Addis Ababa University

Aklilu Lemma Institute of Pathobiology

Determinants of diarrhea, phenotypic and genomic characterization of *Escherichia coli* isolates from diarrheic and non-diarrheic patients attending public primary health facilities in Addis Ababa and Hossana, Ethiopia

BY Deneke Wolde

A Dissertation submitted to Aklilu Lemma Institute of Pathobiology, Addis Ababa University in partial fulfillment for the Degree of Doctor of Philosophy in Tropical and Infectious Diseases

January, 2025

Addis Ababa, Ethiopia

Addis Ababa University

Aklilu Lemma Institute of pathobiology

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Dedication

This thesis is dedicated to the memory of my beloved mother Mulunesh Erido and my dear father Wolde Solomon. I lost both of them in the midst of this journey. Their unwavering love, support, and guidance have been in shaping the person I am today. Though they are no longer with me, their prayers, blessing and wishes remain with me. I am forever grateful for the sacrifices they made and the lesson they taught me. This work is a tribute to their enduring influence and the precious time we shared together.

Statement of author

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I hereby declare, that the submitted thesis entitled "**Determinants of diarrhea, phenotypic and genomic characterization of *Escherichia coli* isolates from diarrheic and non-diarrheic patients attending public primary health facilities in Addis Ababa and Hossana, Ethiopia**" is the result of my own work and all materials used as a sources for this work have been duly acknowledged. The submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

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Abstract

Determinants of diarrhea, phenotypic and genomic characterization of *Escherichia coli* isolates from diarrheic and non-diarrheic patients attending public primary health facilities in Addis Ababa and Hossana, Ethiopia

Deneke Wolde

Addis Ababa University, 2024

Background: Diarrheal diseases are significant public health challenge in low- and middle-income countries. *Escherichia coli* (*E. coli*) is a common cause of diarrhea. It is genetically diverse bacterium, characterized by different strains that are classified into various pathotypes based on their virulence. Many strains of *E. coli* carry mobile genetic elements (MGEs) that encode antimicrobial resistance genes (ARGs) and virulence factors, thus improving their adaptability and survival. The genetic diversity and antimicrobial resistance characteristics of *E. coli* in Ethiopia remain poorly studied despite their important role in human diseases.

Objectives: to assess determinants of diarrhea, investigate antimicrobial resistance characteristics of *E. coli* isolates, and assess the genetic diversity of *E. coli* isolated from stool samples of patients attending public primary healthcare facilities.

Methods: This study was conducted from December 2021 to September 2022 at health facilities in Addis Ababa and Hossana. We used a health facility based matched case-control and cross-sectional study designs. Participants, cases (366 patients with diarrhea) and controls (366 patients without diarrhea) were recruited by convenience sampling technique for assessing determinants of diarrhea, and conditional logistic regression model was used in Stata version 14.0 to examine the determinants of diarrheal diseases. Conventional culture techniques and biochemical tests were used for the isolation and preliminary identification of *E. coli* isolates. A total of 260 E. coli isolates, confirmed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry, were used for further analysis. Phenotypic antimicrobial resistance was determined using the disc diffusion method according to the guidelines of the European Committee on Antimicrobial

Susceptibility Testing (EUCAST). Real-time PCR tests were used to identify the diarrheagenic *E. coli* (DEC) pathotypes. Selected *E. coli* isolates were then subjected to whole genome sequencing (WGS) on the Illumina platform and bioinformatic analysis was performed to characterize the *E. coli* isolates.

Results: Statistical analysis revealed that being partially vaccinated (AOR: 2.70; 95%CI: 1.2, 5.9), use of tap water (AOR: 2.20; 95%CI: 1.1, 4.4), use of protected well/spring water for drinking (AOR: 13.90; 95%CI: 3.7, 51.5)**,** overcrowded sleeping arrangement (AOR: 1.55; 95%CI: 1.2, 1.8), having contact with animal feces/food **(**AOR: 15.10; 95%CI: 4.2, 53.6**),** the cleaning frequency of water-fetching materials (i.e. cleaned sometimes (AOR: 2.40; 95%CI: 1.2, 4.5) and rarely (AOR: 3.03; 95%CI: 1.2, 7.4)), and use of open pit latrine (AOR: 5.61; 95%CI: 1.5, 21.0) were significantly associated with increased odds of having diarrhea. The overall detection rate of DEC-specific virulence-associated genes (VAGs) was 11.5% among the *E. coli* isolates tested. Four common pathotypes were identified, with enteropathogenic *E. coli* (EPEC) being the predominant pathotype. A hybrid pathotype carrying virulence factors from necrotoxic *E. coli* (NTEC2) and Shiga toxin-producing *E. coli* (STEC) strains was detected using whole-genome sequence (WGS) analysis. The studied isolates were distributed across seven phylogenetic groups (A, B1, B2, C, D, E and G). The predominant (49%) *E. coli* isolates belonged to the phylogenetic group B1. The DEC isolates were observed in four of the identified phylogenetic groups. The isolates belonged to 36 different sequence types (STs) and 42 different serotypes, of which 8 isolates (15.7%) were categorized as 'rough,' including five DEC isolates from the pathotypes STEC, EPEC and enterotoxigenic *E. coli* (ETEC). The isolates carried a total of 131 VAGs, including DEC specific genes (*eae*, *aggR*, *eltIAB-8*, *eltIAB-11*, *estah-STa3*, *stx1*, *stx2*). Overall 54.2% of *E coli* isolates were resistant to at least one antimicrobial agent tested. The highest resistance (52.7%) was observed for ampicillin. Out of all isolates, 19.2% were multidrug-resistant (MDR), and 8.5% were extended-spectrum β-lactamase (ESBL) producers. Our results indicated that 82.4% of the sequenced *E. coli* isolates harbored 46 different ARGs that confer resistance to different antimicrobials. The $bla_{TEM\text{-}1B}$ gene was the most commonly detected β-lactamase-encoding

gene in both DEC and non-DEC isolates. No resistance to any of the tested antimicrobial was detected in STEC isolates and no ARGs were detected in these isolates. Seventy percent of the isolates resistant to third-generation cephalosporins and 78.9% of the phenotypically confirmed ESBL-producing *E. coli* isolates carried the $bla_{CTX\text{-}M\text{-}15}$ gene. Mutations in the *gyrA, parC*, and/or *parE* genes were observed in 31.4% of the tested isolates. *E. coli* isolates from all phylogenetic groups contained various ARGs, with the highest frequencies being in phylogroups A and B1. In contrast, *E. coli* isolates from phylogenetic group B2 contained only four ARGs. Except for three isolates, all tested *E. coli* isolates were found to carry between one and eleven plasmids. The IncF group of plasmids with different replicon types and variants was the predominant plasmid group. Additionally, genetic determinants associated with conjugative transfer were identified in 56.9% of the isolates. The IS*Ec1* and IS*609* were the most frequently detected IS elements in the tested *E. coli* isolates. Transposons such as Tn*2*, Tn*7*, Tn*5403*, Tn*6024*, and Tn*6082* were detected in *E. coli* isolates tested.

Conclusion: The occurrence of diarrheal diseases was influenced by various factors, including the vaccination status of children, drinking water sources, crowded sleeping arrangements, exposure to animal feces or food, occasional or infrequent cleaning of water collection items, and the absence of toilet facilities or reliance on open latrines. Considerable genetic diversity was observed among *E. coli* isolates. The isolates had a broad spectrum of antimicrobial resistance and a high proportion of self-transferable conjugative elements, indicating a significant potential for horizontal gene transfer and the spread of clinically important virulence and antibiotic resistance traits.

Recommendations: Implementation of strategies to control the infection and spread of this health threat is essential. Enhanced surveillance covering a large geographical area will help to address this growing public health challenge.

Keywords: Diarrhea, *Escherichia coli*; antimicrobial resistance; genetic diversity; phylogeny, antimicrobial resistance genes; virulence-associated genes; mobile genetic elements; phylogenetic groups; ESBL; whole-genome sequencing; Ethiopia

Acknowledgments

First of all, I would like to praise the Almighty God for all His blessings and perseverance in overcoming all challenges. I would like to express my deepest gratitude to my supervisor Prof. Tadesse Eguale. I cannot find enough words to thank him for his dedicated and highly qualified support, understanding and professional guidance. His advice and encouragement gave me the confidence to think critically and laid the foundation for independent work. Without his close guidance, it would have been difficult to complete this work. It is a great privilege to have him as a supervisor.

I would also like to thank Prof. Marjanca Starčič Erjavec for her close supervision and invaluable guidance throughout the course of this work. Her commitment and dedication were truly incredible and I am deeply indebted for her guidance. I am incredibly grateful that she made it possible for me to come to Slovenia and perform laboratory research in the laboratories at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, Institute of Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, and National Laboratory of Health, Environment and Food in Ljubljana, Slovenia to conduct the necessary tests.

I am grateful for the support and encouragement from my co-supervisors Dr. Girmay Medhin and Dr. Aklilu Feleke. I would like to sincerely thank them for their encouragement and professional guidance, especially during the development of the research protocol and also during the writing up process.

I would also like to thank Marko Erjavec for his warm hospitality and kindness. His kind presence and generosity during my stay in Slovenia were a source of comfort and encouragement. I will always remember his kindness, especially during my first and last days in the country.

My sincere gratitude goes to Dr. Mateja Pirs, Dr. Katja Strašek Smrdel from the Institute of Microbiology and Immunology, Faculty of Medicine, Dr. Jana Avberšek, Dr. Darja Kušar

from the Institute of Microbiology and Parasitology, Faculty of Veterinary Medicine, and Dr. Tjaša Cerar Kišek, Ms. Tea Janko, and Dr. Andrej Steyer from the National Laboratory for Health, Environment, and Food in Slovenia. Their invaluable contributions during the laboratory work and writing manuscripts were immeasurable. Furthermore, I would like to express my sincere gratitude to Nataša Krošelj, Brina Kovačič, Ivana Bohinc, Veronika Katarina Juren, Nuša Koblar and Marko Kolenc, the staff of the Institute of Microbiology and Immunology, University of Ljubljana, for their support throughout the laboratory work. I am deeply indebted to Tom Koritnik from the National Laboratory of Health, Environment, and Food, Ljubljana, Slovenia for his help with DNA sequencing of the isolates.

I would like to sincerely acknowledge the patients who participated in the study, as well as the data collectors. I gratefully acknowledge Mr. Eyosias Sileshi, Mr. Kehabtimer Shiferaw, Dr. Woinshet Hailu, and Mr. Haile Alemayehu for being friendly with me and providing technical support.

I would also like to extend my heartfelt gratitude to my sisters—Selamawit, Belaynesh, and Genet—and my brothers—Samuel, Tesfaye, Muluneh, Fekadu, and Melaku. Each of you has contributed in your own way to my success, and I truly appreciate your support.

Finally, special thanks to my wife Genet Asfaw for her support and encouragement throughout the hard times to make this achievable. Her patience, understanding, and belief in me were instrumental in helping me achieve this goal. I couldn't have done this without her by my side every step of the way. I am deeply grateful for her love.

List of original papers

This dissertation is based on the following three published papers and one paper accepted for publication. They are referred in the text by Paper 1, Paper 2, Paper 3, and Paper 4:

1. **Wolde D**.; Medhin G.; Alemayehu H.; Tilahun G.A.; Kotiso K.S.; Hailu W.; Mihret A.; Regessa F.; Haile A.F.; Eguale T. Determinants of diarrheal diseases among patients attending public health centers in Addis Ababa and Hossana, Ethiopia: A matched case control study (accepted for publication at Tropical medicine and Health journal).

2. **Wolde D**.; Eguale T.; Alemayehu H.; Medhin G.; Haile A/F.; Pirs M.; Strašek Smrdel, K.; Avberšek J.; Kušar, D.; Cerar Kišek T.; Janko T; Steyer A.; Starčič Erjavec M. Diarrheagenic *Escherichia coli* in stool specimens collected from patients attending primary healthcare facilities in Ethiopia: whole-genome sequencing based molecular characterization. Int. J. Mol. Sci. 2024, 25, 10251. https://doi.org/10.3390/ijms251910251

3. **Wolde D**.; Eguale T.; Alemayehu H.; Medhin G.; Haile A.F.; Pirs M.; Strašek Smrdel, K.; Avberšek J.; Kušar, D.; Cerar Kišek T.; Janko T; Steyer A.; Starčič Erjavec M. Antimicrobial susceptibility and characterization of extended-spectrum β-lactamase-producing *Escherichia coli* isolated from stools of primary healthcare patients in Ethiopia. Antibiotics 2024, 13, 93. https://doi.org/10.3390/antibiotics13010093

4. **Wolde D**.; Eguale T.; Alemayehu H.; Medhin G.; Haile A.F.; Pirs M.; Strašek Smrdel, K.; Avberšek J.; Kušar, D.; Cerar Kišek T.; Janko T; Steyer A.; Starčič Erjavec M. Genomic characterization of extended spectrum β-lactamase producing and third-generation cephalosporin resistant *Escherichia coli* isolated from stools of Primary Healthcare Patients in Ethiopia. Antibiotics 2024, 3,851. https://doi.org/10.3390/antibiotics13090851

Table of Contents

Approval sheet ii

Statement of author iii

Abstract iv

Acknowledgments viii

List of Figures xiv

List of Tables xv

Acronyms and abbreviations xvi

Chapter 1: Introduction 1

1.1 Background 1

1.2 Statement of the problem 7

1.3 Significance of the study 9

1.4 Objectives 11

General objective 11

Specific objectives 11

2 Literature review 12

2.1 Determinants of diarrhea 12

2.2 Biology of *Escherichia coli* 13

2.3 Diarrheagenic *E. coli* and other enteropathogens 16

2.4 General characteristics of DEC 19

2.5 Genomic diversity of *Escherichia coli* 23

2.6 Antibiotic-resistant *Escherichia coli* 29

2.7 *Escherichia coli* detection and characterization techniques 32

3 Materials and Methods 36

3.1 Study setting 36

3.2 Study design and study participants 37

3.3 Recruitment of study participants 38

3.4 Sample size estimation 39

3.5 Data collection methods and instruments 39

3.5.1 Demographic data 39
3.5.2 Anthropometric measurements 39
3.5.3 Stool specimen collection and laboratory procedures 40
3.5.4 Isolation and identification of *Escherichia coli* 40
3.5.5 Antimicrobial susceptibility testing 41
3.5.6 DNA extraction and PCR detection of *E. coli* pathotypes genes 41
3.5.7 Whole-genome sequencing 42
3.6 Bioinformatic analysis 42
3.7 Data quality and management 42
3.8 Statistical analysis 43
3.9 Ethical considerations 43
Operational definitions 44
4 Results 45
4.1 Socio-demographic and socio-economic characteristics of study participants 45
4.2 Environmental and behavioral characteristics 47
4.3 Determinants of diarrheal disease among patients attending public health facilities 49
4.4 Molecular identification of DEC isolates 50
4.5 Whole-genome sequencing based detection of DEC isolates 51
4.6 Genetic characteristics of *Escherichia coli* 52
4.6.1 Phylogenetic groups of *E. coli* isolates 52
4.6.2 Sequence types of *E. coli* isolates 53
4.6.3 Serotypes of *E. coli* isolates 54
4.6.4 Virulence-associated gene profile of *E. coli* isolates 56
4.7 Antimicrobial susceptibility of *E. coli* isolates 58
4.8 Phenotypic characterization of ESBL-producing *E. coli* isolates 63
4.9 Antimicrobial resistance genes 63
4.10 Co-occurrence of antimicrobial resistance genes 68
4.11 Plasmids and other mobile genetic elements 68
4.12 Determination of conjugative transferable elements 71

5 Discussion 72
6 Conclusion and recommendations 83
7 References 85
Annexes 126
Annex I. English Version Participants' information sheet 126
Annex II. Amharic Version Participants' information sheet 128
Annex III. English Version Study participant consent form 129
Annex IV. Amharic Version Study participant consent form 130
Annex V. Amharic version Questionnaire 135

List of Figures

Figure 1: Structure of cell envelope. 15

Figure 2: Possible *E. coli* infections sites in humans by different pathotypes 20

Figure 3 The phylogenetic diversity of *E. coli* 24

Figure 4: Mechanisms of antimicrobial resistance 30

Figure 5: Maps of Addis Ababa and Hossana, Ethiopia. 37

Figure 6: Flow diagram. 34

Figure 7: Phylogenetic tree of 51 WGS-confirmed *E. coli* isolates. 55

Figure 8: Number of *E. coli* isolates harboring certain plasmid types. 69

Figure 9: Number of *E. coli* isolates harboring certain types of transposable elements. 70

List of Tables

Table 1:Sample size/isolates that contributed data for the analysis and write-up of various sections of the thesis 45

Table 2: Socio-demographic characteristic of study participants from Addis Ababa city and Hossana town, Ethiopia 46

Table 3: Environmental and behavioral characteristic of study participants from Addis Ababa city and Hossana town, Ethiopia 47

Table 4: Determinants of diarrheal disease among patients attending public health facilities in Addis Ababa city and Hossana town, Ethiopia 49

Table 5: Distribution of DEC isolates determined by PCR among study sites and patient's characteristics 51

Table 6: Distribution among study sites and patient's characteristics of DEC isolates.....52

Table 7: Distribution of different *E. coli* phylogenetic groups among patients from study sites and their relations with other patients' characteristics 53

Table 8: Antimicrobial susceptibility of studied *E. coli* isolates 59

Table 9: Antimicrobial resistance patterns of *E. coli* isolates.....61

Table 10: Distribution of antimicrobial resistance genes and chromosomal mutations among studied *E. coli* isolates 66

Acronyms and abbreviations

ALIPB	Aklilu Lemma Institute of Pathobiology
AMR	Antimicrobial resistance
ARGs	Antimicrobial resistance genes
DAEC	Diffusely adherent *E. coli*
DEC	Diarrheagenic *E. coli*
EMB	Eosin methylene blue
EAEC	Enteroaggregative *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extra-intestinal pathogenic *E. coli*
HGT	Horizontal gene transfer
HE	Hektoen Enteric
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight
MDR	Multi-drug resistant
MLST	Multilocus sequence typing
MGE	Mobile genetic elements
PAIs	Pathogenicity islands
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
STs	Sequence types
STEC	Shiga toxin-producing *E. coli*
T4CP	Type IV Coupling Protein
T4SS	Type IV secretion system
UTIs	Urinary tract infections
WGS	Whole-genome sequencing
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

Chapter 1: Introduction

1.1 Background

Diarrhea is one of the most common clinical signs of gastrointestinal diseases in people of all age groups and regions of the world (Troeger *et al.*, 2018). It is characterized by the passage of abnormally loose or watery stools more frequently than usual within a 24-hour period. It may be accompanied by nausea or vomiting, along with abdominal pain or systemic symptoms such as fever (World Health Organization, 2005).

Infectious diarrhea is usually transmitted through the fecal-oral route, from contact with an infected person or ingestion of contaminated food and drinks (World Health Organization, 2024). It is also acquired via contact with a contaminated surface or animals carrying the pathogens and their contaminated environments (Getachew *et al.*, 2024). It is caused by a wide variety of bacterial, viral, and parasitic pathogens (Riley and Bass, 2008). A single or multiple microbial agents could cause diarrhea (Walker *et al.*, 2010). Diarrhea can also be caused by malabsorption, lactase deficiency, antibiotic or other drug therapy which alters the normal intestinal microbiota, gluten intolerance (Cheesbrough, 2003) and non-gastrointestinal tract illness, including meningitis, septicemia, malaria and bacterial pneumonia (Reisinger *et al.*, 2005).

Pathophysiological mechanisms responsible for the alterations in fluid and electrolyte movement associated with diarrhea are increased luminal osmolality, decreased fluid absorption, increased intestinal secretion, and altered intestinal motility (Binder, 1990; Fernández-Bañares *et al.*, 2016). Diarrhea can be classified into acute and chronic; secretory, osmotic, inflammatory; infectious and non-infectious (Joseph *et al.*, 2017). Whatever the cause or classification, diarrhea results in loss of water and electrolytes and may lead to death due to complications which include dehydration, impaired renal function, acidosis, secondary infections, disseminated intravascular coagulation and hypotension (Yu *et al.*, 1991). Malnutrition is also an important complication of diarrhea, resulting from reduced

feeding and impaired ability to absorb nutrients in the face of increased nutrient requirement as a result of infection during the diarrheic phase (World Health Organization, 2005).

Factors determining the occurrence of this problem are complex, and the relative contribution of each factor varies as a function of interaction between socioeconomic, environmental, and behavioral variables (Boithias *et al.*, 2016; Graf *et al.*, 2008). About 90% of its occurrence is associated with unsafe drinking water and poor sanitation (Melese *et al.*, 2019). More than 2 billion people worldwide lack access to basic sanitation (WHO and UNICEF, 2017; World Health Organization, 2022a), and Ethiopia has the lowest access to safe drinking water and sanitation among sub-Saharan African countries (Gedamu, 2017; Shine *et al.*, 2020). According to the 2019 mini-Ethiopia Demographic and Health Surveys (EDHS), access to basic sanitation services in Ethiopia is only 7.5% (Desye *et al.*, 2023). In 2023, access to basic sanitation and hand-washing facilities in Hossana was reported at 35.1% and 16.8%, respectively (Aydamo *et al.*, 2023). The majority of the population in Addis Ababa lives in polluted environments, exposing them to water and sanitation-related diseases (Beyene *et al.*, 2015). A study conducted in Addis Ababa revealed that 53% of surface water sources and 62% of water source catchments are classified as having very high-risk and high-risk contamination levels, respectively (Sisay *et al.*, 2024). In addition, poor access to latrine, poor housing, crowding, dirty floor, poor food storage condition and cohabitation with domestic animals can result in diarrheal diseases (Agegnehu *et al.*, 2019).

Intestinal pathogenic *Escherichia coli* (*E. coli)*, often designated also as diarrheagenic *E. coli* (DEC) is one of the most common cause of diarrhea among the bacterial etiologies (Spano *et al.*, 2017). This group of bacteria are responsible for about 30–40% of acute diarrhea episodes in children (Ramya *et al.*, 2017). They also play a considerable role in causing diarrhea in adults (Spano *et al.*, 2017). They are classified as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC) (Moyo *et al.*, 2007; Ramadan *et al.*, 2016; Spano *et al.*, 2017). These pathotypes can also be

categorized based on similarities in their pathogenic mechanisms. For example, EPEC and EHEC are grouped as pathogens that induce intestinal lesions known as attaching and effacing lesions (A/E lesions) and encode a type III section system (T3SS) that translocates effector proteins into the infected enterocyte. EHEC is differentiated from EPEC by the production of Shiga toxin, which is cytotoxic and linked to the life-threatening infections, such as hemolytic uremic syndrome (Hartland and Leong, 2013). ETEC and EAEC are common causes of travelers' diarrhea (Zboromyrska *et al.*, 2014). ETEC is characterized by the production of heat-labile enterotoxin (LT) and/or heat-stable enterotoxin (Zhang *et al.*, 2022), while EAEC is identified by its aggregative adherence phenotype on HEp-2 cells (Govindarajan *et al.*, 2020). EAEC can cause enterotoxic and cytotoxic effects, such as dilation of intestinal crypts, rounding of enterocytes, and extrusion (Boisen *et al.*, 2009). EIEC is closely related to *Shigella* spp. and possesses a virulence plasmid (pINV), which is crucial for its invasive characteristics (Miles *et al.*, 2023).

Genetic exchange among various strains promotes the emergence of new pathogenic strains. Hybrid DEC pathotypes harboring specific virulence genes that are characteristic of two or more pathotypes have emerged due to horizontal gene transfer (HGT) among DEC pathotypes in the past few decades. The first hybrid pathotype (EAEC/EHEC or EAHEC) with genetic recombination was reported in Germany in 2011 (Santos *et al.*, 2020).

Pathogenic *E. coli* strains follow a multi-step process of pathogenesis that includes colonizing a mucosal site, evading host defenses, multiplying, and causing damage to the host (Kaper *et al.*, 2004). Even though all pathotypes contribute to diarrhea, they vary considerably with their mechanism of colonization, the presence of diverse virulence factors encoded by specific virulence-associated genes (VAGs), symptoms and clinical outcomes. Different DEC colonize various sites in the human gut. For example, EPEC, ETEC, and DAEC colonize the small intestine, whereas EHEC and EIEC colonize the large intestine; EAEC can colonize both the small and large intestines (Croxen and Finlay, 2010).

E. coli exhibits extreme genomic diversity, reflecting its ability to adapt to various environmental conditions. The *E. coli* pangenome comprises the core genome, which consists of essential genes present in all strains, as well as accessory genes, that are found only in some strains (Hall *et al.*, 2021). The evolution of the *E. coli* pangenome is influenced by the differential acquisition and loss of accessory genes, which can affect the variation in the overall genome sequence. The core genome of this bacterium contains less than 1,000 genes, whereas the accessory genome comprises tens of thousands of genes (Lukjancenko *et al.*, 2010). The size of the *E. coli* pangenome changes over time, with a notable trend of decreasing core genome size. A recent report shows that the pangenome of *E. coli* contains a total of 43,415 genes, including 867 core genes (Yang *et al.*, 2019).

Normally a harmless commensal *E. coli* needs to acquire virulence factors and effectors that can subvert the functions of host cells and become a highly adapted pathogen capable of causing a range of diseases (Leimbach *et al.*, 2013). These virulence factors aid the bacterium in adhesion, invasion, iron acquisition, toxin activity and evasion of the host cell (Pakbin *et al.*, 2021). Many of the virulence factors associated with *E. coli* mediated disease are frequently encoded on genetic elements such as plasmids, bacteriophage, transposons and pathogenicity islands (PAIs) that can be mobilized into different strains to create novel combinations of virulence factors (Kaper *et al.*, 2004). PAIs are a large clusters of virulence genes that can be found on plasmids or integrated into the chromosome in pathogenic bacteria, but they are not found in non-pathogenic bacteria (Desvaux *et al.*, 2020). It is where many of the virulence traits are organized in *E. coli* along with plasmids and prophages (Croxen and Finlay, 2010).

