–2019	[24]
Burkina Faso, West Africa	Health centre	Retrospective	0–5	ND	[1]m-PCR, [2]m-PCR	EPEC, EAEC	7.1	CTX-M	OXA	2018–2019	[23]
Libya, North Africa	Hospital	Prospective	0–5	290	[1]m-PCR, [2]m-PCR	EAEC, EIEC, EHEC	60	CTX-M-15	CTX-8, CTX-M9	2012	[36]
England, Europe	Primary healthcare	Retrospective	0–16	660	[1]PCR, [1,2]Sequencing	EAEC, ETEC, EPEC, EIEC	ND	CTX-M-15	TEM1, CTXM1, CTX-M14, CTX-M27, SHV12	2015–2017	[37]
India, Asia	Hospital	Prospective	0–5	120	[1]m-PCR, [2]Rt-PCR, [2]Sequencing	EPEC, EAEC, ETEC, EHEC	40	CTX-M	TEM, SHV, OXA, NDM-1, IMP, VIM, ACT, DHA and CMY	ND	[38]
Korea, Asia	Hospital	Prospective longitudinal	Children and infants	ND	[1]m-PCR, [2]m-PCR	EPEC, ETEC, EHEC	16	CTX-M-15	CTX-M14, CTX-M27, CTX-M55, CTX-M3, TEM1, PABL*s*, CMY2, DHA1	2007–2016	[39]
Iran, Asia	Hospital	Descriptive cross-sectional study	0–5	321	[1]m-PCR, [1]serotyping, [2]PCR	EPEC	83.3	CTX-M	TEM	2016–2017	[40]

Table 1. *Cont.*

Country and Continent	Setting	Design	Age Group	Sample Size	Detection Methods ([1]*E. coli* and/or Pathotype, [2]ESBL Genes)	Causative Organism	% bla_{CTX-M} Reported	CTX-M Genes Detected	Other ESBLs Genes Detected	Study Period	Reference
Iran, Asia	Hospital	Prospective	0–92	340	[1]PCR, [2]PCR	STEC	69	CTX-M-9	TEM	2014	[41]
Qatar, Asia	Hospital	Prospective	0–10	175	[1]PCR, [2]PCR	EPEC, EAEC	88.2	CTX-M-15	CTX-M-3	2017–2018	[42]
Iran, Asia	ND	Prospective	0–10	1355	[1]PCR, [2]PCR	EPEC	10.9	CTX-M	TEM, SHV, OXA	ND	[20]
China, Asia	Hospital	Prospective	0–5	684	[1]PCR, [1]Serotyping, [2]PCR, [2]Sequencing	EPEC, EAEC, ETEC, EIEC, STEC	20	CTX-M-15	NDM1, KPC2, TEM1, CTX-M-55, CTX-M14, CTXM-65, CTX-M-137	2015–2016	[3]
Iran, Asia	Hospital	Prospective	0–15	395	[1]PCR, [1]phylogrouping, [2]PCR	ETEC, EPEC	ND	CTX-M	TEM	2014–2015	[43]
India, Asia	Paediatric institute	Prospective and retrospective	0–10	900	[1]PCR, [1]Serotyping, [2]PCR	tEPEC, aEPEC	11.5	CTX-M-15	(NDM-1), (VIM)	2012–2013	[30]
Indonesia, Asia	Hospital	Prospective	0–3	133	[1]PCR, 2PCR, [2]Sequencing	EAEC, EPEC	84	CTX-M-15	TEM-1, SHV	2012	[44]
India, Asia	Hospital	Cross-sectional study	0–5	120	[1]PCR,[2]PCR	tEPEC, aEPEC, ETEC, EIEC	ND	CTX-M	SHV, TEM	2015–2016	[31]
Pakistan, Asia	ND	Cross-sectional	0–5	100	[1]PCR, [1]Sequencing, [2]PCR	EPEC	93	CTX-M	TEM	2016–2017	[45]
Japan, Asia	Clinics	Retrospective	ND	167	[1]PCR, [1]Phylogrouping, [2]PCR, [2]Sequencing	EAEC	79	CTX-M-15	CTX-M14, CTX-M55	1992–2010	[46]
India, Asia	Hospital	Prospective longitudinal	0–14	8891	[1]m-PCR, [2]PCR	ETEC, EAEC, EPEC	30.2	CTX-M3	TEM, SHV, OXA1	2012–2019	[47]