The genetic structure of *E. coli* strains can be characterized using various methods, depending on the specific attribute or characteristic of the bacteria that is being examined. *E coli* are classified in eight phylogroups (A, B1, B2, C, D, E, F, and G) based on the presence or absence of *chuA*, *yjaA*, *TspE4.C2*, and *arpA* genes (Clermont *et al.*, 2019). The distribution of bacterial phylogenetic groups is influenced by environmental factors as well as the

characteristics of their host organisms, such as the host's diet, gut structure, and body mass (Tenaillon *et al.*, 2010). Although *E. coli* can be found across various groups, certain pathotypes are often associated with specific phylogroups. Majority of strains responsible for intestinal infections are found in phylogroups A and B1, while extra-intestinal pathogenic *E. coli* (ExPEC) strains are clustered mostly in group B2 and, to a lesser extent, in group D. (Escobar-Páramo, Clermont, *et al.*, 2004).

E. coli are further characterized into more specific groups known as clonal complexes and sequence types by multilocus sequence typing (MLST) method. It involves sequencing of housekeeping genes located at specific loci throughout the bacterial chromosome to assign isolates into different sequence types (STs) based on the combination of alleles. Comparing the sequences of these conserved genes provide a more detailed understanding of the genetic relationships and diversity within a bacterial species (Uelze *et al.*, 2020).

Multiple modalities including antibiotic and non-antibiotic therapies have been used to manage infections of *E. coli*. Antibiotics are advised for invasive intestinal or complicated infections or for treatment of *E. coli* infections outside the intestines, such as urinary tract infections (UTIs) or bloodstream infections (Degebasa *et al.*, 2018). However, treatment for *E. coli* infection has become increasingly complicated due to the emergence of resistance to most antimicrobial agents, resulting in significant morbidity and mortality. Overuse and misuse of antimicrobial agents causes this crisis.

Antimicrobial resistance (AMR) poses a threat to individuals of all ages and sex (Michael *et al.*, 2014). Bacteria may be intrinsically resistant to one or more classes of antimicrobial agents due to inherent structural and functional characteristics, as well as the expression of efflux pumps that efficiently reduce the intracellular concentration of antibiotics (Zeinab *et al.*, 2023). They can also acquire resistance either by mutation of endogenous genes or through the acquisition of resistance genes from other resistant organisms carried on mobile genetic elements (MGEs) (Cesur and Demiroz, 2013; Reygaert, 2018). MGEs can either move within a single cell (e. g. insertion sequences, transposons, and gene

cassettes/integrons) or can be transferred between bacterial cells through self-replication and conjugative elements (e. g. conjugative plasmids and integrative conjugative elements) (Partridge *et al.*, 2018).

The spread of antimicrobial resistance genes (ARGs) in bacteria can occur through both vertical gene transfer (VGT) and horizontal gene transfer (HGT) mechanisms. Vertical gene transfer involves the transmission of these genes from parent to offspring during bacterial division, whereas HGT refers to gene exchange between non-related organisms. Typical mechanisms of HGT are conjugation, transduction, and transformation (Tao *et al.*, 2022). Conjugation is fundamentally important process that facilitates a rapid evolution and adaptation of bacterial strains in diverse environments by enabling the spread of various metabolic traits, such as virulence, biofilm formation, resistance to heavy metals, and antibiotic resistance. Genes needed for the conjugation process, e. g. genes encoding proteins for the conjugative pilus, type IV secretion system (T4SS), and relaxosome, are clustered in the transfer (*tra*) region of conjugative plasmids. The specific DNA sequence, which is cut prior the gene transfer of the single-stranded DNA, is located in the so called *oriT* site. The DNA transfer from the donor cell into the recipient cell happens through a connection with the Type IV coupling protein (T4CP) (Virolle *et al.*, 2020).

There is a scarcity of data regarding the genomic characteristics of *E. coli*, as well as information on molecular data on virulence determinants and markers of antimicrobial resistance in *E. coli* from developing countries like Ethiopia. Therefore, the aim of this study was to assess determinants of diarrhea, to determine the antimicrobial susceptibility of *E. coli* and to perform genomic characterization of DEC isolates and of phenotypically confirmed ESBL-producing and/or third-generation cephalosporin-resistant *E. coli* isolates obtained from stool samples of patients visiting primary healthcare facilities in Addis Ababa and Hossana, Ethiopia, using whole-genome sequencing.

1.2 Statement of the problem

Diarrheal disease is a major public health problem throughout the world. It is responsible for considerably high morbidity and mortality and is among the leading causes of outpatient visit, hospitalization and the global year of life lost (YLL) in people of all ages (Naghavi *et al.*, 2017). It is responsible for more deaths than gastrointestinal cancers, peptic ulcer, or inflammatory bowel disease (Casburn-Jones and Farthing, 2004). Approximately 1.6 million deaths occur each year globally due to diarrhea with the highest burden in developing countries (Troeger *et al.*, 2018). Globally, diarrhea contributed to 15% of all under-five deaths, making diarrheal disease the second leading cause of death next to respiratory disease in the youngest members of society (Black *et al.*, 2010; Lomazzi *et al.*, 2014). It was recognized as a major cause of death in children living in low-and middle income countries in 1970s (Snyder and Merson, 1982). Of all child deaths from diarrhea, 78% occur in the African and Southeast Asian regions (Boschi-Pinto *et al.*, 2008; Farthing *et al.*, 2013; Johansson and Wardlaw, 2009). In these regions, diarrhea accounts for one in eight deaths among children younger than 5 years per annum (Kotloff, 2017). This disease was among the top leading causes of the high disease burden in Ethiopia, both nationally and regionally (Alebel *et al.*, 2018; Gessese *et al.*, 2023; National Data Management Center for Health, 2021).

Pathogenic *E. coli* significantly affects public health and incurs billions of dollars each year across the globe. The worldwide burden of diarrheal diseases associated with DEC pathotypes is astonishing, affecting hundreds of millions of people annually. Pathogenic *E. coli* causes over 160 million cases of diarrhea and one million deaths per year (Wirth *et al.*, 2006). Globally, STEC O157:H7 causes more than 2.8 million acute illnesses annually, with an incidence rate of 43.1 cases per 100,000 people per year. Among those, a total of 10,200 cases of STEC infections occur in Africa with an incidence rate of 1.4 cases per 100,000 people per year (Majowicz *et al.*, 2014).

The evolution and dissemination of antimicrobial resistant bacteria is one of the greatest challenges of the 21st century, with the most severe impact on low- and middle-income countries (Hernando-Amado *et al.*, 2019). The WHO global report on AMR indicates that resistance to common antimicrobials among major bacterial pathogens has reached alarming level in many parts of the world indicating that many of the available treatment options for common infections in some settings are becoming ineffective, posing a therapeutic challenge, leading to increased healthcare costs, treatment failure, morbidity, and mortality (World Health Organization, 2022b). According to WHO report, at least 700,000 people die each year due to antimicrobial-resistant pathogens, with Sub-Saharan Africa and South Asia having the highest attributable death rates from resistant pathogens (World Health Organization, 2014). In 2021, an estimated 4.71 million deaths were associated with AMR in a systematic analysis of the global burden of bacterial AMR from 1990 to 2021, and it is projected that by 2050, about 1.91 million deaths will be attributable to AMR and about 8.22 million deaths will be associated with AMR (Naghavi *et al.*, 2024). The progress in AMR has outpaced the discovery of new antibiotics. The world is approaching a "post-antibiotic era," mainly due to sporadic rise in AMR and lack of new antimicrobials being introduced to combat this issue (Chakraborty *et al.*, 2022).

Antimicrobial resistance in enteric pathogens is of utmost importance in the developing world, where infectious diseases are prevalent (Kowalski *et al.*, 2024). The rise of antibiotic-resistant *E. coli* poses a significant threat to global public health, highlighting the need for proper monitoring to assess its extent and inform effective mitigation strategies. It is one of the "priority pathogens" on the WHO list of antimicrobial-resistant pathogens that pose the greatest threat to human health (World Health Organization, 2021). *E. coli* was the leading cause of AMR related infections, responsible for 829,000 deaths associated with AMR and 219,000 deaths directly attributable to it in 2019 (Murray *et al.*, 2022). In 2021, *E. coli* caused 33,100 deaths attributable to third-generation cephalosporin resistance and 34,100 deaths attributable to fluoroquinolone resistance globally (Naghavi *et al.*, 2024). The resistance of *E. coli* to commonly used antimicrobials has led to a reliance on last-resort antimicrobial

classes, such as carbapenems, leading to increasing rate of resistance to these antibiotics as well (Elshimy, 2022).

Increased resistance of infectious agents to commonly used antimicrobials has been noted in Ethiopia (Alemu *et al.*, 2019; Asrat, 2008; Assefa and Girma, 2019; Gebresilasie *et al.*, 2018; Lamboro *et al.*, 2016). In a recent review, over 50% of *E. coli* isolates exhibit resistance to widely used antimicrobials, including third-generation cephalosporins (Berhe *et al.*, 2021). In the meta-analysis prevalence of *E. coli* resistance varied across different regions of Ethiopia (Tuem *et al.*, 2018). This highlights the importance of studying antimicrobial resistance in *E. coli* to effectively combat its threat. Therefore, this study was designed to explore aforementioned problems.

1.3 Significance of the study

Assessing the determinants of this disease in different geographic locations with varying socioeconomic and demographic characteristics among all age groups is important to develop appropriately targeted prevention and control strategies.

E. coli represents a complex group of bacteria, including both pathogenic and commensal strains. This diversity poses a major public health challenge, as pathogenic strains can cause serious health problems, especially in developing countries (Kalule *et al.*, 2023; Ramos *et al.*, 2020). They have an accessory genome that varies widely between strains and contains numerous MGEs (Zenebe, Sisay and Belay, 2024). Each *E. coli* pathotype has distinct pathogenicity mechanisms and a unique profile of virulence factors encoded by specific gene clusters (Pakbin *et al.*, 2021). Few studies have investigated the prevalence and genetic structure of *E. coli* in Ethiopia using PCR (Addisu *et al.*, 2022; Belina *et al.*, 2023; Getaneh *et al.*, 2021; Zelelie *et al.*, 2023; Zenebe, Sisay, Belay, *et al.*, 2024). However, to the best of our knowledge, no previous study has properly described the genetic structure and genetic markers associated with virulence and resistance in *E. coli* isolated from human stool based on whole-genome sequencing (WGS) in Ethiopia. Understanding the genetic factors that determine a strain's ability to cause infection, as well as the genetic diversity within *E. coli*

populations provides insights into their evolutionary relationships that are crucial for tracking outbreaks and predicting resistance patterns. Identifying and characterizing this bacterium can also help to understand and devise strategies to disrupt transmission pathways, address specific risk factors associated with these pathogens, and provide important insights for the development of therapeutic interventions. In this study, the DEC pathotypes as well as their genetic diversity was investigated in terms of phylogenetic groups, serotypes, STs, VAGs and ARGs using WGS analysis.

Resistance genes and various traits are shared by *E. coli* strains within their ecological niches and pose a risk to public health. In most *E. coli* strains, ARGs are mainly encoded on MGEs that can be transferred between bacteria. These elements facilitate the exchange of resistance genes and other traits with co-residing bacteria in the human gut (Karami *et al.*, 2007). The ability of *E. coli* to share these resistance genes through HGT accelerates the spread of resistance across different bacterial species (Goren *et al.*, 2010). This situation complicates treatment options for infections and heightens the risk of resistant infections in the clinical setting. Therefore, studying the distribution of ARGs in the *E. coli* genome can provide significant insights into the evolution of AMR in this bacterium. In addition, identifying the determinants of conjugative transferability in *E. coli* is critical to understanding how resistance spreads within and between bacterial populations (Smillie *et al.*, 2010). Overall, a comprehensive study of these aspects in *E. coli* is fundamental for the development of targeted strategies to combat pathogenic *E. coli* and associated antibiotic resistance, ultimately improving public health interventions and reducing the impact of antibiotic-resistant infections.

1.4 Objectives

General objective

The general objective of this study was to assess determinants of diarrhea, and conduct phenotypic and genotypic characterization of *E. coli* isolates obtained from stool samples of patients visiting primary healthcare facilities in Addis Ababa and Hossana, Ethiopia.

Specific objectives

- To assess determinants of diarrheal diseases among patients attending public primary healthcare units in Addis Ababa and Hossana
- To determine the burden of DEC pathotypes using real-time PCR from stool specimens of patients attending public primary healthcare units in Addis Ababa and Hossana
- To characterize DEC isolates detected by real-time PCR using whole-genome sequencing
- To investigate the phenotypic antimicrobial resistance of *E. coli* isolated from the stool specimen of patients attending public health facilities in Addis Ababa and Hossana
- To assess the distribution and genetic diversity of phenotypically confirmed ESBL-producing and/or third-generation cephalosporin resistant *E. coli* isolates obtained from patients attending primary healthcare units in Addis Ababa and Hossana using whole-genome sequencing

2 Literature review

2.1 Determinants of diarrhea

Globally, diarrheal disease poses a serious public health challenge. It can be caused by various factors, including environmental, behavioral, and demographic influences (Boithias *et al.*, 2016; Graf *et al.*, 2008). A health facility-based case-control study conducted in the North Shoa Zone of the Amhara Regional State found associations between diarrhea and factors such as family monthly income, hand-washing practices of mothers/caregivers using only water, improper disposal of infant feces, not treating drinking water at home, children consuming leftover food stored at room temperature, and poor knowledge among respondents regarding the associated risks (Derseh *et al.*, 2021). Another case-control study in the Medebay Zana District of Northwest Tigray identified socio-demographic factors associated with diarrhea, including low maternal education levels, older age of the index child, households with three or more children under five, and a maternal history of diarrhea. Environmental and behavioral practices, such as the unavailability of toilet facilities, improper solid waste disposal, and unprotected drinking water, were also significant. Additionally, this study revealed that child feeding practices, including not exclusively breastfeeding and initiating complementary feeding after six months, were predictors of diarrhea (Asfaha *et al.*, 2018).

A community-based comparative cross-sectional study conducted in Awi Zone, Ethiopia, in 2019 found that diarrheal disease is associated with the use of unprotected water sources for drinking and unimproved toilet facilities (Gessesse and Tarekegn, 2022). Similarly, factors such as the type of toilet facility, the use of tap water, the time taken to fetch drinking water, and vaccination with the rotavirus vaccine were independently associated with diarrhea in a cross-sectional study conducted in the Efoulan health district, Cameroon, in sub-Saharan Africa (Ayuk *et al.*, 2018). Moreover, in rural central Tanzania, inappropriate disposal of children's feces, sharing water sources with animals during the dry season, overnight sharing of houses with cats, and keeping chickens inside the house overnight increased the risk of

diarrhea. In contrast, drinking water from public taps and open wells, as well as the older age of children, were found to be protective against diarrhea (Rukambile *et al.*, 2020).

A cross-sectional study in Bosaso, Puntland, Somalia, found that unhygienic drinking water storage and unhygienic homes were statistically significantly associated with the occurrence of diarrheal disease (Kalid *et al.*, 2016). A study in two impoverished suburbs of Johannesburg, South Africa, along with a multi-site study in eight countries, found a significant association between diarrhea and overcrowded dwellings (Mohan *et al.*, 2017; Nkosi *et al.*, 2019). Additionally, partially immunized children were found to be at an increased risk for diarrhea in Ethiopia (Negesse *et al.*, 2021; Wolde, Tilahun, *et al.*, 2022) and West Bengal (Gupta *et al.*, 2015).

2.2 Biology of *Escherichia coli*

The bacterium *E. coli* was discovered by the German-Austrian pediatrician Theodor Escherich (1857–1911), who isolated it from babies' feces in 1885 and named it *Bacterium coli commune* (Shulman *et al.*, 2007). Several decades after the original discovery, Castellani and Chalmers proposed the name *Escherichia coli* for the bacterium in 1919, which was then officially named after its discoverer in 1958 ("Etymologia: *Escherichia coli*", 2015).

E. coli is a gram-negative, non-sporulating, rod-shaped, facultative anaerobic, and coliform bacterium belonging to the genus *Escherichia* in the family *Enterobacteriaceae* (Basavaraju and Gunashree, 2022). This bacterium rapidly colonizes the gastrointestinal tracts of most warm-blooded animals within hours or a few days after birth (Robbens *et al.*, 2014) and co-exists with its host in symbiosis (Dicks *et al.*, 2018). However, some strains have developed the capacity of causing variety of diseases, including diarrhea or dysentery, while other pathotypes lead to extra-intestinal infections, such as urinary tract infections and meningitis (Robbens *et al.*, 2014; Tenaillon *et al.*, 2010).

As it is illustrated in Figure 1, *E. coli* has a complex cell wall structure consisting of several layers. The outer membrane of the cell wall contains lipopolysaccharides (LPS), which are important for the bacterium's interaction with its environment. Beneath the outer membrane is the periplasmic space, which houses a peptidoglycan layer (Caroff and Novikov, 2020). LPS are amphipathic glycoconjugate that consists of lipid A, a hydrophobic glycolipid, core oligosaccharide and the O-specific hydrophilic polysaccharide (Jann and Jann, 1987). The lipid A component is a highly conserved and essential part of the LPS in imparting specific pathogenic attributes to the bacteria, but its specific structure can vary across different bacterial strains, species, and subspecies (Stenutz *et al.*, 2006). The O antigen is highly variable among cell components because of differences in the sugars found in the O unit and the connections within and between these units. Typically, the O antigen comprises 10 to 25 repeating units, each containing two to seven sugar residues. This variability is a key factor in the serotyping of many gram-negative bacteria (Scheutz *et al.*, 2004). The genes that encode O antigen are located on the chromosome in a cluster designated as the O-antigen gene cluster (O-AGC), which is located between two housekeeping genes, *galF* (UTP-glucose-1-phosphate uridylyltransferase) and *gnd* (6-phosphogluconate dehydrogenase). *E. coli* has three known pathways for the assembly and synthesis of the O antigen, the Wzx/Wzy, ABC transporter and synthase pathways each involving a specific gene or set of genes for key steps in processing the O units (Fratamico *et al.*, 2016). Some bacteria produce LPS that lack the O-chain, referred to as lipooligosaccharides (LOS). LPS molecules that contain O-polysaccharides are known as “smooth LPS”, while those that do not have O-polysaccharides are called “rough LPS” (Raina, 2023).

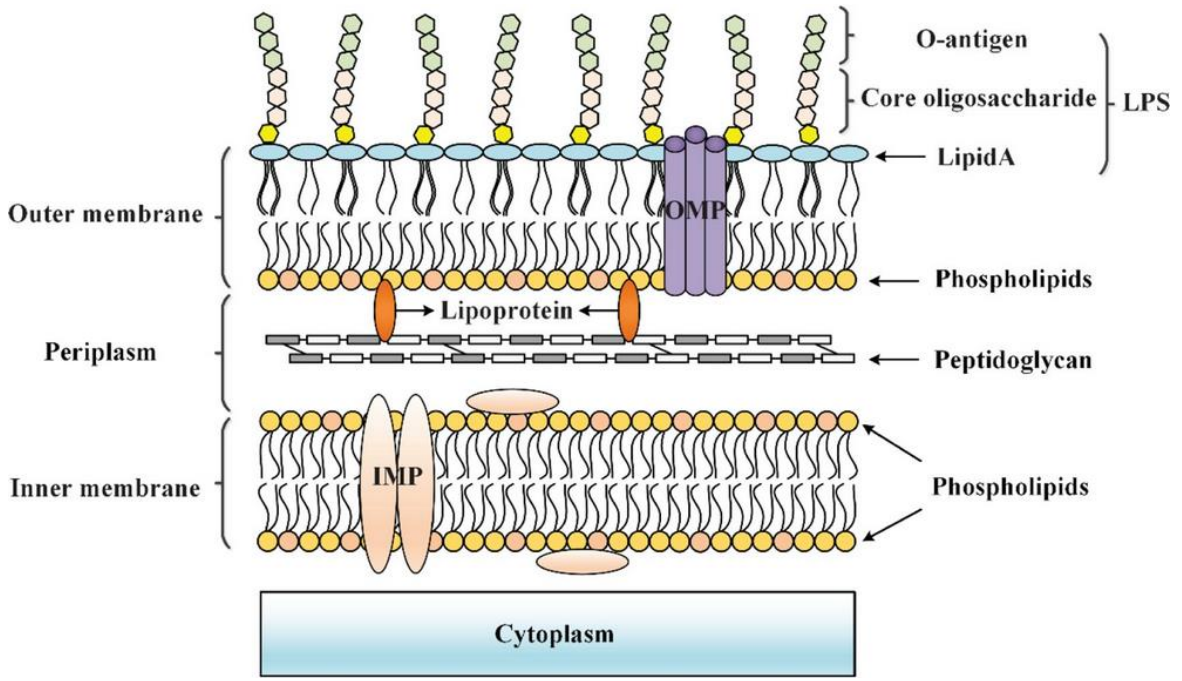

Figure 1: Structure of cell envelope. IMP – inner membrane protein, OMP – outer membrane protein. Source: Reprinted from Meng J. *et al*. (Meng *et al.*, 2021).

Most *E. coli* are motile, and very few strains are non-motile. The primary motility structure, flagellum, can participate in biofilm formation, protein export, and adhesion (Vilas Boas *et al.*, 2024). Flagellum consists of a cytoplasmic export apparatus, a basal body embedded in the cell membrane, a hook that connects the basal body to the filament, and a filament that functions as a propeller (Haiko and Westerlund-Wikström, 2013). The flagelar genes, which include over 50 genes involved in flagellar biogenesis and chemotaxis are organized into three loci (*flg*, *flh* and *fli*). The coordinated expression and regulation of these genes are crucial for the assembly and function of the flagella in *E. coli* (Chilcott and Hughes, 2000). *E. coli* strains also have additional structure such as capsules or microcapsules. They also possess fimbriae, which are hair-like projections on their surface. Some of these fimbriae are host-specific and enable adhesion to receptors found on host's cells. Many strains of *E. coli*, especially the invasive ones, produce a capsule that consists of polysaccharides (K antigens) (Govindarajan *et al.*, 2020).

2.3 Diarrheagenic *E. coli* and other enteropathogens

Globally, diarrheal disease constitutes a serious public health challenge (Ahmed *et al.*, 2008; Anteneh *et al.*, 2017; Joseph *et al.*, 2017; Marie-Cardine *et al.*, 2002; Qu *et al.*, 2012). Various enteropathogens associated with diarrheal disease were detected in studies conducted in different areas at different times. At least one diarrheagenic agent was detected in 92% of Rwandan children under five years of age with acute gastroenteritis visiting health facilities and two or more diarrheagenic agents in 63% of the children. Rotavirus, which was detected in 36.9% of children, was the leading case of gastroenteritis. In addition, adenovirus was found in 39.7%, ETEC with genes for heat-labile (*eltB*) toxin in 31.3%, ETEC with genes for heat-stable (*estA*) toxin in 19.0%, *E. coli* with *eae* gene in 25.2%, *E. coli* with *bfpA* gene in 14.2%, *Shigella* in 17.5% and *Cryptosporidium* in 7.8% of children with gastroenteritis. Severe dehydration due to diarrhea was observed in children infected with rotavirus and ETEC harboring *estA* compared with children with diarrhea due to other causes. Various etiologic agents were identified in different age groups of the study participants. Rotavirus, ETEC with *estA* and *E. coli* with *bfpA* were associated with younger age diarrhea whereas *Shigella* with older age (Kabayiza *et al.*, 2014).

In Burkina Faso, diarrheagenic pathogens were detected in 64% of children with diarrhea compared to 8% of healthy controls. Multiple pathogens were found in 11% of the patients and in 2% of the controls. Among the patients, 40% had a bacterial etiology compared to 8% of controls. Genes indicating *E. coli* pathogroups were found in 24% of the patients versus 7% of the controls, followed by various *Salmonella enterica* ssp. enterica serotypes (9% vs. 2%), *Shigella* spp. (6% vs. 0%), and *Campylobacter* spp. (2% vs. 0%). Viruses were predominantly found in children aged ≤ 2 years, whereas bacteria were equally prevalent across all age groups (Bonkoungou *et al.*, 2013).

In a study conducted in northern Ghana, enteropathogens were isolated from 77% of patients and 53% of controls. Rotavirus was the predominant pathogen identified, followed by adenovirus and norovirus. Bacterial pathogens were rare among the study participants.

Rotavirus was the only pathogen found significantly more frequently in patients than in controls and was associated with young age, fever, and watery stools (Reither *et al.*, 2007). Similarly, in Edo State, Nigeria, rotavirus was the most prevalent viral agent followed by adenovirus and norovirus. Over 42% of children with diarrhea had at least one viral agent, while the control group had none. The prevalence of rotavirus, adenovirus, and norovirus infection was determined in young children with diarrhea and apparently healthy age-matched children. A significant association was noted between age group and infection rate, with the highest infection rate observed in the 7–12 months age group. Viral infection rates were 84.2% and 97.9% in the first and second years of life, respectively. The overall co-infection rate was 7.6%, with rotavirus-adenovirus co-infection being the most common at 5.4% (Imade and Eghafona, 2015).

Diarrheagenic *E. coli* significantly contribute to the burden of diarrheal diseases, particularly in developing countries. However, the frequency of DEC often varies by geographical area. In a study conducted in Kenya, 27% of diarrheic patients were found to have DEC, with all age groups visiting health facilities for diarrhea treatment included. The predominant pathotype was EAEC, followed by EPEC and ETEC. Among the patients with detected DEC, EAEC accounted for 83.3%, while both EPEC and ETEC accounted for 8.3% each (E *et al.*, 2016). In another study in Kisumu County, Kenya, the prevalence of DEC among children under five years old in 2024 was 23%. The most predominant pathotype was EAEC (12%), and mixed infections of EAEC/EPEC and EAEC/ETEC were also observed. The EAEC pathotype was most prevalent in females, while ETEC and mixed infection strains were predominant among males (Yeda *et al.*, 2024).

In Abuja, Nigeria, approximately 13% of children were found to harbor DEC, with prevalence varying by location: 26.4% in urban, 24.8% in peri-urban and 12.2% in rural health centers. The observed pathotypes, in order of frequency, were EPEC, ETEC, EAEC, EHEC, and EIEC. The occurrence of these pathotypes was varied by age group. ETEC, EAEC, and EIEC were detected only in the 0–5 and 25–36 months age groups, while EPEC,

ETEC, EAEC, EHEC, and EIEC were found in the 6–12 months age group. Additionally, ETEC, EPEC, EAEC, and EHEC were isolated from the 13–24 months age group. ETEC was the dominant pathotype in children aged 6–12 months, while EPEC was predominant in those aged 13–24 months. ETEC was identified across all age groups except for the 37–48 months group, where only EHEC was isolated. EAEC was detected in 2% of children and was limited to those under 36 months of age. The study also revealed that EHEC strains were predominantly recovered from children aged 37–48 months. EIEC strains were found only in children aged 0–5, 6–12, and 25–36 months from a hospital in a peri-urban area and a health center in a rural area. All DEC categories, except atypical EPEC, were identified in children aged 6–12 months (Ifeanyi *et al.*, 2015).

A study conducted in Ouagadougou, Burkina Faso, among children under five years of age with diarrhea, found DEC in 7.4% of participants. The predominant pathotypes detected were EAEC and EPEC (both tEPEC and aEPEC). EPEC and ETEC were identified only in children under one year of age (Konaté *et al.*, 2017). In Mozambique, another study found that DEC was present in 48.6% of children with diarrhea, with the predominant pathotypes being EAEC (66.3%) and EPEC (15.8%). The STEC pathotype was not identified in this study, which also showed differences in the frequency of pathotypes across provinces. Multiple DEC pathotypes were isolated from the stool of children, with EAEC and EPEC frequently detected in combination (Manhique-coutinho *et al.*, 2022). In East-Central Gabon, a study involving children under five years old found a prevalence of DEC at 68.5%. Hybrid pathotypes, such as ETEC/EHEC, were identified in 7.5% of diarrheic children and 10.0% of non-diarrheic children, while EAEC/ETEC was found exclusively in diarrheic cases at a rate of 1.9%. The most frequently detected pathotypes among diarrheic children were ETEC and EHEC, each with a detection rate of 28.3%. In contrast, EPEC and EAEC were the least represented, with frequencies of 7.5% and 3.8%, respectively (Mabika *et al.*, 2021).

A study conducted in Ethiopia in Gondar found wide variety of enteropathogens in 86% of HIV positive participated in the study. Multiple enteropathogens were detected in 94% of

stool samples collected. An average of 5 enteropathogens was detected per case. *Shigella* spp./EIEC (80%), ETEC (73%), norovirus (16%) and *Blastocystis hominis* (62%) were detected. Parasites were detected in 28% of stool sample using wet mounts and modified acid fast staining (Seid *et al.*, 2018).

Few studies have assessed the epidemiology of DEC pathotypes in Ethiopia. DEC was detected in 10.3% of diarrheic children under five years old and among human contacts in urban and rural settings of Eastern Ethiopia (Belina *et al.*, 2023), as well as in 24.1% of children under five in Addis Ababa (Gebresilasie *et al.*, 2018). A high proportion of DEC pathotypes was observed in a study conducted in central Ethiopia, where DEC was detected in 40.7% of diarrheic and 28.2% of non-diarrheic children. The predominant pathotypes identified in this study were EAEC (41.5%) and ETEC (21.3%), with approximately 7.1% of participants having hybrid pathotypes, including ETEC/EAEC, ETEC/EPEC, and EPEC/EAEC. Other pathotypes, such as EPEC, EIEC, STEC, and DAEC, were also reported (Zelelie *et al.*, 2023). The present study aimed to investigate the occurrence of DEC pathotypes and their potential determinants of pathogenicity and antimicrobial resistance, to better understand the genetic characteristics of this bacterium, mitigate the risks associated with DEC infections, and improve overall health outcomes of patients visiting healthcare facilities in the study area.

2.4 General characteristics of DEC

Most *E. coli* are harmless and are part of a normal intestinal microbiota of humans and other mammals. It was epidemiologically linked to a human illness for the first time in 1945 after infants' diarrhea cases, which was described as "summer diarrhea" by John Bray in London (Bra, 1945). The species *E. coli* includes several pathotypes responsible for various diseases (Figure 2).

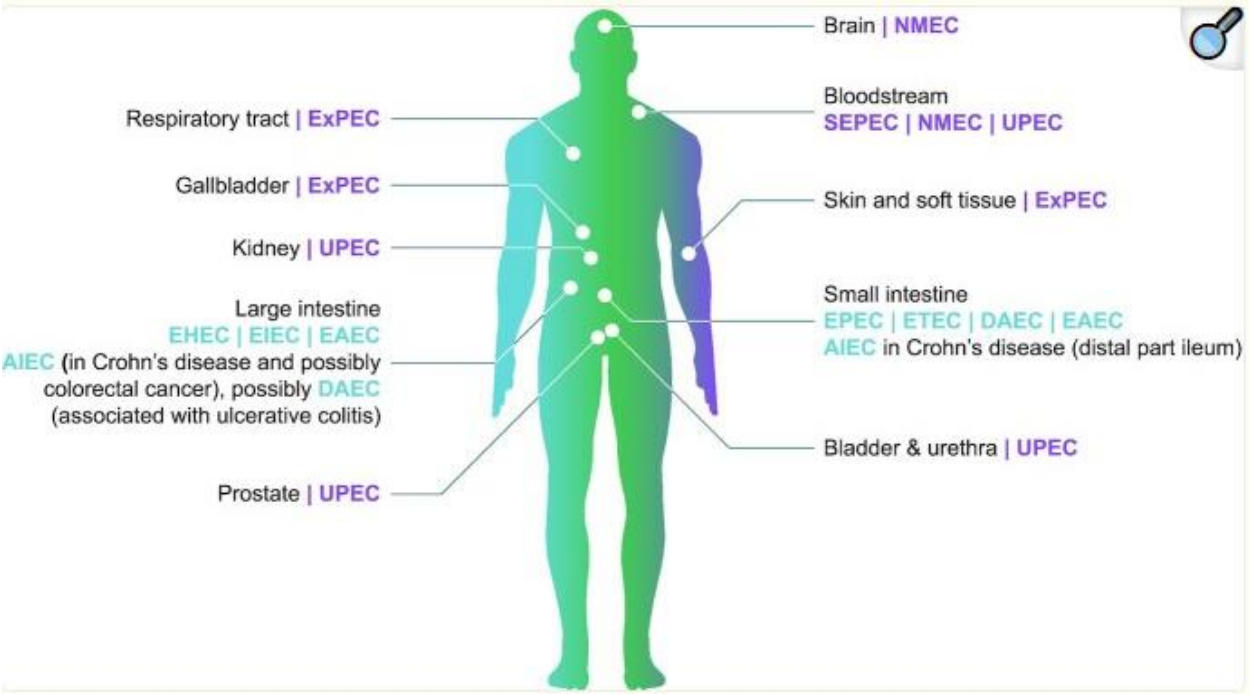

Figure 2: Possible *E. coli* infections sites in humans by different pathotypes (Source: Reprinted from Geurtsen J. *et al* (Geurtsen *et al.*, 2022)).

Enteropathogenic *E. coli* is one of the intestinal pathogenic *E. coli* pathotypes. It was the first pathogenic type of *E. coli* to be associated with human disease and is among the major causes of infantile diarrhea in developing countries (Ochoa and Contreras, 2011) and can be classified as typical and atypical, depending on the presence or absence of the EPEC adherence factor plasmid (pEAF), respectively (Croxen *et al.*, 2013). The hallmark of EPEC infection is the attaching and effacing (A/E) lesion in the gut mucosa of human or animal hosts, leading to diarrheal illness (Kaper *et al.*, 2004). The genes responsible for the A/E lesion formation are located in a chromosomal pathogenicity island of ~35 kb, known as the locus of enterocyte effacement (LEE). LEE encodes an adhesin called intimin, its translocated receptor (Tir), components of a type III secretion system (T3SS) (Jarvis *et al.*, 1995). These virulence factors have a crucial role in A/E lesion formation, and their detection in EPEC strains is an indicator of their potential to produce these lesions (Cleary *et al.*, 2004). Atypical EPEC strains have been associated with diarrhea outbreaks in developed countries (Hedberg *et al.*, 1997; Yatsuyanagi *et al.*, 2003) and with sporadic cases of diarrhea in developing and developed countries (Bueris *et al.*, 2007; Franzolin *et al.*, 2005).

Enterotoxogenic *E. coli* is the other DEC pathotype, which is the most frequent bacterial cause of diarrhea in children and adults living in the developing world where there is inadequate clean water and poor sanitation and also the most common cause of traveler's diarrhea. ETEC is identified by its ability to produce plasmid encoded heat-labile (LT) and/or heat-stable (ST) enterotoxins (Ifeanyi *et al.*, 2018; Oyofo *et al.*, 2001). Both of these enterotoxins play a role in disrupting intestinal fluid homeostasis by increasing secretion of fluids and electrolytes through downstream activation of adenylate cyclase (by LT) or guanylate cyclase (by ST) in the small intestinal epithelial cells. These bacteria colonize the small bowel epithelium by means of thread-like appendages (fimbriae) known as colonization factor antigens (CFA) or coli surface (CS) antigen, after which bacterial toxins stimulate intestinal secretion resulting in diarrhea (Sow *et al.*, 2019). ETEC also expresses a variety of non-classical virulence factors that may be essential for pathogenicity and promising vaccine targets. The *eatA*, *irp2*, and *fyuA* are the most frequently identified non-classical VAGs. These genes are known to be associated with the ETEC high-pathogenicity island (HPI) encoding a yersiniabactin-like iron scavenging system (Guerra *et al.*, 2014).

Enteroaggregative *E. coli* are an emerging enteric pathogen which was described in 1987 and identified in a child from Chile with persistent diarrhea (Harrington *et al.*, 2006). It is a cause of both acute and persistent diarrhea in multiple population groups, in both developing and developed countries (Huang *et al.*, 2006). EAEC strains have been implicated as the second most frequently isolated pathogen, only after ETEC, from travelers' diarrhea patients returning from Latin America and South-East Asia (Ahn *et al.*, 2011; Paredes-Paredes *et al.*, 2011). The ability to produce a 'stacked-brick' appearance on epithelial cells, which is mediated by the aggregative adherence fimbriae (AAF) is a common feature that defines the EAEC strains. This phenotype is derived from the ability of EAEC to attach to other EAEC, to the epithelial cells, and to the surface of tissue culture plates. Numerous adhesins, toxins, and proteins associated with virulence have been described in EAEC strains (Harrington *et al.*, 2006). The aggregative phenotype determinants of EAEC are contained in a large plasmid (pAA) that carries a number of VAGs that are under the control of the master AggR regulator

(Estrada-Garcia and Navarro-Garcia, 2012). EAEC produce at least three fimbrial adhesins encoded on a 60−65 MDa virulence plasmid required for expression of the AA pattern: *aggA* (AAF/I), *aafA* (AAF/II), and *agg-3* (AAF/III) (Huang *et al.*, 2006). In addition to the expression of adhesins and toxins, several other potential virulence factors have been described in EAEC, which are encoded in plasmids or in the chromosome (Weintraub, 2007).

Diffusely adherent *E. coli* (DAEC) strains are heterogeneous group of potentially pathogenic *E. coli* strains, harboring genes encoding for fimbrial or afimbrial adhesins that are responsible for their pathogenicity. They are characterized by the diffuse adherence pattern on cultured epithelial cells HeLa or HEp-2 due to the production of adhesins encoded by a family of *afa*/*dra*/*daa* related operons (Turniak and Sobieszczańska, 2019). Approximately 75% of DAEC harbor adhesins from the Afa/Dr family, responsible for this adherence phenotype (Kaper *et al.*, 2004). Afa/Dr is adhesins family that bind to cell receptors human decay-accelerating factor (hDAF) and carcinoembryonic antigen cell adhesion molecules (hCEACAMs), inducing receptor clustering and finger-like cell projections, resulting in bacteria internalization (Meza-Segura and Estrada-Garcia, 2016).

Shiga toxin-producing *E. coli* (STEC) are also known as "verocytotoxin-producing *E. coli*" (VTEC). These are strains of *E. coli* that are known to produce Shiga toxin (*Stx*). Shiga toxin is one of the most potent bacterial toxins known. They are divided in two major antigenic forms: *Stx1* and *Stx2*. These toxins consist of two major subunits, an A subunit that joins noncovalently to a pentamer of five identical B subunits. The A subunit of the toxin injures the eukaryotic ribosome, and halts protein synthesis in target cells. The function of the B pentamer is to bind to the cellular receptor, globotriaosylceramide, Gb3, found primarily on endothelial cells (Melton-Celsa, 2014). STEC is a recognized group of foodborne pathogens that can lead to various infections, ranging from mild and often unnoticed diarrhea to more severe conditions, including hemorrhagic colitis (HC) and the potentially life-threatening hemolytic uremic syndrome (HUS) (Gould *et al.*, 2013). Currently, there are over 470 STEC serotypes recognized based on *E. coli* O antigen (determination of O serogroup) and H

(flagellar) antigen typing (Ludwig *et al.*, 2020; Mora *et al.*, 2011). More than 130 STEC serotypes are associated with human STEC infections (Valilis *et al.*, 2018). STEC O157:H7 is the most frequent STEC serotype associated with foodborne outbreaks and human infections (Li *et al.*, 2017; Qin *et al.*, 2015).

Enteroinvasive *E. coli* (EIEC) is interacellular pathogen able to enter epithelial cells of colon, multiplicate within them, and move between adjacent cells. They are responsible for bacillary dysentery in humans, which is characterized by invasion and inflammatory destruction of the human colonic epithelium (Pasqua *et al.*, 2017). EIEC infected humans seem to be the major source of infection, as no animal reservoirs have been identified, and transmission uses mainly the oral–fecal route. They have a large F-type plasmid (pINV) containing the genes required for invasion, intracellular survival, and spreading through the intestinal mucosa (Pasqua *et al.*, 2017).

2.5 Genomic diversity of *Escherichia coli*

Bacteria exhibit significant genetic variation within a species, primarily due to the presence or absence of entire genes or gene clusters, often influenced by mobile genetic elements. In *E. coli*, this genetic diversity arises from mutations, horizontal gene transfer, and recombination, allowing adaptation to various environments (Zenebe, Sisay and Belay, 2024). The genetic diversity in *E. coli* has been examined through various traits, including phylogeny, serotypes, sequence types, antibiotic resistance, and virulence (Figure 3).

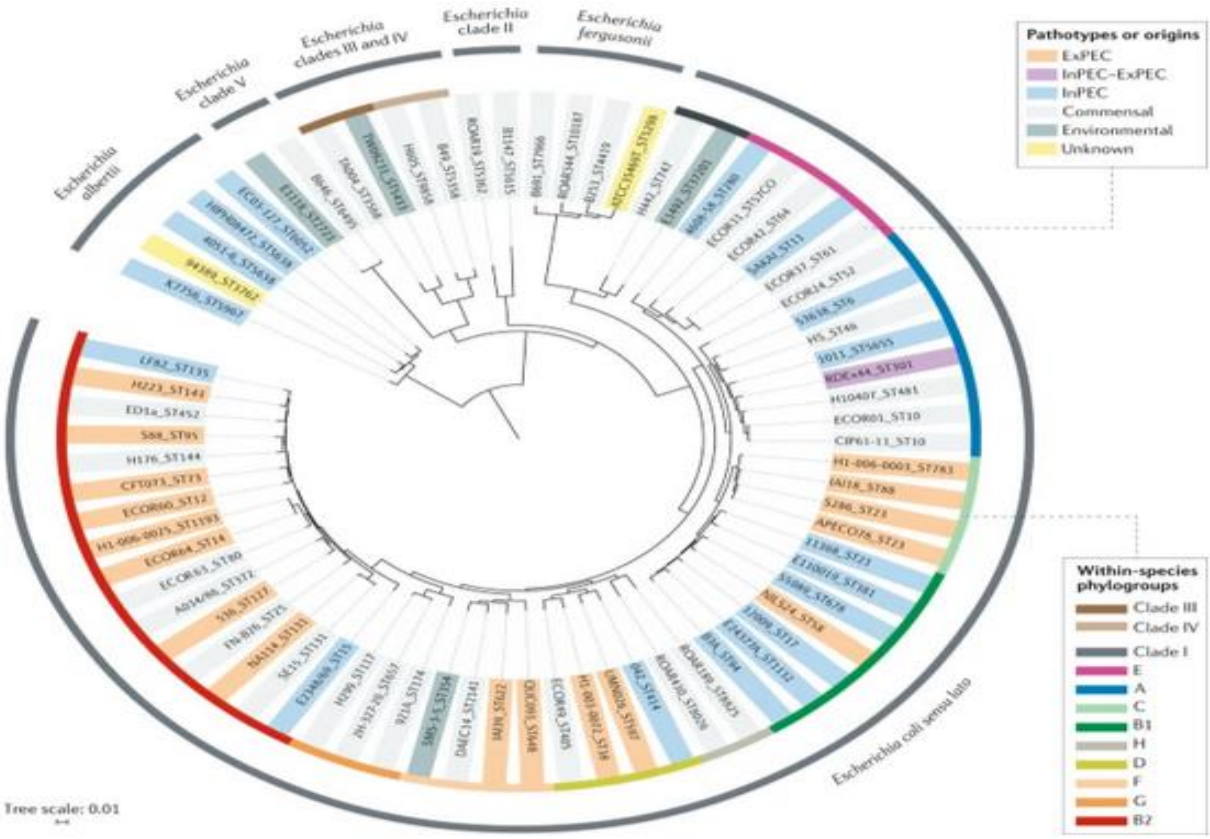

Figure 3 The phylogenetic diversity of *E. coli* (Source: Reprinted from Denamur *et al.* (Denamur *et al.*, 2021))

The proportion and phylogenetic composition of *E. coli* across various studies was different. In an analysis of human commensal *E. coli* isolates from 24 countries, phylogroup A was the most prevalent, accounting for 48.3%, followed by phylogroup D at 30.2%. The frequencies for other phylogroups were 5.2% for B1 and 16.4% for B2. This study revealed the broadest frequency range for group A, varying from 8.7% to 79.3%, while group B2 ranged from 0% to 53.9%, D from 0% to 45.7%, and B1 from 0% to 34% (Stoppe *et al.*, 2017). Similarly study conducted in Abidjan, Côte d'Ivoire, among diarrheic children and adults revealed the predominance of *E. coli* pathovars belonging to phylogenetic group A (53%), followed by phylogroup D (23.5%), B1 (11.7%), and B2 (11.7%) (Adjhi *et al.*, 2014).

Phylogenetic group A was also the most prevalent in both UPEC and DEC strains, followed by group B2, in a study conducted in Egypt in 2019 among patients of different ages presenting with symptomatic UTIs and diarrhea (Khairy *et al.*, 2019). In contrast, a study

conducted in South Africa found DEC pathotypes isolated from diarrheic children predominantly in phylogroup B2 (30.4%), followed by phylogroup B1 (22.8%). Phylogroups C and E were detected in 12.7% each, while phylogroups A and D were detected at rates of 6.3% each. Phylogroup F was also detected in this study but with a relatively lower prevalence (1.3%) (Alfinete *et al.*, 2022). In southwest Nigeria, the majority of *E. coli* isolates from humans, animals, and food products belonged to phylogroup B1 (43.2%) and phylogroup B2 (29.7%). The other phylogroups identified were phylogroup A (18.9%), phylogroup D (5.4%), and phylogroup E (2.7%) (Olowe *et al.*, 2019).

A study aimed at identifying the lineages of EAEC in Nigeria revealed multiple lineages represented by 96 STs. The findings indicated that STs10, 31, and 394 were the most prevalent, accounting for 40% of the total, and highlighted a heterogeneous distribution of virulence factors among the STs. The predominant ST complex, ST10-complex, comprised 21.3% of the isolates. In addition, the study showed that EAEC strains were predominantly belonged to phylogroups A, B1, and D. The study also identified plasmids belonging to diverse incompatibility groups (Okeke *et al.*, 2010).

According to Escobar-Páramo *et al.*, the distribution of *E. coli* phylogenetic groups is influenced by geographical and climatic conditions. In this study, tropical populations appear to preferentially harbor strains from group A, with group B1 present to a lesser extent. In contrast, group B2 isolates were significantly more prevalent in temperate populations than in tropical populations (Escobar-Páramo, Grenet, *et al.*, 2004).

Varieties of virulence and resistance genes that can increase the pathogenicity and the severity of infection with the great possibility of therapy failure have been identified in *E. coli*. Clermont *et al*. found that phylogroup G possesses several virulence genes typically linked to extra-intestinal virulence, including iron uptake systems such as *fyuA* and *irp2* in 84% of the strains, and *iroN*, *iss*, *iucC*/*iutA*, and *sitA* in 72% of the strains. They also identified genes encoding bacteriocins, such as colicin and microcin, with varying frequencies among the phylogroup G sequence type complexes. Seventy percent of these

strains carried resistance genes, with 53% resistant to three or more classes of antimicrobials. Notably, 24% were resistant to third-generation cephalosporins, mainly due to CTX-M-type ESBL, and 25% had mutations in the quinolone-resistance determining region. Resistance was detected in 55% for sulfonamide or trimethoprim, 46% for tetracycline, 16% for chloramphenicol, and 2.7% for colistin (Clermont *et al.*, 2019).

In 2022, *E. coli* isolates from various clinical samples in Egypt were confirmed to carry a diverse array of VAGs. The results indicated that phylogroup B2 (26.67%) was the most prevalent phylogenetic group, exhibiting the highest levels of virulence traits. This study identified VAGs encoding adhesins *afa/dra* (afimbrial/Dr-ahdesin) (14%), *sfaS* (S-fimbriae) (60.67%), *yfcV* (putative fimbriae) (33.33%), *papC* (P-fimbrial usher protein) (49.33%), and *papG* allele II (P-fimbrial tip adhesin II) (21.33%) among the isolates. Additionally, VAGs involved in iron acquisition, including *fyuA*, *iutA*, and *chuA*, were detected at varying frequencies. The study also identified several other VAGs, including *kpsMTII* (the group 2 capsule system), *sta2* (heat-stable enterotoxin ST-I group b), *astA* (EAST1), *stx2* (Shiga toxin II), *cnf1* (cytotoxic necrotizing factor I), *vat* (vacuolating autotransporter toxin), and *eae* (intimin) (El-baz *et al.*, 2022).

Twenty-one distinct ST groups were identified from samples collected between 2012 and 2018 in Malawi. The most prevalent ST was ST131, found in 32.8% of isolates, followed by ST410 at 15.5%. The study identified 69 unique ARGs, with ST131 exhibiting a diverse range of ARGs, particularly the $bla_{CTX\text{-}M\text{-}15}$ gene, which was present at a higher frequency. All ARGs, including $bla_{CTX\text{-}M\text{-}15}$, were widely distributed among the sequence types. Twenty-three genes associated with β-lactam resistance were identified, including 10 ESBL genes. The ESBL genes detected were $bla_{TEM\text{-}113}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}155}$, $bla_{CTX\text{-}M\text{-}159}$, $bla_{CTX\text{-}M\text{-}182}$, $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}3}$, $bla_{EC\text{-}15}$, $bla_{EC\text{-}18}$, and $bla_{EC\text{-}19}$. Quinolone resistance genes, such as *qnrB1* and *qnrS1*, were also noted. Furthermore, other resistance genes, including *aac(3)-IIa*, *aac(3)-IId*, *ant(3")-Ih*, *strA*, *strB*, *catA1*, and *catB3*, were detected (Tegha *et al.*, 2021).

According to Adwan G. *et al*., *E. coli* that have intestinal as well extraintestinal VAGs exist, indicating their ability to adapt to different environments and infect various tissues, potentially causing both intestinal and extraintestinal infections simultaneously (Adwan *et al.*, 2016). Similarly, Nielsen KL *et al*. demonstrated a close genetic relationship between uropathogenic *E. coli* (UPEC) and faecal isolates from both UTI patients and healthy controls through whole-genome sequencing. Their study utilized MLST, SNP-based phylogeny, and accessory genome analyses to compare *E. coli* strains from healthy women who had never experienced a UTI with those from UTI patients. The findings revealed that the faecal *E. coli* strains in healthy women were highly similar to those in UTI patients, suggesting a shared genetic background (Nielsen *et al.*, 2018).