Table 1. *Cont.*

Country and Continent	Setting	Design	Age Group	Sample Size	Detection Methods ([1]*E. coli* and/or Pathotype, [2]ESBL Genes)	Causative Organism	% bla_{CTX-M} Reported	CTX-M Genes Detected	Other ESBLs Genes Detected	Study Period	Reference
Iran, Asia	Hospital	Prospective	0–10	303	[1]m-PCR, [2]PCR	EAEC, EPEC, ETEC, EIEC, STEC	25	CTX-M-15	TEM	2018	[48]
China, Asia	Hospital	Prospective	0–5	1643	[1]PCR, [1]Serotyping, [2]PCR, [2]Sequencing	EPEC	60.3	CTX-M-1	CTX-M9, TEM, SHV	2009	[49]
Iran, Asia	Hospital	Descriptive cross-sectional study	0–81	581	[1]PCR, [2]PCR	EIEC	77.8	CTX-M-15	CTX-M1, TEM1	2016–2017	[50]
China, Asia	ND	Prospective	ND	912	[1]PCR, [2]PCR, [2]Sequencing	ETEC, EPEC, EIEC, EAEC	ND	CTX-M-14	CTX-M79, CTX-M28, TEM	2013–2014	[51]
Iran, Asia	Hospital	Prospective longitudinal	0–10	342	[1]PCR, [1]Serotyping, [2]PCR	EPEC	19	CTX-M-15	TEM, SHV	2011–2013	[4]
Iraq., Asia	ND	Prospective	0–2	656	[1]Serotyping, [2]PCR	EPEC	77.3	CTX-M	TEM, SHV, OXA, AmpC	2009	[52]
Iran, Asia	Referral centre	Prospective	0–14	230	[1]PCR, [1]Serotyping, [2]PCR	EAEC, EPEC, EIEC, ETEC	94.4	CTX-M-15	TEM, AmpC	2015–2016	[53]
Iran, Asia	Hospital	Prospective	0–10	251	[1]PCR, [1]Serotyping, [2]PCR	EPEC	70.6	CTX-M-15	TEM	2015–2016	[54]

ND = no data; DEC= diarrheagenic *E. coli*; EPEC = enteropathogenic *E. coli*; EAEC = enteroaggregative *E. coli*; PCR = polymerase chain reaction; Rt-PCR = real-time PCR; m-PCR = multiplex PCR; tEPEC = typical; aEPEC = atypical; detection methods; Superscript 1 = method for *E. coli* detection, Superscript 2 = method for ESBL detection.

Table 2. Summary of studies on faecal carriage of *bla*CTX-M-15-producing *E. coli* recovered from children with diarrhoea across different continents.

Country and Continent	Setting	Design	Age Group	Sample Size	Detection Methods ([1]*E. coli* and/or Pathotype, [2]ESBL Genes)	Causative Organism	% bla_{CTX-M} Reported	CTX-M Genes Detected	Other ESBLs Genes Detected	Study Period	Reference
South Africa, Sub-Saharan Africa	Community	Prospective longitudinal	0–1	65	[1]Cuture, [2]PCR, [2]Sequencing	*E. coli*	4.9	CTX-M-14	TEM-1, CTX-M-9	ND	[12]
Nigeria, West Africa	Hospital	Prospective	0–5	296	[1]Culture, [2]PCR, [2]Sequencing	*E. coli*	73.3	CTX-M	TEM, SHV	ND	[55]
Libya, North Africa	Clinics	Prospective longitudinal	3–12	243	[1]Culture, [1]Phylogrouping, [2]PCR, [2]Sequencing	DEC: phylogroup B1, D, A and B2	13.4	CTX-M-15	CTX-M1, CTX-M3, TEM, SHV, OXA	2001 and 2007	[28]
France, Europe	Hospital	Prospective	0–16	1118	[1]Culture, [2]PCR, [2]Sequencing	*E. coli*	4.3	CTX-M-15	TEM-24, TEM-19, SHV-5	2010–2011	[56]
Italy, Europe	Community	Prospective	0–6	482	[1]Culture, [1]Phylogrouping, [2]PCR, [2]Sequencing	DEC: Phylogroup A, B1 and D	43	CTX-M	CTX-M1, CTX-M9, CTX-M8, CTX-M2	2011	[33]
Poland, Europe	Hospital	Prospective	0–5	ND	[1]Phylogrouping, [2]PCR	DEC: Phylogroup A, B1, B2 and D	76.6	CTX-M	TEM, SHV	2008–2009	[7]
Iran, Asia	Hospital	Prospective	0–80	216	[1]m-PCR, [1]phylogrouping, [2]PCR	DEC: phylogroup A, D, B1 and B2	25.9	CTX-M-15	OXA1	2013	[32]
Iraq, Asia	Hospital	Prospective cross-sectional	0–8	116	[1]PCR, [2]PCR	DEC	71.4	CTX-M	TEM-1	2019	[2]
Jordan, Asia	Hospital	Prospective	0–1	288	[1]Culture and Biochemical test, [2]PCR, [2]Phylogrouping	*E. coli*	73.2	CTX-M-15	ND	2012	[57]
Malaysia, Asia	Hospital	Prospective	0–5	110	[1]Culture, [2]PCR	*E. coli*	9.1	CTX-M-15	TEM-1, CMY-2	2009–2010	[58]