In another study, a variety of virulence genes were identified in *E. coli* isolates from Egyptian patients across different clinical samples in 2019. The most commonly detected VAGs included *csgA* (78.1%), *crl* (76.5%), and *fimH* (75%). The *colla-lb* gene was prevalent in *E. coli* from various sources, especially in stool isolates (82.6%). Among uropathogenic *E. coli*, the most common VAGs were *fimH* (90.9%), *csgA* (90.9%), *hlyA* (81.8%), and *crl* (86.4%). Additionally, the study reported ARGs such as $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}10}$, and $bla_{OXA\text{-}2}$, as well as the co-occurrence of $bla_{CTX\text{-}M\text{-}15}$ with $bla_{OXA\text{-}2}$-like and $bla_{OXA\text{-}10}$-like genes (El-Baky *et al.*, 2020). Furthermore, a study on uropathogenic *E. coli* in urinary tract infection patients in Addis Ababa, Ethiopia, identified various VAGs, including *fimH*, *aer*, *hly*, *pap*, *cnf*, *sfa*, and *afa*. The majority of *E. coli* isolates belonged to phylogroups B2 (30%), followed by D (27.5%), B1 (24%), and A (18.5%) (Dadi *et al.*, 2020).

Certain virulence factors can induce genetic instability and disrupt the eukaryotic cell cycle, facilitating bacterial invasion and host colonization. These factors are referred to as cyclomodulins. In *E. coli*, notable cyclomodulins include colibactin (*pks*), cytotoxic necrotizing factor (*cnf*), cytolethal distending toxin (*cdt*), cycle inhibiting factor, Shiga toxin, and subtilase toxin. McCoy CS *et al.* characterized cyclomodulin-encoding *E. coli* rectal isolates from healthy and clinically affected marmosets originating from three different

captive sources using PCR-based assays. The finding indicated that nearly all cyclomodulin-positive *E. coli* belonged to phylogenetic group B2 and demonstrated that *E. coli* strains carrying these genes are pathogenic. Additionally, several isolates exhibited virulence factor profiles similar to necrotoxigenic *E. coli* (NTEC) and UPEC strains, including *cnf1*, *hlyA*, *sfaS*, *iss*, and *iroN* (McCoy *et al.*, 2021).

E. coli is a diverse group of bacteria with several different serotypes. In Egypt, *E. coli* strains isolated from human stool and urine samples were serotyped into 64 typable strains. The identified serotypes included O164:K, O126:K71, and O86:K61. The study revealed that each serotype carried different virulence genes. Serotype O164:K carried the *F17*, *hlyA*, and *sta* genes, whereas O126:K71 was confirmed to carry the *stx1* gene, as well as the *F17*, *stb*, and *hlyA* genes. Additionally, serotype O86:K61 carried the *F17*, *stb*, and *hlyA* genes (Osman *et al.*, 2012). Furthermore, another study in Egypt identified seven different serotypes (O26, O44, O55, O151, O125, O145, and O1) among 119 *E. coli* isolates using specific polyvalent and monovalent O antisera. The most predominant serotype was O26. Different virulence genes were also detected in the *E. coli* isolates in this study, and positive and negative correlations were observed between some serotypes and virulence genes. For instance, a positive correlation was found between the *invE* and *stx2* genes and the O145 serotype, while a negative correlation was observed between the *invE* and *stx2* genes and the O44 serotype (Bendary *et al.*, 2022).

A study conducted in Khartoum Sudan confirmed *E. coli* O157:H7 and *E. coli* O55:H as the prevailing strain of *E. coli* causing diarrheal disease. They detected *pal* gene in all samples collected; the *eaeA* and *fimH* genes in 39.1% and 62.6% of *E. coli* strains, respectively. In this study *fliC* was detected in 42.6%, *rfb* in 4.4% and both genes again in 4.4% and *fliC* gene showed suggestive significant association with diarrheal disease (Salih Mohamed *et al.*, 2018). Similarly ETEC with genes for heat-labile (*eltB*) or heat-stable (*estA*) toxin in 31.3% and 19.0%, *E. coli* with *eaeA* or *bfpA* genes in 25.2% were detected in diarrheic patients in Rwanda (Kabayiza *et al.*, 2014). A molecular serotyping study conducted in Morocco on *E.*

coli isolated from a hospital catering service showed that 14 *E. coli* isolates carried the flagellar antigen H7, while no isolates showed O157, indicating that hemorrhagic *E. coli* O157:H7 was not detected in any of the tested samples (Benjelloun Touimi *et al.*, 2020). According to the reviewed studies, *E. coli* has shown genetic diversity, highlighting significant implications for public health. Therefore, assessing the genetic diversity of *E. coli* in Ethiopia is crucial for mapping transmission pathways, assessing risks, and informing clinical management of infections by identifying the presence and distribution of VAGs and ARGs.

2.6 Antibiotic-resistant *Escherichia coli*

2.6.1 Mechanisms of antimicrobial resistance in *Escherichia coli*

Bacteria use various mechanisms of resistance to antibiotics. These include the enzymatic degradation of antibacterial agents, the modification of antimicrobial targets, ribosomal mutations or modifications, the alteration of membrane permeability to antibiotics, the active efflux of a antimicrobial agents, loss of porins and metabolic pathways bypass (Figure 3) (Bennett, 2008).

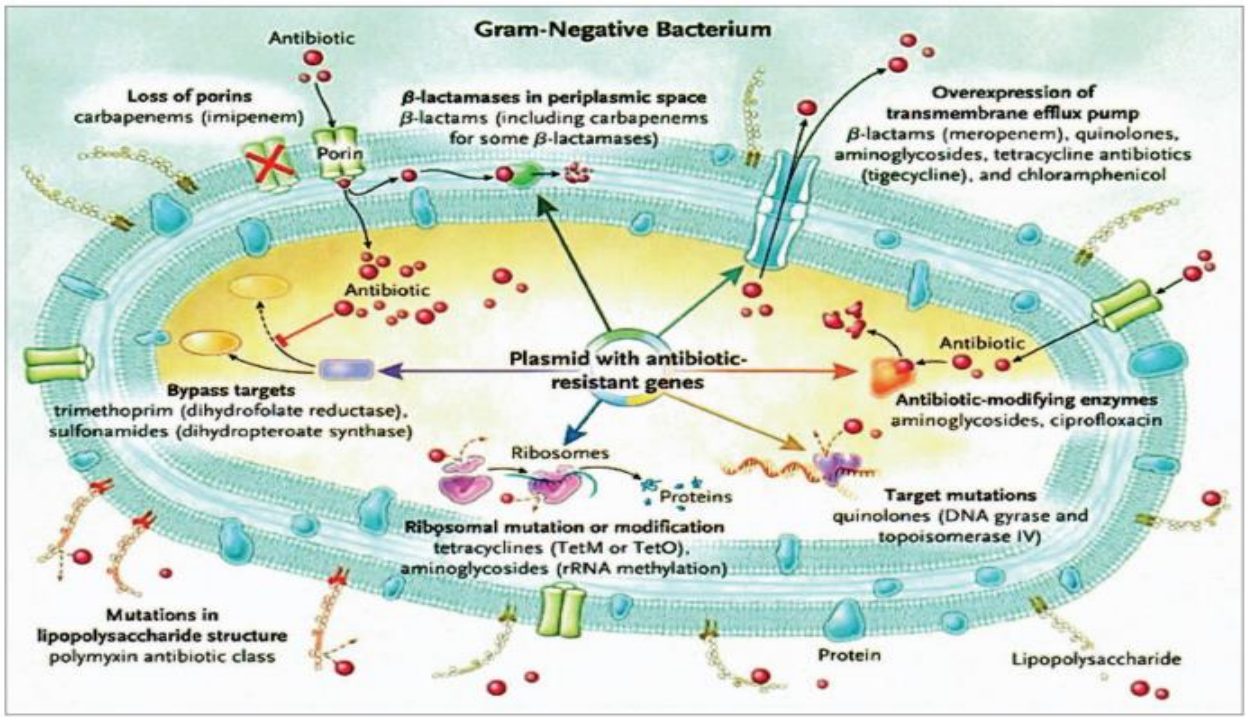

Figure 4: Mechanisms of antimicrobial resistance (Source: Reprinted from Pop-Vicas A and Opal SM (Pop-Vicas and Opal, 2014)).

1.2.4.2 Epidemiology of antimicrobial resistance in *E. coli*

Development of antimicrobial resistance by *E. coli* against easily accessible and commonly prescribed antimicrobials has become a major concern throughout the world, particularly in developing countries. In Lagos, Nigeria, 69.6% of *E. coli* isolates obtained from outpatients attending Lagos State hospitals and animal handlers in abattoirs, poultry farms, and open markets were MDR. The isolates were highly resistant to tetracycline (73.7%), trimethoprim/sulfamethoxazole (61.5%), and ampicillin (59.1%). This study found 1.6% to 15% resistance to the cephalosporins, including the third- and fourth-generation cephalosporins, cefpodoxime (8.1%) and cefepime (1.6%) (Adenipekun *et al.*, 2016). Another study conducted in Lusaka, Zambia, among farm workers in broiler poultry production found a high level of resistance to tetracycline (87.9%), trimethoprim/sulfamethoxazole (48.3%), and ampicillin (46.8%). The isolates also exhibited resistance to nalidixic acid (19.0%), ciprofloxacin (12.1%), cefotaxime (8.6%), and chloramphenicol (5.2%). Of the isolates, 67.2% were resistant to at least one antimicrobial and 29.3% were MDR. No resistance was observed to colistin and imipenem. Additionally, 3.4% of the isolates were identified as ESBL producers, harboring the CTX-M gene. However, neither the TEM nor the SHV gene was detected in any of the isolates tested (Mwansa *et al.*, 2023). Another study in Kenya revealed DEC isolates resistance to ampicillin (92%), trimethoprim-sulfamethoxazole (92%) and amoxicillin-clavulanic acid (85%). All isolates were sensitive to ciprofloxacin and gentamicin in the study area (E *et al.*, 2016).

According to Kebenei *et al.*, DEC isolates were highly resistant to sulfamethoxazole (97%), sulphametoxazole + trimethoprim (96%), and ampicillin (84%) in Trans-Nzoia County, Kenya. The isolates also exhibited varied resistance to chloramphenicol (27%), tetracycline (16%), kanamycin (10%), and streptomycin (9%). Approximately 42.2% of the *E. coli* isolates were resistant to more than one antibiotic, including sulfamethoxazole, co-trimoxazole, ampicillin, chloramphenicol, tetracycline, kanamycin, and streptomycin. The

bla$_{TEM}$ gene was detected in all isolates that were resistant to ampicillin (Kebenei *et al.*, 2016).

Over 71% of *E. coli* isolates were resistant to at least one antibiotic in a study conducted in Eastern Cape, South Africa, among patients attending public health centers. The isolates exhibited resistance against penicillin G (99%), ampicillin (88%), trimethoprim (84%), tetracycline (83%), doxycycline (82%), and erythromycin (71%) (Mkuhlu *et al.*, 2020).

In Ethiopia, Tuem *et al* in 2018 reported an overall antibiotic resistance rate of 48.9% among *E. coli* isolates in a systematic review and meta-analysis. According to this review, the highest resistance rate was recorded in Addis Ababa (62.6%), followed by the Southern Nations, Nationalities, and Peoples of Ethiopia region (58.1%), while relatively low level of resistance was recorded in the Tigray region (27.5%). The isolates were highly resistant to ampicillin (83.8%) and amoxicillin (75.8%), whereas a relatively low level of resistance was reported for nitrofurantoin (13.6%) (Tuem *et al.*, 2018). Another study conducted in Wolaita Sodo, Southern Ethiopia, found a 61.9% resistance rate among *E. coli* isolates obtained from children with diarrhea attending Sodo Christian Hospital. The study identified the highest rate of resistance to neomycin (97.1%), followed by streptomycin (95.6%). The isolates were also resistant to clindamycin (92.6%), ampicillin (70.6%), trimethoprim (67.6%), tetracycline (64.7%), oxytetracycline (58.8%), and sulphonamides (58.8%) (Wolde, Deneke, *et al.*, 2022). In a systematic review and meta-analysis, Gemeda *et al*. reported high rate of MDR in *E. coli* isolates from Ethiopia, with a prevalence of 77% in 2021. This analysis revealed that *E. coli* isolates from different sources exhibited a high rate of resistance to antibiotics from various classes, including cephalosporins, quinolones, aminoglycosides, macrolides, sulfonamides, and tetracycline (Gemeda *et al.*, 2021).

Many isolates of enteric bacteria are known to harbor mobile elements that encode antimicrobial resistance (Wain *et al.*, 2003; Welch *et al.*, 2007). Genetic analysis revealed that all DEC isolates in Zambia were ESBL producers (Chiyangi *et al.*, 2017). The genetic markers associated with resistance to sulphonamides and tetracyclines are reported to be

highly prevalent among phenotypically resistant *E. coli* isolates from Kenya. The *tetA* gene and *sul1* genes were dominant genes detected (Christabel *et al.*, 2012). The study conducted in Eastern Cape, South Africa also identified resistance-determining genes such as *sulII* (12%), *ampC* (22%), bla_{TEM} (25%), and *tetA* (35%) from *E. coli* isolates (Mkuhlu *et al.*, 2020).

2.7 *Escherichia coli* detection and characterization techniques

The detection and characterization of *E. coli* involves a number of techniques due to its diverse phenotypic and genotypic characteristics. Culture methods are commonly employed to isolate *E. coli* from samples, with the choice of method depending on the target strain and the purpose of isolation. One of the most commonly used media is MacConkey agar, which differentiates *E. coli* from non-lactose fermenting gram-negative bacteria. This medium contains lactose and indicators that allow *E. coli* to form pink colonies during lactose fermentation, while non-lactose fermenters remain colorless (Lupindu, 2017). Another selective medium is eosin methylene blue (EMB) agar, which also isolates and differentiates *E. coli* based on lactose fermentation. EMB uses the dyes eosin Y and methylene blue to differentiate between lactose-fermenting and non-fermenting organisms and at the same time inhibits gram-positive bacteria. On EMB agar, *E. coli* colonies are characterized by a distinct dark center and a metallic sheen, making them easy to identify (Lal and Cheeptham, 2007). In addition, Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric (HE) agar are selective media for *E. coli*. HE agar is specifically designed to isolate gram-negative bacteria and differentiate them on the basis of their biochemical properties. *E. coli* ferments lactose on HE agar, resulting in yellow colonies, while other enteric pathogens such as *Salmonella* and *Shigella* form green or colorless colonies because they cannot ferment lactose. Furthermore, *E. coli* can ferment xylose on XLD agar and form yellow colonies that aid in identification (Cappuccino and Welsh, 2018). Biochemical methods such as the IMViC tests and Analytical Profile Index 20E (API 20E) systems as well as enzymatic methods such as Brilliance *E. coli*

Agar or Petrifilm select *E. coli* counting plates and molecular methods such as MALDI-TOF are used to confirm *E. coli* isolates (Lupindu, 2017).

Cell culture-based adherence assays can also be used to diagnose and characterize various *E. coli* pathotypes based on their adherence properties to human epithelial cells. These assays typically involve cultured mammalian cell lines, such as HEp-2, and HeLa, which simulate the human epithelial environment (Nataro *et al.*, 1987; Scaletsky *et al.*, 1984)

The evaluation of pathotypes, antimicrobial resistance, virulence and clonal relatedness in *E. coli* requires a thorough characterization of the bacterium. A commonly used method for this characterization is serotyping, which follows the Kauffman classification scheme to identify the O (somatic) polysaccharides and H (flagellar) surface antigens (Prager *et al.*, 2003). Establishing genetic relatedness is a crucial point when using molecular genetic data to draw conclusions about the spread of pathogens. Pulsed field gel electrophoresis (PFGE) is a tool for typing, assessing genetic relatedness and detecting outbreaks of *E. coli* strains (Ejrnaes *et al.*, 2006). It is a robust genotyping technique used to separate large genomic DNA fragments after the DNA has been digested with specific restriction enzymes and then applied to a gel matrix in which an electric field periodically changes direction to facilitate separation (Sharma-Kuinkel *et al.*, 2016). If the DNA banding patterns of two isolates are identical, they are considered to be the same strain. However, if the isolates belong to different strains, the sites at which the restriction enzymes cut the DNA and the distances between these sites vary, resulting in distinct DNA banding patterns (Goering, 2010). Multi-Locus Variable Number of Tandem Repeat Analysis (MVLA) is another high-resolution genotyping method that can serve as an effective alternative to traditional MLST for epidemiological surveillance of *E. coli* (Naseer *et al.*, 2012). These methods have proven to be effective in the epidemiological investigation of pathogenic *E. coli* (Schürch *et al.*, 2018) and the assessment of the emergence and dissemination of MDR clones. However, none of these methods can accurately define the evolutionary relationships between *E. coli* strains; hence there is a need for a tool with a higher resolution. The advent of whole-genome sequencing (WGS) technologies has greatly

enhanced not only the epidemiological investigation of outbreaks and the global spread of multidrug resistant and pathogenic clones of *E. coli* (Joensen *et al.*, 2014), but also our understanding of the physiology and evolutionary history of how some pathogenic strains evolve from commensal *E. coli* strains. WGS of bacteria has become an effective method for investigating the information contained in the genome sequence of the organism. This method has highly discriminative power enabling the comparison of genetic relatedness between bacteria even on a sub-species level (Uelze *et al.*, 2020). Multilocus sequence typing (MLST) is a sequence-based method used for detecting and characterizing bacteria. Traditionally, it involves amplifying seven housekeeping gene loci through PCR, followed by sequencing the resulting fragments. These sequences are then compared to established allelic profiles. A single nucleotide change at any locus signifies a different allele, contributing to the overall sequence type (ST), which MLST defines by assigning unique integer numbers to each allele and a specific integer to each unique combination of alleles. Unrelated STs typically share few or no alleles, while STs that differ by only one or two alleles are considered closely related. Nowadays, MLST can be applied to fully sequenced bacterial genomes. In this modern approach, short sequence reads are first assembled into draft genomes, and the MLST alleles for each locus are aligned to the genome using BLAST. The closest matching MLST allele is chosen, and the ST is determined based on the combination of these alleles (Larsen *et al.*, 2012). Finally, MLST analyses can be transformed into a phylogenetic tree using various methods, such as single-linkage hierarchical clustering, neighbor-joining (NJ), or minimum spanning (MS) trees (Zhou *et al.*, 2018).

Some of aforementioned studies described the determinants of diarrheal diseases, the distribution and genetic characteristics of DEC pathotypes, and the phenotypic and genotypic characteristics of antimicrobial susceptibility of *E. coli*. In Ethiopia, some studies also attempted to determine the determinants of diarrheal diseases among specific age groups in a single area. Only a few studies have been conducted on genomic characterization of *E. coli*, mainly using PCR. To this end, comprehensive genomic data on the diversity of *E. coli* are important to clarify the evolutionary relationship and genetic relatedness between different

strains (Edward Alexander Catoiu *et al.*, 2023). This information can then be used for public health investigations, such as tracing the source and transmission patterns, identifying the emergence and spread of new, potentially more virulent or antibiotic-resistant strains, and to monitor the evolution and spread of strains in different geographical regions and populations.

3 Materials and Methods

3.1 Study setting

Ethiopia, the oldest independent country, located in the eastern part of Africa, lies between 3 and 15 degrees North latitude and 33 and 48 degrees East longitude. It is the second most populous nation in Africa after Nigeria and the tenth largest country in Africa, covering 1,104,300 square kilometer ("Ethiopia country factsheet", 2024). The country is divided into fourteen politically autonomous regional states and two chartered cities, the latter being Addis Ababa and Dire Dawa. The regional states are subdivided into zones and special "woredas" (districts).

The current study is conducted at Addis Ababa city, the capital of Ethiopia, and Hossana town, Hadiya zone, Central Ethiopia Region. Addis Ababa is an important administrative center not only for Ethiopia but also for the whole of Africa. The city was founded in 1886 and it is 7,726 feet (2,355 meters) above sea level and it is the third highest capital in the world (Uli Wessling Tolon, 2008). According to world population review, Addis Ababa has estimated population of 5,461,000 in 2023. The city is home to 23.8% of all urban dwellers in Ethiopia, and has an estimated density of 5936 per square kilometer. Almost all the Ethiopian ethnic groups are represented in Addis Ababa due to its position as capital of the country. The majority of the populations, mainly the urban poor, are living in a polluted environment, exposed to water and sanitation-related diseases (Beyene *et al.*, 2015). There were a total of 83 government-owned health centers (thirty-three in the sub-cities selected for the study) and 10 government hospitals in the Addis Ababa City Administration.

The other study area is Hossana town which is located in Hadiya zone. Hadiya zone is one of the central zones in Central Ethiopia Region. According to The World Fact book estimates as of March 2018 Hadiya population was projected to be 1.8 million inhabited in 3542.66 sq km area. Hossana town is the administrative center of the Hadiya Zone and Central Ethiopia Regional State. Hossana has a latitude and longitude of 7°33′N 37°51′E with an elevation of 2177 meters above sea level. The town is 230 km far from the capital Addis Ababa to South

West, with a total estimated population of 75,963. The zone is one of the most densely populated areas in Ethiopia. The population density of the zone is 415 people per square kilometer. The town has one government comprehensive specialized hospital, one private owned hospital, three health centers and eight urban health extension workers offices. Map of the study area is shown in Figure 4.

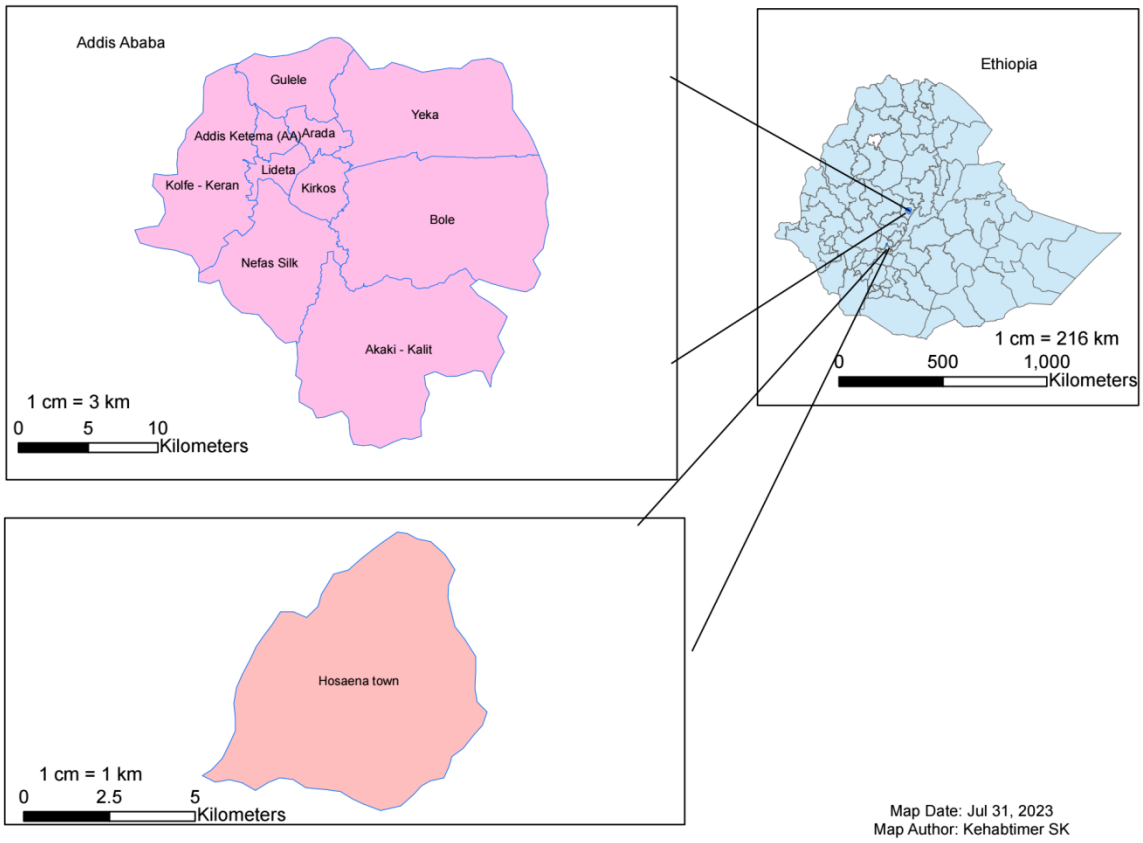

Figure 5: Maps of Addis Ababa and Hossana, Ethiopia.

3.2 Study design and study participants

In this study two study designs, namely, matched case-control and cross-sectional, were employed. A health institution-based matched case control study design with 1:1 ratio was used to identify determinants of diarrheal diseases, whereas a cross-sectional study design was employed to conduct the other objectives. Case and control were matched by age (maximum of three years of age difference). Patients of any age who had diarrhea and attended health centers (primary healthcare facilities) selected for the current study were

considered as cases. Patients with no symptom of diarrhea or gastrointestinal complaint for two weeks before enrolment in the study attending the same health facility were recruited as a control. Diarrhea was defined as a passage of three or more loose or liquid stools per day according to WHO guideline (World Health Organization, 2005). Consecutive cases of diarrheic patients of any age and sex group who visited the health facilities during the data collection period were included. The cases that constitute comparison groups were selected from the same health facilities that do not have symptoms of diarrhea, who visited the facilities for other health purposes during the study period, and for each case was age match control. Controls were recruited at the same date of the cases. Any participants who had a sibling recruited as a case or control and who had taken any antimicrobial agents in the last fifteen days prior to the study were excluded from the study. Potential controls who had diarrhea in the previous 7 days before initiation of the study were also excluded.

We used the isolates obtained from the participants used to address the other four objectives. In terms of the study design, this goes with cross-sectional design because the isolates were identified from a stool sample of patients who came to the health facility at a single point in time.

3.3 **Recruitment of study participants**

Multi-stage sampling method was used to select study participants in Addis Ababa. First, four sub-cities from Addis Ababa city administrative (which is 40% of the total) were selected. Then, 13 health centers (3 from Addis ketema, 4 from Arada, 4 from Kirkos, and 2 from Gulele) were randomly selected based on the number of health centers available in the sub-cities. Finally, study participants from the health facilities were recruited proportionally according to patient flow in the randomly selected health facilities. In Hossana town all three health centers were included and participants were recruited proportionally according to patient flow like Addis Ababa.

3.4 Sample size estimation

The sample size for identification of determinants of diarrhea was calculated by using Stata version 14.0 by considering 95% confidence level, power 90%, case to control ratio of 1:1, OR=2.68, design effect=2 and proportion of controls with exposure 35%. The odds ratio and proportion for control were taken from solid waste disposal category of a study conducted in Ethiopia by Mosisa *et al*., in 2021 (Mosisa *et al.*, 2021). After adding a 10% non-response rate, the final sample size for cases and controls in Addis Ababa was 488 (244 cases and 244 controls). The same sample size of 244 (122 cases and 122 controls) was used for Hossana without considering the design effect, as there was no multistage sampling process for selecting individual study participants. Overall, the total sample size used for assessing determinants of diarrhea was 732 (366 cases and 366 controls).

3.5 Data collection methods and instruments

3.5.1 Demographic data

Data on socio-demographic, clinical data and other risk factors were collected through face-to-face interview using a structured questionnaire. The questionnaire was prepared in English, translated into Amharic, and finally, translated back to English to check its consistency.

3.5.2 Anthropometric measurements

Weight and height of study participants were measured at enrollment to assess the influence of anthropometry on occurrence of diarrheal diseases. The standard procedure and measuring scale was utilized to determine the height. The height of infant age 6–23 months was measured in recumbent position while height of the other study participants was measured in standing position. The study participants were requested to takeoff their shoes before taking height measurement. Height measurement was taken to the nearest of 0.1cm. The calibrated beam balance was employed to determine the weight of the participants, and prior to each measurement, the scale was verified to ensure it was set to zero. Weight was measured to the

nearest 10g by removing heavy cloths. For a child who cannot stand alone, the mother was weighed together with a child and then without the child. The difference between the two measurements was taken as the child's weight.

3.5.3 Stool specimen collection and laboratory procedures

Patients who met the inclusion criteria were informed of the study's purpose and procedures and provided their informed consent before submitting a fresh stool sample in a dry, sterile, wide mouthed container with tight fitting leak proof lid containing Cary–Blair transport medium. Each stool specimen was carefully labeled with unique identifier and put in a cold box (4°C) containing ice packs to transport to Microbiology laboratory of Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University for processing.

3.5.4 Isolation and identification of *Escherichia coli*

About 1 g of stool specimen was pre-enriched in 9 ml of buffered peptone water (BPW) (Becton-Dickinson, Sparks, MD, USA) and incubated overnight at 37°C. It was then streaked onto eosin methylene blue agar (EMB) (Oxoid, Basingstoke, UK) and the plates were incubated at 37 °C for 24 hours. Colonies that showed a metallic green sheen were subjected to the different biochemical tests for identification including indole, citrate, urease, and oxidase tests. After preliminary confirmation, the *E. coli* isolate from each positive sample was stored at –80 °C in 20% glycerol until further testing.

For further testing, randomly selected isolates (260 isolates in total) were exported on slant agar to Slovenia. A computer-generated random sampling technique was used to select the isolates. Each strain was streaked on MacConkey agar (BD MacConkey II Agar, Becton Dickinson, Germany) at the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, for revitalization. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Microflex LT with regularly updated BruckerMS library, Brucker Daltonics, Bremen, Germany) was used to confirm *E. coli* before further analysis.

3.5.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was conducted using the disk diffusion method in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek *et al.*, 2014). Morphologically identical colonies were picked using a sterile cotton swab to make a suspension of the organism in saline to the density of a 0.5 McFarland turbidity standard. Colonies were suspended in pure saline until they reached the 0.5 McFarland standard. The McFarland densitometer was used to adjust the density of the suspension. The suspension was then inoculated on a Mueller-Hinton Agar (BD BBL MH II Agar, Becton Dickinson, USA) plate using an automatic plate rotator, and antimicrobial disks were placed onto the surface of the agar using disc dispenser. The following antimicrobial discs from BD-Becton Dickinson, USA were used: ampicillin (Am 10 μg), amoxicillin-clavulanic acid (AMC 20/10 μg), piperacillin-tazobactam (TZP 30/6 μg), cefuroxime (CXMP 30 μg), cefotaxime (CTX 5 μg), ceftriaxone (CRO 30 μg), ceftazidime (CAZ 10 μg), cefepime (FEP 30 μg), ertapenem (ETP 10 μg), imipenem (IPM 10 μg), meropenem (MEM 10 μg), gentamicin (GM 10 μg), amikacin (AN 30 μg), ciprofloxacin (CIP 5 μg), levofloxacin (LVX 5 μg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75 μg), aztreonam (ATM 30 μg), and tobramycin (NN 10 μg). Zones of inhibition were measured with a digital caliper. The results were interpreted according to EUCAST guidelines (Leclercq *et al.*, 2013; The European Committee on Antimicrobial Susceptibility testing, 2023). Multidrug-resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial groups (Magiorakos *et al.*, 2012). Extended-spectrum β-lactamase (ESBL) production among the strains was phenotypically determined using the double disk diffusion method suggested by the EUCAST. This involved a synergy test between a disk with amoxicillin-clavulanic acid and cephalosporins (cefotaxime and ceftazidime), which were separated by 2 to 3 cm from one another (Giske *et al.*, 2017). The presence of ESBL was confirmed if there were augmented inhibition zones around the cephalosporin disks or if a 'keyhole' appearance is observed towards the disk containing clavulanic acid.

3.5.6 DNA extraction and PCR detection of *E. coli* pathotypes genes

Genomic DNA was extracted using STARMag 96X4 Universal Cartridge Kit (Seegene Inc, Walnut Creek, CA, USA) on the automated system Seegene STARlet (Seegene) according to manufacturer's instructions. Multiplex real-time PCR was used to determine the presence of EPEC (*eaeA*), STEC (*stx1/stx2*), EAEC (*aggR*) and ETEC (*lt/st*) genes. The PCR reaction

was prepared instantly on the same automated system Seegene STARlet (Seegene) according to the manufacturer's instructions (Allplex™ GI-Bacteria (II) Assay, Seegene). Briefly, the 25 µl of PCR reaction contained 5 µl 5× MuDT Oligo Mix (amplification and detection reagent), 10 µl of RNase-free water, 5 µl of premix with DNA polymerase, Uracil-DNA glycosylase (UDG), and Buffer containing dNTPs and 5 µl of DNA template. As a negative control 5 µL of RNase-free water was used, whereas 5 µL of GI-B(II) PC was used as a positive control. The amplification and detection procedures were carried out on a CFX96™ Real-time PCR Detection System (CFX Manager™ Software-IVD v1.6) with steps: 20 min at 50 °C, 15 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 1 min at 60 °C and 30 sec at 72 °C (Seegene, 2022). The determination of EIEC was performed by real-time PCR, namely by detecting the invasion plasmid antigen H (*ipaH*) gene. The primers and probe targeting the *ipaH* gene were previously described by Wang *et al*. (2010). In brief, the 10-µl PCR reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 1× assay mix (mixture of 500 nM of both PCR primers and 200 nM of TaqMan probe labeled with FAM dye), and 1 µl of DNA template. PCR amplification (2 min at 50 °C, 10 min at 95 °C, followed by 35 cycles of 15 s at 95 °C and 1 min at 60 °C) and detection were performed using the QuantStudio 5 real-time PCR system (Applied Biosystems, USA) (Mainda *et al.*, 2019).

3.5.7 Whole-genome sequencing

All DEC isolates detected by real-time PCR and those resistant to third generation cephalosporins and producing ESBL were subjected to whole genome sequencing (WGS). A total of 30 DEC pathotypes were identified, but 5 failed quality assessment and 1 lacked DEC-specific genes, leaving 24 DEC isolates for final analysis. In addition, 2 DEC isolates were confirmed as ESBL producers. Of the isolates selected for WGS, 12 were resistant to third generation cephalosporins and 19 were ESBL producers. A total of 19 ESBL-producing and 10 cephalosporin-resistant isolates were included in the final analysis; whereas 2 of the cephalosporin-resistant isolates were excluded as they did not meet the quality criteria. After consideration of duplicates, 51 unique *E. coli* isolates were used for further analysis (Figure 1). Illumina DNA Prep (Illumina) was used to prepare the sequencing libraries and the NextSeq 2000 system (Illumina) with 2 × 150bp paired-end reads chemistry was used to sequence the isolates (Illumina, 2020). Trimmomatic was used to trim raw reads from adapter sequences and low-quality reads (Bolger *et al.*, 2014). The quality of both raw and trimmed

reads was assessed using FastQC v0.11.9 (Babraham Bioinformatics, n.d.). Species identification was confirmed with KmerFinder v3.0.2 based on trimmed reads. The trimmed reads were assembled into contigs with SPAdes v3.15.3 (Prjibelski *et al.*, 2020) using the default Kmer values and the parameters "--isolate". The quality assessment of the assemblies was performed with Quast v5.2.0 (Mikheenko *et al.*, 2018). An assembly coverage depth of > 30x, N50 > 15 kbp and < 500 contigs in the assembly were set as preliminary quality assessment criteria to consider the sequenced *E. coli* data for further bioinformatic analysis. To determine the assembly coverage, the fastq files were converted to .bam using samtools. Subsequently, »samtools depth« was used to determine base coverage, which was then averaged and reported. The laboratory workflow of the study is presented in Figure 5.

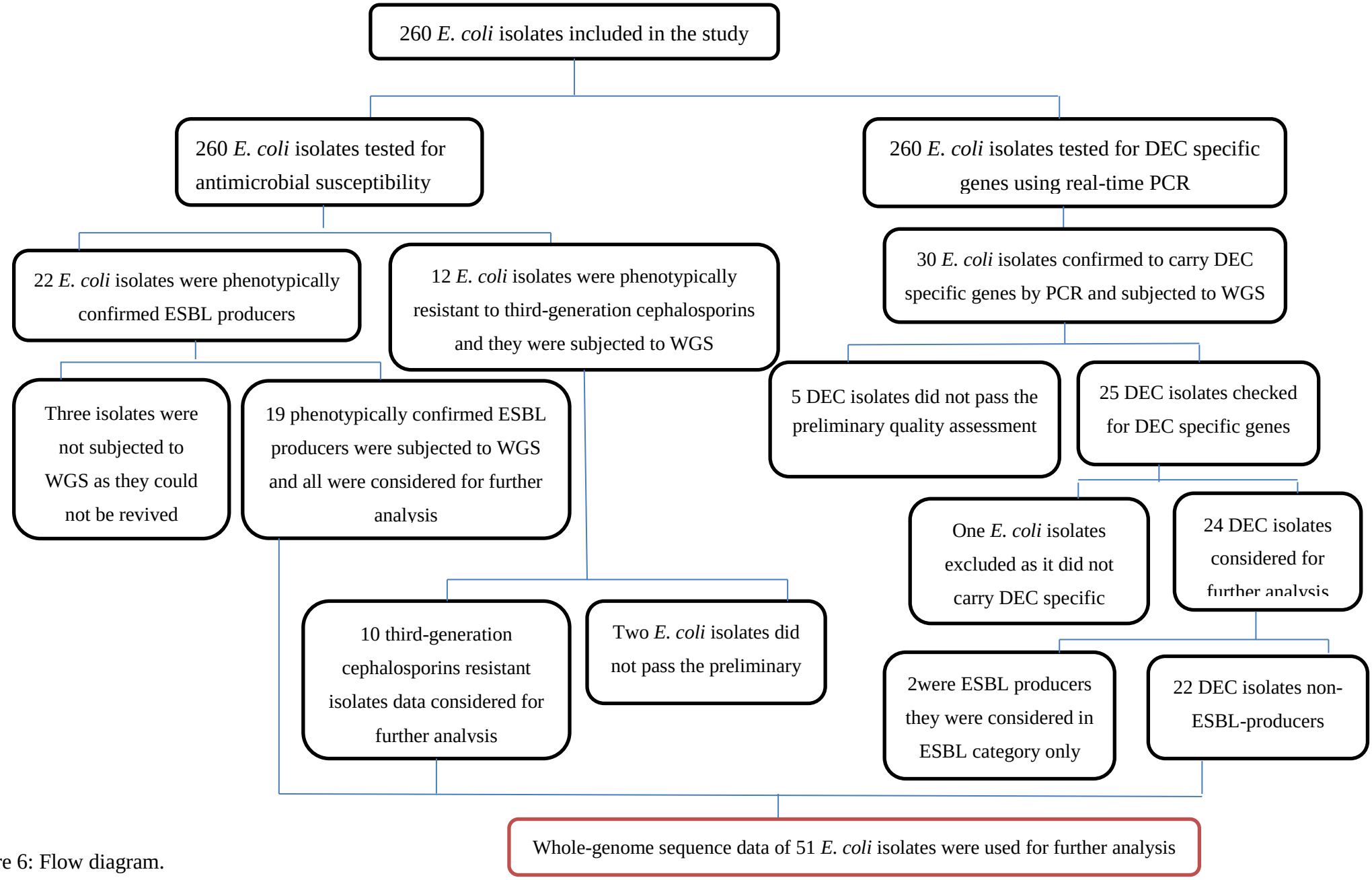

Figure 6: Flow diagram.

3.6 Bioinformatic analysis

The Clermon Typing tool available at http://clermontyping.iame-research.center/ was utilized to determine the phylogenetic groups of *E. coli* isolates (Beghain *et al.*, 2018). Sequence typing was conducted using the seven-gene Achtman scheme via https://pubmlst.org/ (Jolley *et al.*, 2018) and EnteroBase https://enterobase.warwick.ac.uk/ (Zhou *et al.*, 2020). The assembled genomes were compared using a curated core genome MLST scheme in SeqSphere+ (version 10.0.2, Ridom), available at https://www.ridom.de). A phylogenetic tree was generated using the neighbor-joining algorithm with the software's default settings (Saitou and Nei, 1987). Detection of *E. coli* VAGs and serotypes was performed using VirulenceFinder 2.0 and SerotypeFinder, accessible at www.genomicepidemiology.org, (Joensen *et al.*, 2014, 2015). Additionally, acquired antimicrobial resistance genes and chromosomal point mutations were identified using ResFinder 4.0 available at http://genepi.food.dtu.dk/resfinder with default settings (Bortolaia *et al.*, 2020). Plasmid replicons were detected using PlasmidFinder 2.1 at https://cge.food.dtu.dk/services/PlasmidFinder/ with default parameters (Carattoli *et al.*, 2014). Integrated mobile genetic elements in the assembled genomes were predicted using MobileElementFinder version 1.0 with MGEdb v1.0.2 available at https://cge.food.dtu.dk/services/MobileElementFinder/(Johansson *et al.*, 2021). OriTfinder was employed to identify conjugative transferable elements, available at https://tool2-mml.sjtu.edu.cn/oriTfinder/oriTfinder.html. The *stx*-carrying prophages from whole-genome sequenced STEC isolates were identified using the PHASTEST web-based tool at https://phastest.ca/ (Wishart *et al.*, 2023).

3.7 Data quality and management

Quality of the data was assured by different quality assurance activities, including standardization of procedures and providing training for data collectors. All laboratory tests were done by strictly following the standard operation procedures (SOPs) and manufacturer instructions. Two days training was given for data collectors and supervisors by expert who has experience on ODK. Reagents were checked for their expiry date before any analysis is started and a reference strains *E. coli* ATCC 25922 and negative controls (sterile distilled water) was used to check the performance of culture media for supporting the growth of

bacteria and checking the potency of antibiotics discs. Culture media were prepared by strict aseptic technique and 5% of these prepared media were checked for sterility by keeping them in the incubator at 37 °C for 24 hours.

3.8 Statistical analysis

Data analysis was done by Stata version 14.0. Continuous variables were described based on their distribution, either using the mean (standard deviation) or median (interquartile ranges), and categorical variables were reported as a number and percentage. The Shapiro-Wilk test was used to evaluate the normality of the quantitative variable data, and the variance inflation factor (VIF) test was used to check multi-collinearity. Conditional logistic regression was used to identify independent predictors after checking all the preliminary assumptions of the model. Variables with p-value less than 0.25 in a bivariable analysis were identified as candidate for multivariable model. In multivariable analysis, variables with p-value < 0.05 were reported as being statistically significant and AOR with 95% CI was reported as measures of the strength of the associations.

The laboratory data was captured in Microsoft Excel and analyzed using SPSS software (Version 25. IBM SPSS Inc, United States). Descriptive analyses using frequency and percentages were used to summarize categorical variables. Continuous variables were described based on their distribution, either using the mean (standard deviation) or median (interquartile ranges). Chi-square or Fisher exact test was used to investigate association between different variables. A p-value of < 0.05 was considered as indicator of statistically significant association.

3.9 Ethical considerations

Ethical approval was obtained from the institutional review board of ALIPB, AAU. Permission to conduct the study was obtained from the Addis Ababa Health Bureau and the Haddiya Zone Health Department. Informed consent or assent was obtained from each study participant. Patients were informed that they had the right to withdraw from the study at any

time and that participation in the study was completely voluntary. All information obtained from participants during the study was kept confidential. The collected stool samples were used only for the stated objectives.

Operational definitions

Diarrhea: is defined as having three or more loose or watery stool in a 24 hour period.

Completely vaccinated: children who received all childhood vaccines according to Ethiopia national immunization program.

Partially vaccinated: children who have received one or more immunizations according to the National Immunization Schedule, but have not completed all of them.

Cleaning frequency of water fetching devices:

Frequently: cleaning fetching materials four to six times per week.

Occasionally: cleaning fetching materials one to three times per week.

Rarely: not cleaning fetching materials at least once a week.

4 Results

The key findings of this study are published in three articles (Paper 2, Paper 3, and Paper 4) and one paper accepted for publication (Paper 1). Additional results which are not included in the articles are also added in the dissertation. The findings from Paper 2 and Paper 4 regarding phylogeny, serotypes, STs, and VAGs have been merged. Results from Paper 2 and Paper 3 concerning phenotypic antimicrobial resistance, as well as the genotypic antimicrobial resistance characteristics from all three published papers are merged and presented in this thesis. The term phylogroup and phylogenetic group are used interchangeably both in published papers and in the dissertation.

Table 1: Sample size/isolates that contributed data for the analysis and write-up of various sections of the thesis.

Type of analysis	**Total sample size/ number of isolates**	**Sample size/ number of isolates Addis Ababa**	**Sample size/ number of isolates Hossana**
Risk factor analysis	732	488	244
Molecular identification of DEC isolates	260	147	113
Phenotypic antimicrobial susceptibility testing	260	147	113
Whole-Genome Sequencing based analysis of DEC isolates, phenotypically confirmed ESBL-producing and/or third-generation cephalosporin resistant *E. coli* isolates	51	23	28

4.1 Socio-demographic and socio-economic characteristics of study participants

A total of 732 participants (366 cases and 366 controls) were included in the current study. The median age (IQR) was 18.0 (7.0–30.0) years for cases and 20.0 (8.0–30.0) years for controls. Significant proportions of participants were from 0-14 years of age (42.9% in the cases and 41.5% in the controls). The mean (±SD) number of rooms the house had 2.16 (±1.1) in the cases group and 2.40 (±1.1) in the controls group. Regarding household family

size, 66.4% of cases and 68.1% of controls had at least 4 members. On average, 4.14 (±1.5) persons live in the house of the cases and 4.25 (±1.5) persons live in the house of controls. Similarly, 2.48 (±1.1) persons in the house of cases and 2.44 (±1.1) persons in the house of controls share a single sleeping room (Table 2) (**Paper 1**).

Table 2: Socio-demographic characteristic of study participants from Addis Ababa and Hossana, Ethiopia

Characteristics	**Total**		**Addis Ababa**		**Hossana**	
	No. Cases (%)	**No. Control (%)**	**No. Cases (%)**	**No. Control (%)**	**No. Cases (%)**	**No. Control (%)**
Sex						
Male	193 (52.7)	196 (53.5)	131 (53.7)	139 (57.0)	62 (50.8)	57 (46.7)
Female	173 (47.3)	170 (46.5)	113 (46.3)	105 (43.0)	60 (49.2)	65 (53.3)
Marital status						
Never married	157 (42.9)	153 (41.8)	112 (45.9)	106 (43.4)	46 (37.7)	47 (38.5)
Single	86 (23.5)	87 (23.8)	62 (47.0)	65 (47.1)	24 (31.6)	22 (29.3)
Married	115 (31.4)	124 (33.9)	63 (47.7)	72 (52.2)	51 (67.1)	52 (69.3)
Divorced	8 (2.19)	2 (0.55)	7 (5.3)	1 (0.7)	1 (1.3)	1 (1.3)
Educational status						
No formal education	30 (8.2)	22 (6.01)	22 (9.0)	10 (4.1)	8 (6.6)	12 (9.8)
Pre-school	46 (12.57)	39 (10.66)	34 (13.9)	28 (11.5)	12 (9.8)	11 (9.0)
Primary school (1–8)	121 (33.1)	142 (38.8)	73 (29.9)	88 (36.1)	48 (39.3)	54 (44.3)
Secondary school (9–12)	95 (25.96)	84 (22.95)	66 (27.0)	63 (25.8)	29 (23.8)	21 (17.2)
Above secondary school	74 (20.22)	79 (21.58)	49 (20.1)	55 (22.5)	25 (20.5)	24 (19.7)
Occupation						
Student	145 (39.6)	163 (44.5)	117(48.0)	124 (50.8)	28 (23.0)	39 (32.0)
Government employee	55 (15.0)	67 (18.3)	35 (14.3)	47 (19.3)	20 (16.4)	20 (16.4)
NGO employee	5 (1.4)	4 (1.1)	2 (0.8)	1 (0.4)	3 (2.5)	3 (2.5)
Merchant	53 (14.5)	49 (13.4)	29 (11.9)	21 (8.6)	24 (19.7)	28 (23.0)
Other	108 (29.5)	83 (22.7)	61 (25.0)	51 (20.9)	47 (38.5)	32 (26.2)
Under-five year old child in the household						
No	209 (57.1)	220 (60.1)	150 61.5)	152 (62.3)	59 (48.4)	68 (55.7)

Yes	157 (42.9)	146 (39.9)	94 (38.5)	92 (37.7)	63 (51.6)	54 (44.3)
Immunization status						
Not vaccinated	2 (1.27)	1 (0.66)	2 (1.8)	0	0	1 (2.2)
Partially vaccinated	56 (35.67)	40 (26.32)	38 (35.2)	28 (26.4)	18 (39.1)	12 (27.3)
Totally vaccinated	99 (63.06)	111(73.03)	70 (64.8)	78 (73.6)	28 (60.9)	32 (72.7)

4.2 Environmental and behavioral characteristics

The environmental and behavioral characteristics of the study participants are presented in Table 3. The presence of domestic animals in the households was reported by 26.2% of cases and by 18.4% of controls in Addis Ababa, and 38.5% of cases and 22.1% of controls in Hossana. Of the total animals reported, cats accounted for 70.3% of cases and 65.3% of controls. Similarly, dogs were reported by 42.3% of cases and 34.7% of controls. In Addis Ababa, only 1.2% of cases and 0.4% of control reported using protected well or spring water for drinking. In Hossana, 23.0% of cases and 7.4% of controls reported using protected well or spring water as a source of drinking water. Overall, 18.0% of cases and 18.9% of controls treat their drinking water at home using various methods. More than half (56.5%) of cases and 36.9% of controls in Hossana used either an open pit latrine or a bush for defecation, while only 3.7% of cases and 1.6% of controls in Addis Ababa used these facilities for defecation. The average number of people sharing a toilet was 19.3 persons in the case group and 11.5 persons in the control group (**Paper 1**).

Table 3: Environmental and behavioral characteristic of study participants from Addis Ababa city and Hossana town, Ethiopia.