ND = no data; DEC= diarrheagenic *E. coli*; PCR = polymerase chain reaction; m-PCR = multiplex PCR; *E. coli* = *E. coli* not categorised as DEC; Superscript 1 = method for *E. coli* detection, Superscript 2 = method for ESBL detection.

3.3. Age Distribution

Only 36% (14/38) of the studies reported on the 0–5 years age group, 11% (4/38) of the studies assessed children under the age of 3 years; 40% (15/38) of the studies reported on the age groups between 0 and 16 years and 8% (3/38) of the studies investigated a mixed population between birth and 92 years of age. All the studies reporting a wide age range (0–100 years) were conducted in Iran and Western Asia (Tables 1 and 2).

3.4. Distribution of Studies by E. coli Pathotype

Overall, about 21% (8/38) of studies reported specifically on EPEC. The prevalence of CTX-M producers among the EPEC-positive isolates ranged between 10 and 78%. Only two studies specified the existence of $bla_{CTX\text{-}M\text{-}15}$-positive EPEC isolates (Table 3). Enteroaggregative *E. coli* (EAEC) was investigated in two studies in Asia (Japan) and North Africa (Egypt), respectively. The prevalence of CTX-M producers among the EAEC-positive isolates ranged between 19 and 50%. In both studies, only one CTX-M-15-producing EAEC isolate was observed among all the CTX-M producers [35,46]. On the other hand, STEC was only reported in one study conducted in Asia (Table 1).

Table 3. Summary of studies on CTX-M-producing Enteropathogenic *E. coli* (EPEC) recovered from paediatric diarrhoea cases.

No. of EPEC Isolates	Prevalence of CTX-M Producers (%)	Prevalence of $bla_{CTXM\text{-}15}$	Reference
87	13 (15)	ND	[45]
59	7 (12)	7	[30]
58	31 (56)	ND	[49]
192	21 (11)	ND	[20]
22	17 (77)	ND	[52]
14	10 (71)	ND	[40]
42	8 (19)	8	[4]
17	12 (71)	ND	[54]

ND = No data on *bla*CTX-M-15.

3.5. Prevalence of CTX-M and Other ESBLs

In addition to the CTX-M gene variants, TEM was reported in 79% (30/38) of studies followed by SHV, which was reported in 34% (13/38) of the studies. Another ESBL, which was reported in 18% (7/38) of the studies was OXA, while CMY was reported in 8% (3/38) of the studies. Consequently, while 50% (19/38) of the studies reported on the CTX-M-15 variant, 37% (14/38) of the studies generalised the report as CTX-M. The other variants that were reported as part of the investigation included CTX-M-14 (5%; 2/38), CTX-M-9 (2%; 1/38), CTX-M-1 (2%; 1/38) and CTX-M-3 (2%; 1/38) (Tables 1 and 2). Sequencing of the CTX-M gene was reported in 34% (13/38) of the studies (Tables 1 and 2).

The prevalence of the CTX-M gene including the CTX-M-15 variant ranged between 1% and 94%, and the mean and standard deviation were 48% and 29%, respectively. The lowest prevalence rate was reported in Europe (1% and 4%). In Asia, the lowest rate (9%) was reported in Malaysia while the highest rate (94%) was reported in Iran. The mean rate of CTX-M-producing *E. coli* in Asia was 56%. Of the three common countries reporting on CTX-M-producing *E. coli* in Asia, the highest rate was reported in Iran (94%) followed by China (60%) and India (40%).