Variables	**Total**		**Addis Ababa**		**Hossana**	
	No. Cases (%)	**No. Control (%)**	**No. Cases (%)**	**No. Control (%)**	**No. Cases (%)**	**No. Control (%)**
Animals living in the house with people	111 (30.3)	72 (19.7)	64 (26.2)	45 (18.4)	47 (38.5)	27 (22.1)
Where do animals stay at night?						
Inside dwelling	32 (28.8)	29 (40.3)	13 (20.3)	14 (31.1)	19 (40.4)	15 (55.6)
Outside dwelling	30 (27.0)	16 (22.2)	14 (21.9)	7 (15.6)	16 (34.0)	9 (33.3)
Both outside and inside	49 (44.1)	27 (37.5)	37 (57.8)	24 (53.3)	12 (25.5)	3 (11.1)

Children had close contact with animals	42 (65.6)	13 (31.0)	26 (63.4)	8 (29.6)	16 (69.6)	5 (33.3)
Child plays with animal feed, animal dung	45 (70.3)	10 (23.8)	25 (61.0)	7 (25.9)	20 (87.0)	3 (20.0)
Drinking water source						
Tap water	309 (84.4)	304 (83.1)	217 (88.9)	200 (82.0)	92 (75.4)	104 (85.2)
Protected well/spring	31 (8.5)	10 (2.7)	3 (1.2)	1 (0.4)	28 (23.0)	9 (7.4)
Bottled water	26 (7.1)	52 (14.2)	24 (9.8)	43 (17.6)	2 (1.6)	9 (7.4)
Households do something to make drinking water safer to drink	66 (18.0)	69 (18.9)	50 (20.5)	47 (19.3)	13 (10.7)	12 (9.8)
What do you usually do to the water to make it safer to drink?						
Boil	7 (10.6)	8 (11.6)	4 (7.7)	7 (13.0)	3 (21.4)	1 (6.7)
Add liquid chlorine	17 (25.8)	16 (23.2)	17 (32.7)	16 (29.6)	0	0
Use a water filter	41 (62.1)	44 (63.8)	30 (57.7)	31 (57.4)	11 (78.6)	13 (86.7)
Other	1 (1.5)	1 (1.5)	1 (1.9)	0	0	1 (6.7)
Household have rack for drying dishes in home	136 (37.2)	162 (44.3)	96 (39.3)	90 (36.9)	40 (32.8)	52 (42.6)
Toilet facility						
No facilities or use open pit latrine	78 (21.3)	49 (13.4)	9 (3.7)	4 (1.6)	69 (56.5)	45 (36.9)
Flush toilet	15 (4.1)	16 (4.4)	9 (3.7)	16 (6.6)	6 (4.9)	0
Ventilated improved pit (VIP) latrine	8 (2.2)	6 (1.6)	5 (2.0)	3 (1.2)	3 (2.5)	3 (2.5)
Pit latrine with slab	265 (72.4)	295 (80.6)	221 (90.6)	221 (90.6)	44 (36.1)	74 (60.7)
How do you usually dispose of household rubbish?						
Rubbish pit	38 (10.4)	39 (10.7)	9 (3.7)	4 (1.6)	29 (23.8)	35 (28.7)
Put in garden	54 (14.8)	63 (17.2)	44 (18.0)	50 (20.5)	10 (8.2)	13 (10.7)
Put in bush	23 (6.3)	31 (8.5)	20 (8.2)	27 (11.1)	3 (2.5)	4 (3.3)
Open burning	91 (24.9)	99 (27.1)	37 (15.2)	39 (16.0)	54 (44.3)	60 (49.2)
Other	160 (43.7)	134 (36.6)	134 (54.9)	124 (50.8)	26 (21.3)	10 (8.2)
Patients who had known comorbidities	9 (2.5)	8 (2.2)	4 (1.6)	7 (2.9)	5 (4.1)	1 (0.8)
Patents who frequently wash hands	361 (98.6)	357 (97.5)	243 (99.6)	242 (99.2)	118 (96.7)	115 (94.3)
Patients who had habit of consuming raw vegetable	260 (71.0)	242 (66.1)	180 (73.8)	163 (66.8)	80 (65.6)	79 (64.8)
Patients who had habit of consuming raw milk	171 (46.7)	164 (44.8)	103 (42.2)	99 (40.6)	68 (55.7)	65 (53.3)
Household who had a habit of eating cooked food after its	239 (65.3)	240 (65.6)	138 (56.6)	139 (57.0)	101 (82.8)	101 (82.8)

overnight storage

4.3 Determinants of diarrheal disease among patients attending public health facilities

Results from multivariable logistic regression showed that use of tap water and use of protected well/spring water for drinking, child being partially vaccinated, a higher number of people sleeping in one room, contact with animal feces/feed, frequency of cleaning water fetching materials, BMI level, and unavailability of toilet facility or use of open pit latrine as independent determinants of diarrhea (Table 4) (**Paper 1**).

Table 4: Determinants of diarrheal disease among patients attending public health facilities in Addis Ababa city and Hossana town, Ethiopia.

Characteristics	Number (%) without diarrhea	Number (%) with diarrhea	AOR (95% CI)	p-value
Sex				
Male	196 (53.6)	193 (52.7)	1	
Female	170 (46.5)	173 (47.3)	1.38 (0.91, 2.10)	0.128
Income	6679.6(±4614.7)[#]	6129.1(±4582.5)[#]	1.00 (0.99, 1.00)	0.378
Children participants Immunization status				
Fully vaccinated	111 (30.3)	99 (27.1)	1	
Partially vaccinated	40 (10.9)	56 (15.3)	2.70 (1.23, 5.91)	**0.013***
Not vaccinated	1 (0.27)	2 (0.55)	11.53 (0.25, 320.3)	0.210
Education status				
No formal education	22 (6.0)	30 (8.2)	0.53 (0.20, 1.40)	0.202
Pre-school	39 (10.7)	46 (12.6)	0.48 (0.16, 1.42)	0.186
Primary (1-8)	142 (38.8)	121 (33.1)	0.35 (0.18, 0.69)	0.126
Secondary (9-12)	84 (23.0)	95 (26.0)	0.69 (0.39, 1.22)	0.198
Above secondary	79 (21.6)	74 (20.2)	1	
Number of living room	2.40 (±1.14)[#]	2.16 (±1.12)[#]	0.94 (0.77, 1.15)	0.523
Number of People sleeping in one room (mean ±SD)	2.8 (±1.2)[#]	2.4 (±0.9)[#]	1.55 (1.23, 1.92)	**0.001***
Presence of animals living in a household				
No	294 (80.3)	255 (69.7)	1	
Yes	72 (19.7)	111 (30.3)	1.80 (0.95, 3.40)	0.070

Children Playing with animals, feeds or dung				
No	32 (76.2)	19 (29.7)	1	
Yes	10 (23.8)	45 (70.3)	15.1 (4.23, 53.6)	**0.001***
Source of drinking water				
Tap water	304 (83.1)	309 (84.4)	2.20 (1.10, 4.40)	**0.027***
Protected well/spring	10 (2.7)	31 (8.5)	13.90 (3.74, 51.50)	**0.001***
Bottled water	52 (14.2)	26 (7.1)	1	
Type of toilet				
Flush toilet	16 (4.4)	15 (4.1)	1	
Ventilated improved pit (VIP) latrine	6 (1.6)	8 (2.2)	5.02 (0.74, 34.10)	0.100
Pit latrine wit slab	295 (80.6)	265 (72.4)	1.52 (0.50, 4.70)	0.467
No toilet facility or use open pit latrine	49 (13.4)	78 (21.3)	5.61 (1.50, 21.0)	**0.010***
Having rack for drying dishes				
No	208 (56.8)	224 (61.2)	0.58 (0.34, 1.00)	0.051
Yes	158 (43.2)	142 (38.8)	1	
Frequency of cleaning water fetching material				
Daily	74 (20.2)	43 (11.8)	1	
Frequently	58 (15.9)	46 (12.6)	1.10 (0.45, 2.70)	0.834
Occasionally	205 (56.0)	247 (67.5)	2.35 (1.22, 4.51)	**0.010***
Rarely	29 (7.9)	30 (8.2)	3.03 (1.24, 7.43)	**0.015***
Contact with diarrheic patient				
No	362 (98.9)	352 (96.2)	1	
Yes	4 (1.1)	14 (3.8)	2.10 (0.56, 7.74)	0.276
BMI	18.6 ($\pm$4.2)[#]	19.7 ($\pm$3.5)[#]	0.75 (0.65, 0.83)	**0.001***
Habit of eating raw vegetables				
No	124 (33.9)	106 (29.0)	1	
Yes	242 (66.1)	260 (71.0)	1.35 (0.88, 2.10)	0.170

*statistically significant ($p<0.05$); [#] mean (+SD)

4.4 Molecular identification of DEC isolates

The distribution and occurrence of DEC isolates obtained from patients attending public primary healthcare facilities is presented in **Paper 2**. Among 260 *E. coli* isolates, DEC pathotype specific genes were detected in 30 (11.5%) isolates by real-time PCR. No isolates were positive for *ipaH* gene (EIEC pathotype). Seventeen (56.7%) isolates were obtained

from stool specimens of diarrheic patients. The predominant DEC pathotype was EPEC (12/30; 40%), followed by EAEC (8/30; 26.7%) and ETEC (5/30; 16.7%). Four (13.3%) of *E. coli* isolates belonged to STEC pathotype. One isolate was determined as a hybrid strain EPEC/ETEC (3.3%). All STEC isolates were detected in non-diarrheic patients; however, the other pathotypes were detected from both diarrheic and non-diarrheic patients. The DEC pathotypes were detected more frequently in Addis Ababa than in Hossana. However, there was no significant difference in the distribution of DEC between Addis Ababa and Hossana (Fisher exact p = 0.491). In the age group 20–45 years, in general a high proportion of DEC isolates (46.7%) was detected (Table 5). The prevalence of DEC isolates was slightly higher (53.3 %) in females.

Table 5: Distribution of DEC isolates determined by PCR among study sites and patient's characteristics.

Characteristics	**DEC isolates** (n = number of isolates, %)				
	EPEC (n = 12)	EAEC (n = 8)	STEC (n = 4)	ETEC (n = 5)	EPEC/ETEC (n = 1)
Study site					
Addis Ababa	6 (20)	5 (16.7)	3 (10)	2 (6.7)	0
Hossana	6 (20)	3 (10)	1 (3.3)	3 (10)	1 (3.3)
Sex					
Male	5 (16.7)	4 (13.3)	2 (6.7)	1	0
Female	7 (23.3)	4 (13.3)	2 (6.7)	4 (13.3)	1 (3.3)
Participants					
Diarrheic	9 (30)	3 (10)	0	4 (13.3)	1 (3.3)
Non-diarrheic	3 (10)	5 (16.7)	4 (13.3)	1 (3.3)	0
Age group					
0–4	2 (6.7)	0	0	0	1 (3.3)
5–9	1 (3.3)	1 (3.3)	0	1(3.3)	0
10–14	3 (10)	0	1 (3.3)	0	0
15–19	1 (3.3)	1 (3.3)	0	1 (3.3)	0
20–45	5 (16.7)	5 (16.7)	2 (6.7)	2 (6.7)	0
46–65	0	1 (3.3)	1 (3.3)	1 (3.3)	0

4.5 Whole-genome sequencing based detection of DEC isolates

The distribution of DEC isolates based on WGS analysis is summarized in Tables 6. The twenty-four *E. coli* isolates carried DEC specific genes. DEC specific genes of four major pathotypes, including *eae-a01-α*, *eae-b01a-β*, *eae-e08-π*, *eae-g02-θ*, *eae-e02-ε* or *eae-e06-η* for EPEC, *aggR* for EAEC, *eltIAB-8*, *eltIAB-11*, or *estah-STa3* for ETEC, and *stx1*, *stx2*, *stx1a*, *stx1c*, *stx1-S*, or *stx2c* for STEC were detected. In addition, cytotoxic necrotizing factor 2 (*cnf2*), which is linked to cell death or tissue damage and recognized as a key virulence factor of necrotoxigenic *E. coli* (NTEC2), was found in an isolate that carried the *stx1* gene. This indicates that the isolate exhibits characteristics of a hybrid pathotype, incorporating

virulence factors from NTEC and STEC strains. The predominant DEC pathotype was EPEC (10/24; 41.7 %), followed by EAEC (6/24; 25 %) and ETEC (5/24; 20.8 %). Two (8.3%) of *E. coli* isolates belonged to STEC pathotype. Of the 24 DEC isolates, 14 (58.3%) were isolated from stool specimens of diarrheic patients (**Paper 2**).

Table 6: Distribution among study sites and patient's characteristics of DEC isolates.

Characteristics	**DEC isolates** (n = number of isolates, %)				
	EPEC (n = 10)	EAEC (n = 6)	ETEC (n = 5)	STEC (n = 2)	STEC/NTEC (n = 1)
Study site					
Addis Ababa	4 (16.7)	3 (12.5)	2 (8.3)	2 (8.3)	1 (4.2)
Hossana	6 (25)	3 (12.5)	3 (12.5)	0	0
Sex					
Male	6 (25.0)	3 (12.5)	0	1 (4.2)	0
Female	4 (16.7)	3 (12.5)	5 (20.8)	1 (4.2)	1 (4.2)
Participants					
Diarrheic	7 (29.2)	3 (12.5)	4 (16.7)	0	0
Non-diarrheic	3 (12.5)	3 (12.5)	1 (4.2)	2 (8.3)	1 (4.2)
Age group					
0-4	2 (8.3)	0	1 (4.2)	0	0
5-9	1 (4.2)	1 (8.3)	0	0	0
10-14	2 (8.3)	0	0	0	1 (4.2)
15-19	0	1 (4.2)	1 (4.2)	0	0
20-45	5 (20.8)	3 (12.5)	2 (8.3)	1 (4.2)	0
46-65	0	1 (4.2)	1 (4.2)	1 (4.2)	0

4.6 Genetic characteristics of *Escherichia coli*

The genome size of the 51 sequenced *E. coli* isolates ranged from 4,590,218 to 5,379,764 base pairs, with an average GC content of 50.65%. The number of contigs varied from 47 to 361, with an average of 138.43 contigs. The phylogenetic tree showed that *E. coli* isolates have significant genetic diversity (Figure 6).

4.6.1 Phylogenetic groups of *E. coli* isolates

This study revealed that 51 WGS *E. coli* isolates were distributed across seven phylogenetic groups (A, B1, B2, C, D, E and G). Phylogroup B1 (n = 25; 49%) was the predominant group, followed by phylogroup A (n = 17; 33.3%). Only two isolates belonged to phylogroups D and G (**Paper 2** and **4**). The DEC isolates were observed in four identified phylogenetic groups. The isolates belonging to phylogroup B1 contained more number of EPEC pathotype at 20.8% and low number of the STEC/NTEC hybrid pathotypes (4.2%).

None of the isolates from Addis Ababa belonged to phylogroups B2, C, D and G, while all phylogenetic groups were identified among isolates from Hossana. Phylogroups A, B1, C and E were distributed among both non-diarrheic and diarrheic patients. Twenty-five (49%) of the sequenced *E. coli* isolates were found in individuals aged 20 to 45 years, with these isolates distributed across various phylogenetic groups, excluding phylogroups D and G (Table 7) (**Paper 2** and **Paper 4**).

Table 7: Distribution of different *E. coli* phylogenetic groups among patients from study sites and their relations with other patients' characteristics.

Characteristics	Phylogroup (N = 51)						
	A N (%)	B1 N (%)	B2 N (%)	C N (%)	D N (%)	E N (%)	G N (%)
Study site							
Addis Ababa (*n* = 23)	11 (47.8)	10 (43.5)	0	0	0	2 (8.7)	0
Hossana (*n* = 28)	6 (21.4)	15 (53.6)	2 (7.1)	2 (7.1)	1 (3.6)	1 (3.6)	1 (3.6)
Sex							
Female (*n* = 25)	7 (28)	13 (52)	2 (8)	1 (4)	1 (4)	1 (4)	0
Male (*n* = 26)	10 (38.5)	12 (46.2)	0	1 (3.8)	0	2 (7.7)	1 (3.8)
Participants							
Diarrheic (*n* = 27)	11 (40.7)	12 (44.4)	0	1 (3.7)	1 (3.7)	2 (7.4)	0
Non-diarrheic (*n* = 24)	6	13	2	1	0	1	1
Age groups							
0–4 (*n* = 5)	3 (60)	1 (20)	0	1 (20)	0	0	0
5–9 (*n* = 6)	1 (16.7)	3 (50)	0	0	1 (16.7)	0	1 (16.7)
10–14 (*n* = 6)	3 (50)	2 (33.3)	0	0	0	1 (16.7)	0
15–19 (*n* = 3)	2 (66.7)	1 (33.3)	0	0	0	0	0
20–45 (*n* = 25)	7 (28)	13 (52)	2 (8)	1 (4)	0	2 (8)	0
46–65 (*n* = 6)	1 (16.7)	5 (83.3)	0	0	0	0	0

4.6.2 Sequence types of *E. coli* isolates

A total of thirty-six distinct STs were identified among 51 WGS *E. coli* isolates. The most frequently identified ST was ST10 (n = 8; 15.7%), followed by ST48, ST224, ST314, ST345, ST410, ST517 and ST1136 (n = 2; 3.9% each). DEC isolates were belonged to 17 different STs. Only ST10 was shared between DEC and other *E. coli* isolates. This study identified a novel sequence type designated as ST15980, which belongs to the isolates from Hossana. ST131 was also found in an *E. coli* isolate from a non-diarrheic patient in Hossana. Of the total STs identified, only four ST10, ST48, ST155 and ST314 were detected in *E. coli* isolates from both Addis Ababa and Hossana. The EPEC pathotypes were associated with eight different STs, while EAEC, ETEC and STEC were associated with five, four and two different STs, respectively (**Paper 2** and **Paper 4**).

4.6.3 Serotypes of *E. coli* isolates

A total of 42 distinct serotypes were identified among the 51 *E. coli* isolates examined. The DEC isolates were divided into 21 different serotypes, while the EPEC isolates belonged to nine different serotypes. Eight isolates (15.7%) were categorized as 'rough,' including five DEC isolates from the pathotypes STEC, EPEC and ETEC (**Paper 2**). Of the total *E. coli* isolates analyzed, one (1.96%) was confirmed to lack expression of the H antigen and was classified as a non-DEC isolate. The most frequently identified serotype was H30 (n = 3; 5.9%), which was not detected in DEC isolates. Six serotypes, namely H21, H34, O134:H53, O8:H21, O112ab:H21, and O9a:H30, were each detected in two isolates. The serotype O153:H4 identified in this study was classified as ST131. Three serotypes, H21, H30, and O112ab:H21, were detected among isolates from both Addis Ababa and Hossana. All serotypes were unique to DEC and other *E. coli* isolate (**Paper 2** and **Paper 4**).

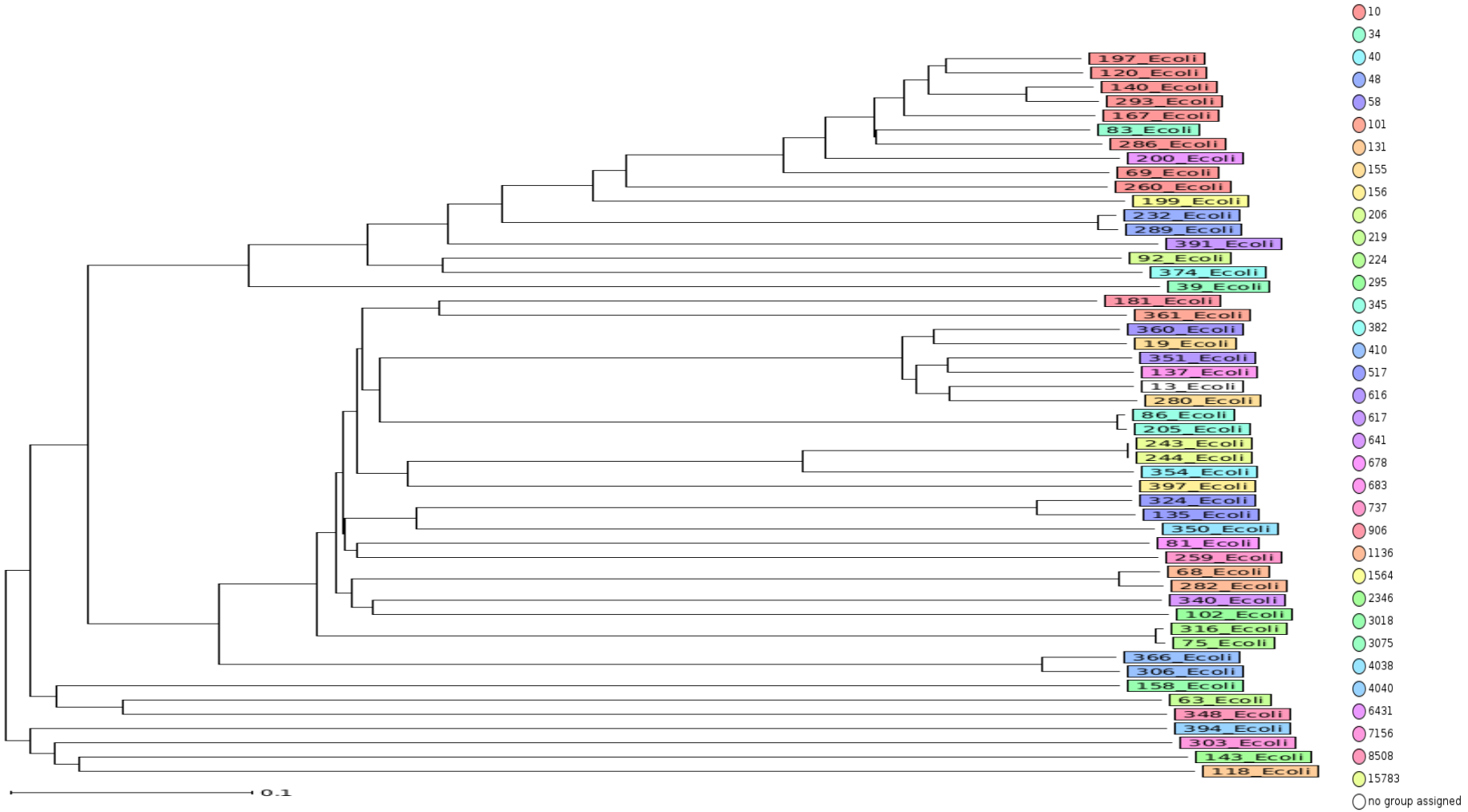

Figure 7: Phylogenetic tree of 51 WGS-confirmed *E. coli* isolates.

4.6.4 Virulence-associated gene profile of *E. coli* isolates

Overall, 131 different VAGs were detected in 51 whole-genome sequenced *E. coli* isolates in this study. Among these, 48 were adhesion-encoding genes, 50 were protectins-encoding genes, 23 were toxin-encoding genes, and 10 were iron acquisition genes. Of these, 55 genes including *aalF*, *aaiC*, *aap*, *aatA*, *cba*, *cea*, *cma*, *cswR*, *cvaC*, *iroN*, *tsh*, *etpC*, and *faeC*, were detected exclusively in DEC isolates. On average, each tested *E. coli* isolates carried 18.9 different VAGs, with a minimum of 8 and a maximum of 37 VAGs. In addition, all tested *E. coli* isolates carried the *terC* gene, which encodes for tellurite resistance, as well as the *nlpI* gene, which encodes an outer membrane lipoprotein. Forty-eight (94.1%) of the isolates had the *yehC* gene (chaperone, YhcD fimbrial cluster), 47 (92.2%) had *yehD* (major pilin subunit, YHD fimbrial cluster), and 46 (90.2%) had both *hlyE* (avian *E. coli* hemolysin) and *yehB* (usher, YhcD fimbrial cluster). Additionally, 45 (88.2%) of the isolates carried the *csgA* gene (curlin major subunit CsgA). Thirty-seven VAGs were detected in three or more *E. coli* isolates from Addis Ababa, while 38 VAGs were detected in three or more isolates from Hossana. Eighty-seven VAGs were detected in isolates from both Hossana and Addis Ababa. Twenty genes, including the *cdt-IIIB* gene (which encodes the CdtB subunit of the cytolethal distending toxin complex), the *cnf2* gene (which encodes cytotoxic necrotizing factor 2), *stx1* and *stx2*, *ehxA* (enterotoxin), and the *tsh* gene (which encodes serine protease autotransporter of *Enterobacteriaceae*, or SPATE), were exclusively detected in isolates from Addis Ababa. Conversely, 24 different genes, including capsular polysaccharide-related genes (*kpsE*, *kpsMII*, and *kpsMII_K5*), the *toxB* gene (which encodes toxin B), *hlyA*, *hlyF*, and the *usp* gene (uropathogenic specific protein), were found exclusively in isolates from Hossana. Bacteriocin and microcin encoding genes, namely *cba*, *cea*, *cia*, *cib*, *cma*, *colE2*, *colE5*, and *cva*, were detected in various *E. coli* isolates in this study (**Paper 2** and **Paper 4**).

A total of 117 different VAGs were identified among DEC isolates. VAGs were detected more frequently in the EPEC strains compared to other DEC pathotypes. All genes involved in toxin production identified in this study were found in DEC isolates. However, genes such as Shiga toxin-encoding genes (*stx1* or *stx2*), enterotoxin-encoding genes (including *eltIAB-8*, *eltIAB-11*, *estah-STa3*, *pet*, and *pic*), as well as *cnf2*, *ehxA*, *hlyA*, *hlyF*, *toxB*, and *vat* genes were detected exclusively in DEC isolates. Notably, none of the DEC isolates carried the *kpsE*, *kpsMII*, *kpsMII_K5*, or *usp* genes. The *hlyE* gene (91.7%), *yehB* (91.7%), *yehC*

(91.7%), *yehD* (87.5%), *csgA* (87.5%), and *fimH* (83.3%) were the most frequently detected genes among DEC isolates **(Paper 2**).

Phages associated with the *stx* genes were identified in STEC isolates using PHASTEST. This analysis revealed that the *stx1* gene was carried by a complete, intact phage in one isolate (isolate 259); while the *stx2* gene was associated with an incomplete phage in another isolate (isolate 280). No phage sequences were identified in the contig harboring the *stx1* gene in a third STEC isolate (isolate 361), despite the contig being sufficiently long (**Paper 2**).

The *afaB*, *afaE*, and *kpsMII_K5* genes were only found in the O153:H4 serotype. This serotype also possessed genes involved in the biosynthesis and uptake of yersiniabactin, aerobactin, and salmochelin siderophores. The *faeC*, *colE2*, *cma*, *cba*, *aalF*, *ehxA*, and *vat* genes were exclusively detected in serotype O81:H21. The KpsE gene was also detected in this serotype (**Paper 2** and **Paper 4**).

Many VAGs were found in all phylogroups, with these genes being more prevalent in strains from phylogroup B1 compared to those from other groups. Eight VAGs (*csgA*, *fimH*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC* and *yehD*) were detected in isolates from all phylogenetic groups. Except in phylogroup B2, *gad* gene was also detected in all phylogenetic groups. Phylogroup A and B1 shared 57 similar VAGs, including *aggR*, *cif*, *tir*, *cdt-IIIB*, *hlyE*, *pic*, and *sat* genes. Thirty different VAGs were detected in phylogroup B2 strain. The *afaB*, *afaC*, *afaE*, *eae-a01-α*, espC, *kpsMII_K5*, perA, and yfcV genes were only detected in phylogroup B2 isolates. Eight VAGs such as *hlyE*, *nlpI*, *terC*, *yehB*, and *yehC* were identified in the entire phylogroup B1. The *neuC* gene, which encodes a polysialic acid capsule biosynthesis protein was detected only in phylogroup B1 and C, while the *kpsMII* gene, which encodes a polysialic acid transport protein group 2 capsule and the *usp* gene, which encodes a uropathogenic specific protein, were only detected in phylogroups D, and G, respectively (**Paper 2** and **Paper 4**).

Various virulence-associated genes (VAGs) were identified in specific STs of *E. coli*. Thirty-seven were detected in only a single *E. coli* isolate of particular STs. ST131 carried 29 different VAGs, including *sat*, *senB*, *fyuA*, *irp2*, *iucC*, *iutA*, *kpsE*, *kpsmII-k5*, *aslA*, *iha*, *iss*, and *sitA*.

4.7 Antimicrobial susceptibility of *E. coli* isolates

The overall antimicrobial susceptibility pattern of *E. coli* isolates is summarized in Table 8. *E. coli* isolates showed different susceptibility patterns to the tested antibiotics. Of the total *E. coli* isolates included in the study, 119 (45.8%) were susceptible to all tested antimicrobials, including 12 (40%) of the DEC isolates. Higher resistance rates among the *E. coli* isolates was observed for ampicillin and trimethoprim-sulfamethoxazole, followed by amoxicillin-clavulanic acid, cefuroxime, ceftriaxone, and cefotaxime. All isolates were sensitive to carbapenems (ertapenem, imipenem, and meropenem) as well as amikacin. All DEC isolates were also susceptible to eight antimicrobials tested, carbapenems (ertapenem, imipenem, and meropenem), aminoglycosides (amikacin, gentamicin, and tobaramycin), levofloxacin, and piperacillin-tazobactam. In addition, DEC isolates showed high susceptibility to ciprofloxacin (96.7%), ceftazidime (93.3%), and cefepime (93.3%). A higher resistance rate was observed in *E. coli* isolates from patients in Hossana town for all other antimicrobials, with the exception of ampicillin and amoxicillin-clavulanic acid. The chi-square test showed a statistically significant difference in antimicrobial susceptibility patterns in Addis Ababa and Hossana ($p < 0.05$). Resistance to aztreonam ($p = 0.045$), ciprofloxacin ($p = 0.023$), cefotaxime ($p = 0.033$), cefepime ($p = 0.041$), and levofloxacin ($p = 0.020$) was significantly higher among *E. coli* isolates from Hossana compared to those from Addis Ababa (**Paper 3**).

Table 8: Antimicrobial susceptibility of studied *E. coli* isolates.

Antibiotic	**S (Susceptible, Standard Dosing Regimen)**				**I (Susceptible, Increased Exposure)**				**R (Resistant)**			
	Total isolates (*n* = 260)	**DEC isolates (*n* = 30)**	**Addis Ababa isolates (*n* = 147)**	**Hossana isolates (*n* = 113)**	**Total isolates (*n* = 260)**	**DEC isolates (n = 30)**	**Addis Ababa isolates (*n* = 147)**	**Hossana isolates (*n* = 113)**	**Total isolates (*n* = 260)**	**DEC isolates (*n* = 30)**	**Addis Ababa isolates (*n* = 147)**	**Hossana isolates (*n* = 113)**
	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
AM	123 (47.3)	13 (43.3)	73 (49.7)	50 (44.2)	0	0	0	0	137 (52.7)	17 (56.7)	74 (50.3)	63 (55.7)
AMC	223 (85.8)	26 (86.7)	123 (83.7)	100 (88.5)	0	0	0	0	37 (14.2)	4 (13.3)	24 (16.3)	13 (11.5)
TZP	257 (98.8)	30 (100)	145 (98.6)	112 (99.1)	0	0	0	0	3 (1.2)	0	2 (1.4)	1 (0.9)
CXMp	0	0	0	0	226 (86.9)	27 (90)	133 (90.5)	93 (82.3)	34 (13.1)	3 (10)	14 (9.5)	20 (17.7)
CTX	226 (86.9)	27 (90)	133 (90.5)	93 (82.3)	1 (0.4)	0	1 (0.7)	0	33 (12.7)	3 (10)	13 (8.8)	20 (17.7) *
CRO	226 (86.9)	27 (90)	133 (90.5)	93 (82.3)	0	0	0	0	34 (13.1)	3 (10)	14 (9.5)	20 (17.7)
CAZ	232 (89.2)	28 (93.3)	137 (93.2)	95 (84.1)	6 (2.3)	1	1 (0.7)	5 (4.4)	22 (8.5)	1 (3.3)	9 (6.1)	13 (11.5)
FEP	234 (90.0)	28 (93.3)	138 (93.9)	96 (85.0)	8 (3.1)	1	3 (2.0)	5 (4.4)	18 (6.9)	1 (3.3)	6 (4.1)	12 (10.6) *
ETP	260 (100)	30 (100)	147 (100)	113 (100)	0	0	0	0	0	0	0	0
IPM	260 (100)	30 (100)	147 (100)	113 (100)	0	0	0	0	0	0	0	0
MEM	260 (100)	30 (100)	147 (100)	113 (100)	0	0	0	0	0	0	0	0
ATM	227 (87.3)	27 (90)	133 (90.5)	94 (83.2)	13 (5.0)	2 (6.7)	7 (4.8)	6 (5.3)	20 (7.7)	1 (3.3)	7 (4.8)	13 (11.5) *
AN	260 (100)	30 (100)	147 (100)	113 (100)	0	0	0	0	0	0	0	0
GM	255 (98.1)	30 (100)	146 (99.3)	109 (96.5)	0	0	0	0	5 (1.9)	0	1 (0.7)	4 (3.5)
NN	257 (98.8)	30 (100)	147 (100)	110 (97.3)	0	0	0	0	3 (1.2)	0	0	3 (2.7)
CIP	238 (91.5)	29 (96.7)	140 (95.2)	98 (86.7)	3 (1.2)	1 (3.3)	1 (0.7)	2 (1.8)	19 (7.3)	0	6 (4.1)	13 (11.5) *
LVX	240 (92.3)	30 (100)	140 (95.2)	100 (88.5)	2 (0.8)	0	2 (1.4)	0	18 (6.9)	0	5 (3.4)	13 (11.5) *
SXT	183 (70.4)	21 (70)	109 (74.1)	74 (65.5)	0	0	0	0	77 (29.6)	9 (30)	38 (25.9)	39 (34.5)

AM—ampicillin, AMC—amoxicillin-clavulanic acid, AN—amikacin, ATM—aztreonam, CAZ—ceftazidime, CIP—ciprofloxacin, CRO—ceftriaxone, CTX—cefotaxime, CXMp—cefuroxime-parenteral, ETP—ertapenem, FEP—cefepime, GM—gentamicin, IPM—imipenem, LVX—levofloxacin, MEM—meropenem, NN—tobramycin, SXT—trimethoprim-sulfamethoxazole, TZP—piperacillin-tazobactam and * indicates statistically significant resistance ($p < 0.05$).

Among the 18 tested antibiotics, a total of 54.2% *E. coli* isolates in this study showed resistance to at least one antibiotic from nine antibiotic classes, with 51.7% from Addis Ababa and 57.5% from Hossana. Among the DEC isolates, 18 (60%) were resistant to at least one of the tested antimicrobials. Resistance to a single agent, such as ampicillin or trimethoprim-sulfamethoxazole, was observed in 24.1% of the tested *E. coli* isolates. Multidrug resistance (MDR) was detected in 50 (19.2%) isolates, including 3 (6%) DEC isolates. Sixteen isolates from Hossana and 11 from Addis Ababa were resistant to six or more of the tested antimicrobials, accounting for 10.4% of the total. Thirteen (5.0%) isolates were resistant to all tested cephalosporins, and none of these were DEC isolates (**Paper 3**).

The findings of this study revealed that the prevalence of MDR *E. coli* was significantly higher in isolates from Hossana compared to those from Addis Ababa ($X^2 = 5.27$, $p = 0.022$). The majority (59.2%) of MDR *E. coli* isolates were obtained from male patients. In addition, individuals aged 20 to 45 years contributed to 44.9% of the MDR *E. coli* isolates. All MDR isolates were resistant to ampicillin, 75.5% were resistant to trimethoprim-sulfamethoxazole, 71.4% were resistant to ceftriaxone, and 69.4% were resistant to cefotaxime. Varied resistance patterns were observed in *E. coli* isolates in this study. A total of 33 resistance patterns were identified, including 6 among DEC isolates: 20 in Addis Ababa, 25 in Hossana, and 12 that were common to both locations. The most frequently observed MDR pattern was AM, AMC, SXT, followed by AM, CIP, LVX, SXT (Table 9) (**Paper 3**).

Table 9: Antimicrobial resistance patterns of *E. coli* isolates.

Antimicrobial Resistance Pattern	Resistant Isolates (*n* = 141), Resistant DEC isolates (n = 18) Addis Ababa (*n* = 77); Hossana (*n* = 64)					ESBL-Producing Isolates (*n* = 22), ESBL-producing DEC (n = 2) Addis Ababa (*n* = 9); Hossana (*n* = 13)			
	No. of Antimicrobial Groups in Resistance Pattern	No. of Isolates with This Resistance Pattern *n* (%)	No of DEC isolates with this resistance pattern *n* (%)	No. of Isolates with This Resistance Pattern from Addis Ababa *n* (%)	No. of Isolates with This Resistance Pattern from Hossana *n* (%)	No. of ESBL-Producing Isolates with This Resistance Pattern *n* (%)	No of DEC isolates with this resistance pattern *n* (%)	No. of ESBL Isolates with This Resistance Pattern from Addis Ababa *n* (%)	No. of ESBL Isolates with This Resistance Pattern from Hossana *n* (%)
AM	1	30 (21.3)	5 (27.8)	16 (20.8)	14 (21.9)	0	0	0	0
SXT	1	4 (2.8)	1 (5.6)	2 (2.6)	2 (3.1)	0	0	0	0
AM, SXT	2	37 (26.2)	6 (33.3)	22 (28.6)	15 (23.4)	0	0	0	0
AM, AMC	2	20 (14.2)	3 (16.7)	17 (22.1)	3 (4.7)	0	0	0	0
AM, AMC, TZP	3	1 (0.7)	0	1 (1.3)	0	0	0	0	0
AM, AMC, SXT	3	7 (5.0)	0	3 (3.9)	4 (6.3)	0	0	0	0
AM, CIP, LVX, SXT	3	5 (3.5)	0	1 (1.3)	4 (6.3)	0	0	0	0
AM, SXT, CXMp, CRO	4	1 (0.7)	0	1 (1.3)	0	1 (4.5)	0	1 (11.1)	0
AM, AMC, GM, SXT	4	1 (0.7)	0	0	1 (1.6)	0	0	0	0
AM, ATM, SXT, CAZ	4	1 (0.7)	0	1 (1.3)	0	0	0	0	0
AM, GM, CXMp, CTX, CRO	4	1 (0.7)	0	0	1 (1.6)	1 (4.5)	0	0	1 (7.7)
AM, SXT, CXMp, CTX, CRO	4	4 (2.8)	1 (5.6)	2 (2.6)	2 (3.1)	2 (9.1)	0	1 (11.1)	1 (7.7)
AM, CIP, GM, LVX, NN	3	1 (0.7)	0	0	1 (1.6)	0	0	0	0
AM, CXMp, CTX, CRO, FEP	3	1 (0.7)	0	0	1 (1.6)	1 (4.5)	0	0	1 (7.7)
AM, SXT, GM, CTX, CXMp, CRO	5	1 (0.7)	0	1 (1.3)	0	1 (4.5)	0	1 (11.1)	0
AM, AMC, CXMp, CTX, CRO, FEP	4	2 (1.4)	1 (5.6)	1 (1.3)	1 (1.6)	2 (9.1)	1 (50)	1 (11.1)	1 (7.7)
AM, ATM, CXMp, CTX, CRO, CAZ	4	2 (1.4)	0	1 (1.3)	1 (1.6)	0	0	0	0
AM, SXT, CXMp, CTX, CRO, CAZ	4	2 (1.4)	0	1 (1.3)	1 (1.6)	1 (4.5)	0	0	1 (7.7)
AM, CIP, LVX, CXMp, CTX, CRO	4	1 (0.7)	0	1 (1.3)	0	1 (4.5)	0	1 (11.1)	0
AM, SXT, CXMp, CTX, CRO, CAZ, FEP	4	1 (0.7)	0	1 (1.3)	0	0	0	0	0
AM, ATM, SXT, CXMp, CTX, CRO, CAZ	5	2 (1.4)	1 (5.6)	0	2 (3.2)	2 (9.1)	1 (50)	0	2 (15.4)
AM, ATM, CXMp, CTX, CRO, CAZ, FEP	4	1 (0.7)		0	1 (1.6)	1 (4.5)	0	0	1 (7.7)
AM, ATM, SXT, CXMp, CTX, CRO, CAZ, FEP	5	3 (2.1)		1 (1.3)	2 (3.2)	2 (9.1)	0	1 (11.1)	1 (7.7)
AM, ATM, CIP, LVX, CXMp, CTX,	5	1 (0.7)		0	1 (1.6)	1 (4.5)	0	0	1 (7.7)

CRO, FEP								
AM, AMC, SXT, CIP, LVX, CXMp, CTX, CRO	6	1 (0.7)	0	1 (1.6)	0	0	0	0
AM, ATM, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	5	1 (0.7)	0	1 (1.6)	0	0	0	0
AM, AMC, TZP, ATM, CXMp, CTX, CRO, CAZ, FEP	6	1 (0.7)	1 (1.3)	0	1 (4.5)	0	1 (11.1)	0
AM, AMC, ATM, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	6	1 (0.7)	0	1 (1.6)	0	0	0	0
AM, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	6	3 (2.1)	2 (2.6)	1 (1.6)	3 (13.6)	0	2 (22.2)	1 (7.7)
AM, AMC, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ	7	1 (0.7)	1 (1.3)	0	0	0	0	0
AM, AMC, TZP, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	8	1 (0.7)	0	1 (1.6)	1 (4.5)	0	0	1 (7.7)
AM, AMC, ATM, SXT, CIP, LVX, NN, CXMp, CTX, CRO, CAZ, FEP	8	1 (0.7)	0	1 (1.6)	1 (4.5)	0	0	1 (7.7)
AM, AMC, ATM, SXT, CIP, LVX, GM, NN, CXMp, CTX, CRO, CAZ, FEP	8	1 (0.7)	0	1 (1.6)	0	0	0	0

AM—ampicillin, AMC—amoxicillin-clavulanic acid, AN—amikacin, ATM—aztreonam, CAZ—ceftazidime, CIP—ciprofloxacin, CRO—ceftriaxone, CTX—cefotaxime, CXMp—cefuroxime-parenteral, ETP—ertapenem, FEP—cefepime, GM—gentamicin, IPM—imipenem, LVX—levofloxacin, MEM—meropenem, NN—tobramycin, SXT—trimethoprim-sulfamethoxazole, and TZP—piperacillin-tazobactam.

4.8 Phenotypic characterization of ESBL-producing *E. coli* isolates

Twenty-two (8.5%) of *E. coli* isolates were phenotypically confirmed to produce ESBL (**Paper 3**). Among these, 2 strains (9.1%) were classified as DEC isolates (**Paper 2**). All ESBL-producing *E. coli* isolates were resistance to ampicillin, ceftriaxone, and cefuroxime, with 95.5% also resistant to cefotaxime. The ESBL-producing strains exhibited resistance rates ranging from 22.7% to 63.6% against amoxicillin-clavulanic acid, levofloxacin, ciprofloxacin, aztreonam, ceftazidime, cefepime, and trimethoprim-sulfamethoxazole. Lower resistance rates were noted for piperacillin-tazobactam (9.1%), gentamicin (9.1%), and tobramycin (4.5%) among the ESBL-producing *E. coli*. Over half (59.1%) of the ESBL-producing *E. coli* isolates were from the Hossana. However, no significant difference was observed in the distribution of ESBL-producing *E. coli* between the isolates from Addis Ababa and Hossana (X^2 = 2.39, p = 0.122). The ESBL-producing strains showed sixteen different resistance patterns for the tested antimicrobials (Table 9) (**Paper 3**).

4.9 Antimicrobial resistance genes

The frequency of ARGs among *E. coli* isolates is reported in Table 10. A total of 46 different ARGs were detected in 42 (82.4%) of the *E. coli* isolates tested. Of the total *E. coli* isolates tested, 72.5% carried resistance genes conferring resistance to β-lactam antibiotics, and 54.9% carried resistance genes for quinolone resistance. Among the DEC isolates, 23 different ARGs conferring resistance to β-lactams, aminoglycosides, quinolones, sulfonamides, trimethoprim, macrolides and tetracyclines were identified. The $bla_{TEM\text{-}1B}$ gene was the predominant gene detected in both DEC and non-DEC isolates (**Paper 2** and **Paper 4**). The findings showed that the EPEC and ETEC isolates carried many ARGs that confer resistance to different antimicrobials. In contrast, the STEC isolates exhibited no resistance to any of the tested antibiotics and lacked any of the ARGs (**Paper 2**). Of the total *E. coli* isolates carrying ARGs, 24 carried $bla_{TEM\text{-}1B}$, 22 $bla_{CTX\text{-}M\text{-}15}$ and 17 *tet(A)* genes. Of the phenotypically confirmed ESBL-producing *E. coli* isolates, 78.9% possessed the $bla_{CTX\text{-}M\text{-}15}$ gene. In addition, 70% of the third-generation cephalosporin-resistant *E. coli* isolates also had the $bla_{CTX\text{-}M\text{-}15}$ gene. On average, the *E. coli* isolates tested carried 5 ARGs, with a maximum of 14 ARGs. In particular, two *E. coli* isolates from Hossana contained 14 different resistance

genes conferring resistance to different classes of antibiotics. Nine (90%) of *E. coli* isolates resistant to third-generation cephalosporins carried genes encoding for β-lactamase and quinolone resistance (**Paper 4**) (Table 10).

Plasmid-mediated quinolone resistance (PMQR) determinants, including *qnrS1*, *aac(6')-Ib-cr*, *qepA1*, *qepA2*, and *qepA4*, were identified, with *qnrS1* being the most prevalent, detected in 14 (27.5%) of the isolates. Among the *E. coli* isolates tested, 31.4% exhibited chromosomal mutations in the *gyrA*, *parC*, and/or *parE* genes, which are linked to resistance against quinolones such as ciprofloxacin and nalidixic acid. Double mutations in *gyrA* (S83L + D87N) were found in 8 isolates (15.7%), and these isolates also had an additional mutation in the *parC* (S80I) and *parE* (S458A) genes. Single amino acid substitutions were identified in the *gyrA* gene, including S83L (n = 3), S83A (n = 1), and S83V (n = 1). In the *parC* gene, mutations S80I (n = 6), E84K (n = 1), and A56T (n = 1) were observed, while in *parE*, mutations S458A (n = 7), L416F (n = 1), and I529L (n = 1) were noted. Additionally, mutations in the QRDR and PMQR genes co-occurred in 5 (18.5%) of the *E. coli* isolates that carried genes associated with quinolone resistance. Mutations in *gyrA*, *parC* and *parE* were associated with resistance to fluoroquinolones, ciprofloxacin and nalidixic acid. The presence of PMQR alone was associated with resistance to ciprofloxacin.

The macrolide ARGs, such as *mph(A)* and *erm(B)* genes, were detected in 21.6% and 7.8% of the *E. coli* isolates, respectively. Two isolates carried both *mph(A)* and *erm(B)* genes. The chloramphenicol resistance gene *catA1* was detected in three *E. coli* isolates. In addition, the sulfonamide resistance genes *sul1* and *sul2* as well as eight trimethoprim resistance genes, namely *dfrA1*, *dfrA7*, *dfrA8*, *dfrA12*, *dfrA14*, *dfrA15*, *dfrA17* and *dfrB4*, were detected in the *E. coli* isolates in this study. In addition, tetracycline resistance genes (*tet(A)* or *tet(B)*) were detected in 24 *E. coli* isolates. Sixteen isolates carried *tet(A)* alone, while seven isolates carried the *tet(B)* gene. Only one strain carried both *tet(A)* and *tet(B)*. The *tet(A)*-carrying isolates were resistant to tetracycline and doxycycline, while the *tet(B)*-positive isolates were resistant to tetracycline, doxycycline and minocycline (**Paper 2** and **Paper 4**).

E. coli isolates from all phylogenetic groups carried a variety of ARGs, with the highest frequency detected in phylogroups A and B1. In contrast, only four ARGs, such as $bla_{CTX\text{-}M\text{-}15}$, *gyrA:p.S83L*, *parE:p.I529L*, and *mph(A)* were identified in *E. coli* isolates belonging to phylogenetic group B2. Phylogroups A and B1 were confirmed to share 21 different ARGs. *E. coli* serotypes (H30, O138:H48, O101:H10, O174:H28, O134:H53, O9a:H30, O8:H21) were found to harbor between 10 and 14 different ARGs, with the O9a:H30 and O8:H21

serotypes carrying the highest number (14) of ARGs. This study identified a high proportion of ARGs in ST224 and ST410 (**Paper 1** and **Paper 3**).

Table 10: Distribution of antimicrobial resistance genes and chromosomal mutations among studied *E. coli* isolates.

Antimicrobial Class	Antimicrobial Resistance Genes/Mutations Detected	No (%) of ESBL-Producing Isolates Possessing the Gene/Mutation (*n* = 19)	No (%) of 3rd-Generation-Cephalosporin-Resistant Isolates Possessing the Gene/Mutation (*n* = 10)	DEC isolates Possessing the Gene/Mutation (*n* = 24*)	Total no (%) of Isolates Possessing the Gene/Mutation (*n* = 51)
β-lactams	*bla*$_{\text{CTX-M-15}}$	15 (78.9)	7 (70)	2 (8.3)	24 (41.1)
	bla$_{\text{CTX-M-3}}$	4 (21.1)	0	1 (4.2)	4 (7.8)
	bla$_{\text{TEM-1B}}$	10 (52.6)	5 (50)	12 (50)	26 (50.96)
	bla$_{\textit{TEM-122}}$	0	0	1 (4.2)	1 (1.96)
	bla$_{\textit{TEM-163}}$	0	0	1 (4.2)	1 (1.96)
	bla$_{\text{TEM-169}}$	2 (10.5)	0	0	2 (3.9)
	bla$_{\text{TEM-33}}$	2 (10.5)	0	0	2 (3.9)
	bla$_{\text{SHV-12}}$	0	1 (10)	0	1 (1.96)
	bla$_{\text{OXA-1}}$	1 (5.3)	1 (10)	0	2 (3.9)
Aminoglycosides	*aph(6)-Id*	8 (42.1)	3 (30)	6	17 (33.3)
	aph(3")-Ib	8 (42.1)	3 (30)	6	17 (33.3)
	aac(3)-IIa			1 (4.2)	1 (1.96)
	aac(6')-Ib-cr	1 (5.3)	1 (10)	0	2 (3.9)
	aac(3)-IId	2 (10.5)	0	0	2 (3.9)
	aadA1	2 (10.5)	0	2 (8.3)	4 (7.8)
	aadA2	5 (26.3)	0	0	5 (9.8)
	aadA5	2 (10.5)	2 (20)	1 (4.2)	5 (9.8)
	aadA24	0	0	1 (4.2)	1 (1.96)
Quinolones	*qnrS1*	8 (42.1)	5 (50)	2 (8.3)	15 (29.4)
	qepA1	0	1 (10)	0	1 (1.96)
	qepA2	0	1 (10)	0	1 (1.96)
	qepA4	1 (5.3)	1 (10)	0	2 (3.9)
	gyrA:p.S83L	7 (36.8)	4 (40)	0	11 (21.6)
	gyrA:p.D87N	5 (26.3)	3 (30)	0	8 (15.7)
	gyrA:p.S83A	1 (5.3)	0	0	1 (1.96)
	gyrA:p.S83V	1 (5.3)	0	0	1 (1.96)
	parC:p.S80I	5 (26.3)	2 (20)	0	7 (13.7)
	parC:p.E84K	0	1 (10)	0	1 (1.96)
	parC:p.A56T	1 (5.3)	0	0	1 (1.96)
	parE:p.S458A	5 (26.3)	2 (20)	0	7 (13.7)
	parE:p.L416F	0	1 (10)	0	1 (1.96)
	parE:p.I529L	0	1 (10)	0	1 (1.96)

Sulfonamides	*sul1*	7 (36.8)	3 (30)	2 (8.3)	12 (23.5)
	sul2	8 (42.1)	3 (30)	8 (33.3)	19 (37.3)
Trimethoprim	*dfrA1*	2 (10.5)	0	2 (8.3)	4 (7.8)
	dfrA7	0	0	1 (4.2)	1 (1.96)
	dfrA8	0	0	3 (12.5)	3 (5.9)
	dfrA12	5 (26.3)	0	0	5 (9.8)
	dfrA14	4 (21.1)	2 (20)	2 (8.3)	8 (15.7)
	dfrA15	0	0	1 (4.2)	1 (1.96)
	dfrA17	2 (10.5)	2 (20)	1 (4.2)	5 (9.8)
	dfrB4	0	1 (10)	0	1 (1.96)
Tetracyclines	*tet*(A)	9 (47.4)	5 (50)	4 (16.7)	18 (35.3)
	tet(B)	4 (21.1)	1 (10)	3 (12.5)	8 (15.7)
Macrolides	*mph*(A)	5 (26.3)	2 (20)	6 (25)	13 (25.5)
	erm(B)	1 (5.3)	2 (20)	1 (4.2)	4 (7.8)
Amphenicols	*catA1*	2 (10.5)	1 (10)	0	3 (5.9)

* two DEC isolates are ESBL-producers, total isolate calculation considered this to avoid double counting of these two isolates

4.10 Co-occurrence of antimicrobial resistance genes

In the current study, several ARGs were identified together in the majority of isolates. Among the *E. coli* isolates, those with at least one quionolone ARGs or chromosomal mutation also carried β-lactam resistance genes in 92.9% of cases and sulfonamide resistance genes in 67.9% of cases. In addition, isolates with at least one aminoglycoside resistance gene also had β-lactam (90.9%), quionolone (77.3%), sulfonamide (90.9%) and trimethoprim resistance genes (86.4%). Furthermore, *E. coli* isolates that had at least one β-lactam resistance gene also had quinolone resistance mutation/genes and sulfonamide resistance genes in 70.3% and 64.9% of cases, respectively. The β-lactam resistance genes were detected in 59.5% of *E. coli* isolates with at least one trimethoprim resistance gene, 32.4% of those with at least one macrolide resistance gene and 8.1% of those with at least one amphenicol resistance gene (**Paper 2** and **Paper 4**).