In the African continent, the prevalence of CTX-M-producing *E. coli* ranged between 5% and 73%, the lowest rate was reported in Sub-Saharan Africa (South Africa), while the highest rate was reported in West Africa. Most studies (4/7) in Africa were reported in North African countries, Egypt, and Libya. Only three studies reported CTX-M-15-producing *E. coli* associated with diarrhoea in children in Africa. It is evident that recent

information on CTX-M-15-producing *E. coli* is scarce in Africa since only one study was conducted within the last 5 years between 2018 and 2019 [25]. Only one study confirmed the production of ESBL in isolates using the double disc synergy test [25] and only one study used sequencing [29]. There is a huge gap regarding standard approaches to surveillance due to resource constraints in Africa. Nevertheless, all three studies reported a low number of (8–15) CTX-M-15-producing *E. coli* isolates. Commensal isolates have been implicated as CTX-M-15 producers in one study [36] and thus *E. coli* is a prominent reservoir for ESBL genes.

In Europe, the literature on CTX-M-15-producing *E. coli* associated with diarrhoea in children is limited. Only two studies included in this review implemented sequencing to detail the epidemiology of CTX-M-15-producing *E. coli*. The prevalence of CTX-M-producing isolates ranged between 60% and 80%. In the studies included, the most common phylogroups were A, D and B1. The current review observed that CTX-M-producing *E. coli* was prominent among phylogroups A and D [7,28,32]. In addition, the CTX-M-15 variant was mostly associated with phylogroup D [28,32].

4. Discussion

This review describes the epidemiology of CTX-M-producing *E. coli* associated with diarrhoea in children based on studies published between 2012 and 2022. The prevalence of CTX-M gene varied between countries across the continents. The CTX-M gene was more common in Asian countries such as China, Iran and India and the highest prevalence (94%) of CTX-M was reported in Iran among MDR *E. coli* [53].

Most of the studies included in this review were conducted in clinical settings such as hospitals and clinics (Tables 1 and 2). The impact of *E. coli* pathotypes such as EPEC and EAEC in causing hospitalisation of children suffering from diarrhoea has been reported [42]. In developing countries, EPEC is the leading cause of infantile diarrhoea [4]. The latter report explains the current observations in this review that EPEC was the most *E. coli* pathotype investigated for CTX-M resistance genes and to a lesser extent, CTX-M genes were also reported in EAEC and STEC. Thus, the tendency of EPEC and EAEC to carry CTX-M resistance genes is a cause of concern towards the management of diarrhoea in children because CTX-M-producing *E. coli* has been reported to be associated with increased resistance to first-line antibiotics, quinolone antibiotics as well as beta-lactam antimicrobials with an oxyimino side chain such as cephalosporins (cefotaxime, ceftriaxone and ceftazidime) and the oxyimino-monobactam (aztreonam) [33,59].

Understanding the epidemiology of CTX-M-producing *E. coli* is important in clinical practice. This review has shown that very few studies are being conducted in Africa on the surveillance of CTX-M-producing *E. coli* associated with diarrhoea in children. While Africa and Asia are flagged as regions with high morbidity and mortality rates in young children due to diarrhoea, it is important to uncover the epidemiology of antibiotic-resistant bacteria such as ESBL-producing *E. coli* that are more likely to complicate the treatment and management of diarrhoea in children. More studies on phenotypic resistance are conducted in developing countries whereas molecular surveillance of ESBL-producing *E. coli* is lacking [24]. The current review established that Sub-Saharan Africa, which is a hot spot of paediatric diarrhoea, is lagging regarding surveillance of CTX-M-producing *E. coli* unlike in Asia where such studies are being conducted across different regions. The literature suggests that Asian countries where at least 70% of the world population inhabits are epicentres for antimicrobial resistance [60]. A previous review in 2015 also reported that CTX-M-producing *E. coli* is the dominant multi-drug resistant (MDR) *E. coli* in Asian countries [10].

The most common CTX-M gene variant reported in North Africa was CTX-M-15 [28], which agrees with the findings of this study, especially in countries such as Egypt and Libya. No studies from East Africa were identified in this review. On the other hand, only one study from Southern Africa was identified. While East Africa and Southern Africa are key regions of Sub-Saharan Africa, which is known to experience the majority of childhood

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12010171/s1, File S1: Database of studies on CTX-M-Producing *E. coli* between 2012 and 2022.

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