4.11 Plasmids and other mobile genetic elements

A variety of mobile genetic elements (MGEs) were identified in *E. coli* isolates, including plasmids, insertion sequences (IS), integrative conjugative elements (ICEs), and transposons. In total, 113 MGEs were detected among the tested *E. coli* isolates, comprising 30 plasmids, 65 IS elements, 16 transposons, 1 ICE, and 1 miniature inverted repeat transposable element (MITEEc1) (**Paper 2** and **Paper 4**). Among the DEC isolates, 82 unique MGEs were identified, with the highest frequency detected in the EPEC strain, followed by the EAEC strain (**Paper 2**).

All *E. coli* isolates tested were confirmed to carry one to eleven plasmids, with the exception of three isolates (**Paper 2** and **Paper 4**). Of the DEC isolates, 22 (91.7%) carried one or more plasmids. The IncF plasmid with different replicon types and replicon variants was the most abundant plasmid. Other incompatibility group plasmids detected in the *E. coli* isolates included *IncI1* (n = 11), IncI2 (n = 3), *IncY* (n = 9), *IncX1* (n = 4), *IncX3* (n = 1) and *IncX4* (n = 2). Col plasmids such as Col(MG828), Col156, ColRNAI, Col(BS512) and ColpVC were also identified in the isolates. However, eight plasmids, including IncY, IncX3, IncX4, IncFII(pAMA1167-NDM-5), IncFIB(S), IncHI1A, IncHI1B(CIT) and p0111, were not found in the DEC isolates. Of the total plasmids detected, 15 (29.4%) were identified in EPEC

isolates, while only five (9.8%) were found in STEC isolates. Fourteen isolates had two plasmids and eleven isolates had three plasmids. In addition, fourteen isolates carried four or more plasmids, with one isolate from a patient in Hossana having a total of eleven plasmids (Figure 7) (**Paper 2** and **Paper 4**).

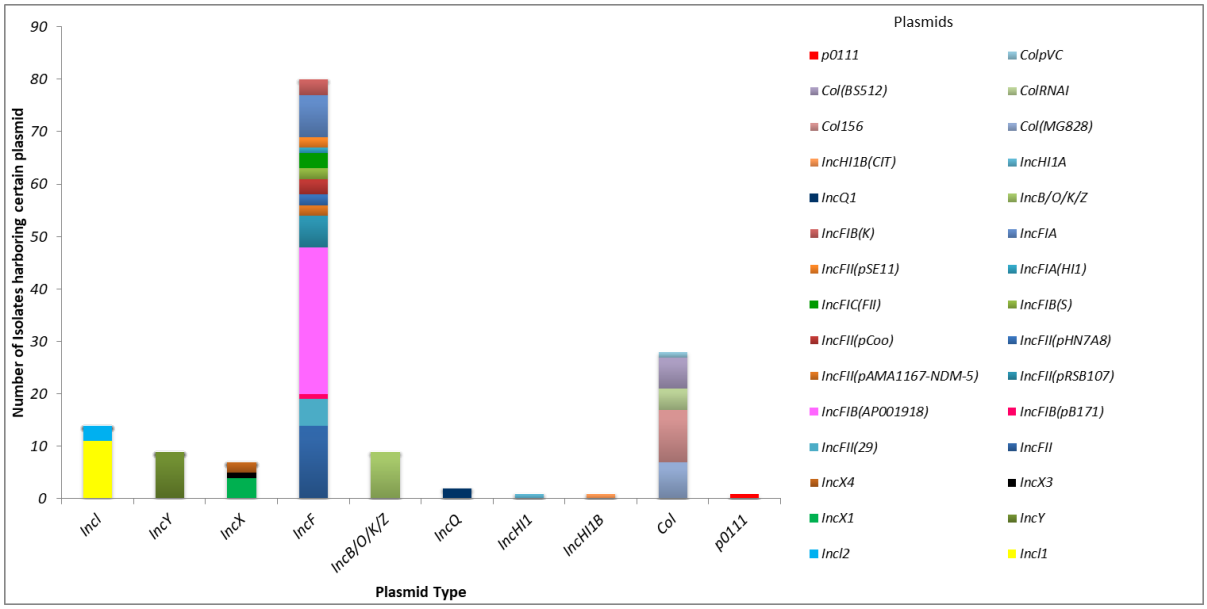

Figure 8: Number of *E. coli* isolates harboring certain plasmid types.

A variety of plasmids were identified in *E. coli* isolates that carry ARGs. Among the plasmids identified in this study, 96.7% were detected in *E. coli* isolates carrying β-lactam resistance genes, 86.7% in isolates carrying sulfonamide resistance genes, and 83.3% in those carrying aminoglycoside and quinolone resistance genes. The lowest number (33.3%) of plasmids was detected in *E. coli* carrying amphenicol resistance genes. The IncFIB(AP001918) plasmid type was the predominant plasmid detected across all classes of antibiotic resistance gene-carrying *E. coli* isolates. In contrast, the IncHI1A, IncFII(pSE11), and IncHI1B(CIT) plasmids were found only in *E. coli* isolates harboring genes resistant to β-lactams (**Paper 4**).

Several IS elements, MITEEc1, ICEs and transposons were also detected in *E. coli* isolates (Figure 8). On average, 9.3 MGEs other than plasmids were detected, with some isolates carrying up to 17 (**Paper 2** and **Paper 4**). DEC isolates had an average of 7 MGEs (**Paper 2**). All isolates harbored MITEEc1. The most prevalent IS elements, IS*Ec1* and IS*609*, were detected in 68.6% and 64.7% of all *E. coli* isolates tested, respectively. These two IS elements were also dominant in DEC isolates, with a frequency of 66.7% for IS*609* and 62.5% for IS*Ec1*. Approximately 51% of *E. coli* isolates had 10 or more different MGEs

(excluding plasmids), with those with seven different MGEs being the most abundant (13.7%). Transposons such as Tn*2*, Tn*7*, Tn*5403*, Tn*6024* and Tn*6082* were identified in *E. coli* isolates tested, with Tn*2*, Tn*7* and Tn*6024* detected in 16.7% of DEC isolates (**Paper 2** and **Paper 4**). Among the DEC pathotypes, EPEC carried 37 (44.6%) of the MGEs other than plasmids, while ETEC carried 29 (34.9%) and 11 (13.3%) were detected in the STEC isolates (**Paper 2**).

This study found a significant association between the presences of ARGs in *E. coli* isolates and IS elements. There was a significant association between the presence of genes conferring resistance to β-lactam antibiotics and IS*Ec9* (Fisher exact p = 0.045), as well as aminoglycoside resistance genes and IS*5075* (Fisher exact p = 0.034), IS*3* (Fisher exact p = 0.005), and IS*629* (χ^2 = 4.83, p = 0.027). Additionally, a significant association was observed between quinolone resistance genes and IS*Ec9* (Fisher exact p = 0.01). There was also a significant association between sulfonamide resistance genes and IS*5075* (Fisher exact p = 0.004) and IS*629* (χ^2 = 9.848, p = 0.002). Furthermore, a significant association was noted between tetracycline resistance genes and IS*629* (Fisher exact p = 0.043) and IS*5075* (Fisher exact p = 0.042). Similarly, there was a significant association between amphenicol resistance genes and IS*Cfr1* (Fisher exact p = 0.002) and IS*Ec10* (Fisher exact p = 0.002), as well as between macrolide and IS*6100* (Fisher exact p = 0.001) and trimethoprim resistance genes and IS*5075* (Fisher exact p = 0.034) (**Paper 4**).

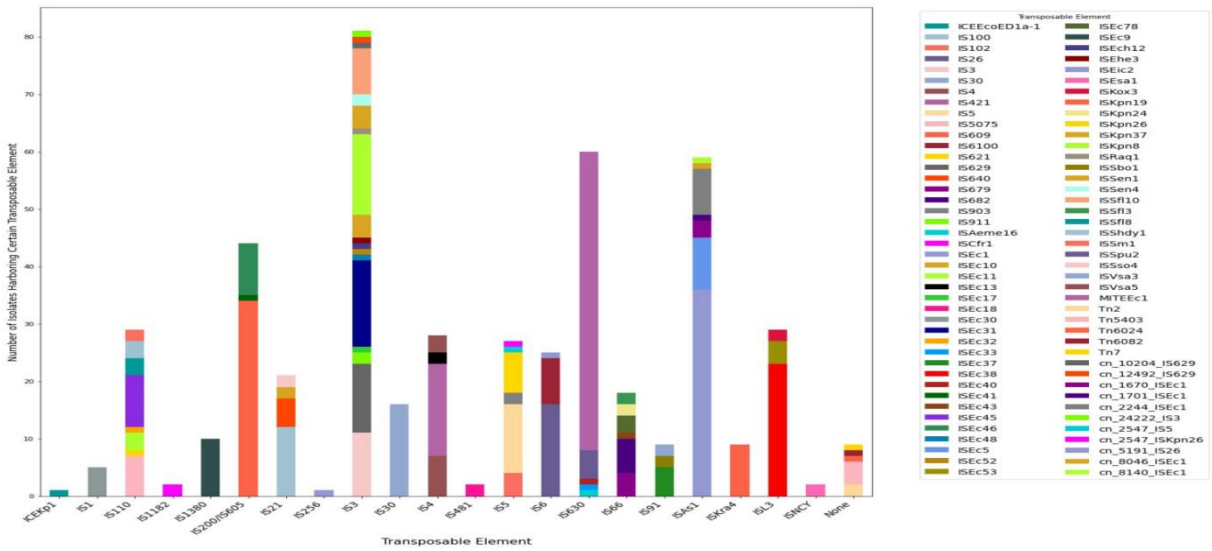

Figure 9: Number of *E. coli* isolates harboring certain types of transposable elements.

4.12 Determination of conjugative transferable elements

All essential components required for conjugation, such as *oriTs*, relaxases, T4CPs and T4SSs, were detected in 29 (56.9%) of the 51 *E. coli* isolates tested, indicating their potential for self-transferability. Among these, 9 (17.6%) were DEC isolates. The *E. coli* isolates with conjugative transferable elements were obtained from nine diarrheic patients and seven non-diarrheic patients in Hossana, while the remaining thirteen (five from diarrheic patients and eight from non-diarrheic patients) were from Addis Ababa. Three *E. coli* isolates (5.9%) were found to carry neither relaxase nor T4CP or T4SS. Eight isolates (15.7%) lacked T4CP, while T4SS was not detected in 4 (7.8%) isolates. In two isolates, only *oriT* and relaxases were detected. Among the *E. coli* with conjugative transferable elements, 28 isolates (96.6%) had acquired ARGs. Virulence-associated genes were found in all *E. coli* isolates with self-transferable and non-self-transferable MGEs (**Paper 4**).

5 Discussion

Although, diarrheal diseases can be significantly prevented by the provision of clean water, hundreds of millions of people in developing countries do not have access to clean water (Hunter *et al.*, 2013). People in Ethiopia use both tap water and protected wells/springs as sources of drinking water. However, the current study found significant association between diarrhea cases and use of both tap water and protected wells/springs. This finding is consistent with a community-based comparative study among children under five years of age in Jawi district, Awi Zone Ethiopia (Gessesse and Tarekegn, 2022), a study conducted among children under five years of age in Efoulan health district in Cameroon (Ayuk *et al.*, 2018), and in a household in Mopani district in South Africa (Kapwata *et al.*, 2018). In contrast, a study among children under five years of age in three districts in central Tanzania (Rukambile *et al.*, 2020) showed a protective effect of drinking tap water against diarrhea. This may be related to the absence of a regular drinking water quality control system in Ethiopia, which highlights the risk of water contamination with a range of pathogenic microorganisms that cause diarrheal diseases (**Paper 1**).

In addition, the frequency of washing water fetching material was another important factor in determining diarrhea occurrence. This study found that patients who occasionally clean their water fetching materials had a 2.40 times greater risk of developing diarrhea compared to patients who daily wash their water fetching materials. Likewise, the odds of developing diarrhea was 3.03 times higher among patients who rarely clean their water fetching materials compared to patients who daily wash their water fetching materials (**Paper 1**). This is in agreement with the study conducted in under-five children in Bosaso district, Puntland-Somalia (Kalid *et al.*, 2016). This may be due to unhygienic practices that can lead to water contamination and create environments where harmful microorganisms proliferate, further increasing the risk of diarrhea transmission (Freeman *et al.*, 2017; Gil *et al.*, 2004)

Defecating in open fields puts individuals at risk of acquiring diseases transmitted through the fecal-oral route. Another sanitation-related risk factor for the occurrence of diarrheal disease identified in this study was the unavailability of toilet facilities or the use of open pit latrines.

Patients who used an open-pit latrine were 5.61 times more likely to have diarrhea than patients who used a flush toilet, which is consistent with other studies conducted in Benishangul Gumuz Regional State, northwestern Ethiopia (Mihrete *et al.*, 2014); Dakahlia, Egypt (El-Gilany and Hammad, 2005); Accra Metropolitan Area, Ghana (Kwasu .O.B, 2005); and slum areas of the 5th subdivision of Douala, Cameroon (Mabvouna *et al.*, 2023). The possible explanation may be the likelihood of fecal contamination in the environment, which facilitates the spread of pathogens responsible for diarrheal diseases (**Paper 1**).

Adequate space is a fundamental element to support the maintenance of clean indoor air and reduce the risk of disease transmission. One of the independent risk factors for the development of diarrheal disease identified in this study was the number of people sharing a bedroom. As the number of people sharing bedrooms increased by one, the odds of developing diarrhea increased by 55%. This is consistent with the study conducted in two impoverished suburbs of Johannesburg, South Africa (Nkosi *et al.*, 2019), in children presenting to a health facility in the city of Salvador in northeastern Brazil (Ferrer *et al.*, 2008), and with a multisite study conducted in 8 countries (Mohan *et al.*, 2017). The reason for this may be crowding creates an optimal environment for transmission of diarrheal pathogens (**Paper 1**).

Most human diarrheal pathogens are zoonotic, and pathogen transmission can occur through contaminated food, water, the environment, and direct contact with animals. In low- and middle-income countries, animals that harbor pathogens that can infect humans live in close proximity to them in domestic settings (Delahoy *et al.*, 2018). These conditions increase the potential for fecal contamination from animals in the home environment and subsequent zoonotic transmission of enteric pathogens carried by these food-producing animals. Animals in particular have been implicated as a source of fecal contamination of soil (Pickering *et al.*, 2012). Closely related pathogens have been isolated from humans and animals, suggesting possible cross-infection between humans and domestic animals (Shrivastava *et al.*, 2020). These pathogens are excreted in the feces of animals. Consequently, animal feces may contribute to the incidence of diarrheal disease in humans by introducing zoonotic pathogens that cause the disease or by increasing the transmission of pathogens found in both animals and humans (Zambrano *et al.*, 2014). Exposure of children to animal feces/feed was associated with an increased risk of diarrheal disease in this study. Compared with their counterparts, children exposed to animal feces/feed had a 15.1-fold

higher risk of developing diarrheal disease. Similar to this finding, a systematic review by Penakalapati *et al* (Penakalapati *et al.*, 2017) also showed that exposure to animal feces was associated with diarrheal disease. In addition, a concordant result was obtained in a case-control study matched for age, sex, and place of residence done in children younger than five years in western Kenya (Conan *et al.*, 2017). This is possibly because enteric pathogens are often shed in the animal feces; contact of human with animal feces can lead to fecal-oral transmission of these pathogens through fecal contamination of fingers (Heredia and García, 2018) (**Paper 1**).

Vaccines are considered the safest and most cost-effective measures to reduce morbidity and mortality in children. Diseases that vaccines can prevent in infants and children in Ethiopia include diphtheria, pertussis, tetanus, tuberculosis, polio, measles, hepatitis B, influenza (caused by *Haemophilus influenzae* type b), pneumococcal pneumonia and diarrhea (caused by rotaviruses) (Ministry of Health-Ethiopia, 2022). However, these diseases continue to pose a major public health challenge in Ethiopia. Childhood immunization status was also identified in the current study as a determinant of diarrheal disease among patients attending public health facilities. The odds of having diarrhea is about three times higher among partially vaccinated children compared to fully vaccinated children according to national immunization program. This finding is consistent with previous studies in children under five years of age in different regions of Ethiopia (Damene *et al.*, 2020; Negesse *et al.*, 2021; Wolde, Tilahun, *et al.*, 2022), Nyarugenge district in Rwanda (Nsabimana *et al.*, 2017), Bankura slums in West Bengal (Gupta *et al.*, 2015), and Epworth Township in Harare in Zimbabwe (Chari *et al.*, 2023). This may be because vaccination significantly reduces morbidity and mortality from serious infections that disproportionately affect children (Rodrigues and Plotkin, 2020). Accordingly, vaccines developed targeting the most common etiologies of diarrheal disease contributed significantly to the decline in diarrheal disease (Das *et al.*, 2013) (**Paper 1**).

The relationship between diarrhea and malnutrition is bidirectional. Malnutrition leads to increased frequency and duration of diarrheal illness. This study found an association between BMI level and diarrheal disease in patients attending public health facilities. A 1 kg/m^2 increase in BMI decreased the likelihood of developing diarrhea by 25% (**Paper 1**). This is consistent with previous studies in children under five years of age, which found that malnourished people were more likely to have diarrhea in West Africa (Owusu *et al.*, 2023) and Namibia (Bauleth *et*

al., 2020). This is because malnutrition can predispose to infection because of several physiologic vulnerabilities, including immunologic dysfunction, disruption of the intestinal barrier, dysbiosis of the gut microbiota, and deficiency of essential nutrients (Tickell *et al.*, 2020).

DEC pathotypes are among the most common causative agents of diarrhea in developing countries, with varied prevalence by region and population. The present study detected DEC-specific genes in 11.5% of the patients attending public primary healthcare facilities in Addis Ababa and Hossana, with the predominance of EPEC and EAEC (**Paper 2**). However, several previous studies have found differences in the predominant pathotypes in different geographical locations. Studies conducted in central Ethiopia in children with and without diarrhea and in Bahir Dar town in northwestern Ethiopia in children with diarrhea who had contact with calves revealed predominance of EAEC, ETEC and EPEC, and ETEC, STEC and EPEC, respectively (Addisu *et al.*, 2022; Zelelie *et al.*, 2023). Another study in four provinces of Mozambique showed that EAEC, EPEC and STEC were dominant pathotypes in children with diarrhea (Manhique-coutinho *et al.*, 2022). A recent study among under-five-year-old children with close contact with food animals in Kenya found that the most common pathotypes found in the children were EAEC, ETEC and EPEC (Yeda *et al.*, 2024). A study conducted in East Central Gabon also showed that ETEC and EHEC were the most common pathotypes (Mabika *et al.*, 2021). The differences in the distribution of DEC isolates may be attributed to the heterogeneity of factors that influence their distribution in various geographic locations, such as the health status of the host and environmental, geographic, and social conditions (Escobar-Páramo *et al.*, 2006; Gordon and Cowling, 2003).

STEC is one of the most common causes of gastrointestinal illness worldwide. It is often associated with severe infections such as haemorrhagic colitis and haemolytic uraemic syndrome (Majowicz *et al.*, 2014). However, there is significant variability in the virulence potential among different STEC serotypes, and many of them may not be pathogenic (Mathusa *et al.*, 2010). In our study, all STEC pathotypes were detected in patients without diarrhea. This finding is consistent with a case-control study conducted in rural Mozambique involving children under five (Garrine *et al.*, 2020). In contrast, in a study conducted in central Ethiopia among children with and without diarrhea, STEC were detected only in patients with diarrhea (Zelelie *et al.*,

2023). This may be due to the coexistence of *eae* and *stx* genes in contrast to our study, which is likely to increase the pathogenicity of the strains (Food and Agriculture Organization of the United Nations and World Health Organization, 2018). In addition, all strains identified in our study were assigned to phylogroup B1, in which commensal *E. coli* strains are frequently identified (Escobar-Páramo *et al.*, 2006) and were sensitive to all antimicrobials tested and did not carry ARGs due to the fact that STEC infections are not usually treated with aggressive antimicrobial therapy (Pokharel *et al.*, 2023; Rahal *et al.*, 2015) (**Paper 2 and Paper 4**).

Hybrid strains harboring virulence genes characteristic of two or more DEC pathotypes, or virulence genes from intestinal pathogenic *E. coli* (IPEC) and ExPEC, are emerging worldwide. These strains exhibit diverse and potentially enhanced pathogenicity. The identification of such hybrid strains presents a significant public health concern (Santos *et al.*, 2020). In the current study, an NTEC2/STEC hybrid pathotype was detected, carrying the *cnf2* gene, which encodes cytotoxic necrotizing factor 2, and the *stx1* gene, which encodes Shiga toxin (**Paper 2**). Both cytotoxic necrotizing factor and Shiga toxin are cyclomodulins known to modulate cellular differentiation, apoptosis, and proliferation (McCoy *et al.*, 2021). They are exotoxins capable of causing severe damage to cells and tissues, contributing to disease severity in pathogenic strains of *E. coli*. Specifically, CNF2 has been associated with the *E. coli* Vir plasmid (Chaoprasid and Dersch, 2021). Additionally, this isolate carried another cyclomodulin-encoding gene, the *cdt-IIIB* gene, which is located on pVir-like conjugative plasmids and has the ability to induce DNA damage and cell cycle arrest, leading to cytotoxic effects and facilitating the entry of other toxins (Pérès *et al.*, 1997). The isolate also contained a gene encoding a serine protease with proteolytic activity, capable of cleaving human coagulation factor V (*espP* gene) (Brunder *et al.*, 1997). The combination of these virulence factors suggests that the strain possesses sophisticated mechanisms for causing disease, indicating a significant health risk for affected individuals. Hence, stringent infection control practices should be enforced to prevent the spread of this strain and manage the associated risks of this pathogenic *E. coli* strain.

E. coli exhibits a high level of genetic diversity across its various strains, significantly influencing its pathogenicity, ecological adaptability, and interactions with hosts (Kaas *et al.*, 2012). This genetic variability enables *E. coli* to thrive in diverse environments and evolve rapidly in response to selective pressures, such as antibiotic use and changes in host immunity

(Croxen *et al.*, 2013). Phylogenetic group analysis is instrumental in understanding genetic variation and identifying potential outbreaks, thereby facilitating the development of targeted interventions (Stoppe *et al.*, 2017). The phylogenetic analysis in the current study reveals that *E. coli* is a complex and diverse species, comprising seven distinct phylogenetic groups (A, B1, B2, C, D, E, and G) (**Paper 2** and **Paper 4**). Phylogroup B1 (49%) was the predominant group, similar to a finding from a study conducted in southwestern Nigeria on *E. coli* isolates from humans (Olowe *et al.*, 2019). In contrast, a study in Côte d'Ivoire involving diarrheic children and adults found that phylogroup A was the most prevalent, accounting for 53% of cases (Adjhi *et al.*, 2014). Additionally, two studies in Egypt on *E. coli* isolated from various clinical sources including patients aged 10 to 65 with symptomatic urinary tract infections (UTIs) and diarrhea also revealed a predominance of phylogroup A at 53% (El-baz *et al.*, 2022; Khairy *et al.*, 2019). Furthermore, a study conducted in South Africa among diarrheic children confirmed a predominance of phylogroup B2 (Alfinete *et al.*, 2022). The differences in the distribution of *E. coli* phylogenetic groups can be explained by differences in socio-economic characteristics (Stoppe *et al.*, 2017).

The results of the current study indicated that 82.4% of the *E. coli* isolates belonged to phylogroups A and B1, which include both commensal strains and those associated with intestinal infections (Picard *et al.*, 1999) (**Paper 2** and **Paper 4**). The isolates belonged to these phylogroups exhibited a higher number of VAGs compared to other phylogroups. This finding is consistent with a systematic review and meta-analysis of human commensal *E. coli* from various global regions, which also demonstrated the predominance of phylogroups A and B1 (Stoppe *et al.*, 2017). The presence of a high number of VAGs may enhance the ability of these strains to cause intestinal infections, potentially acquired through horizontal gene transfer (Javadi *et al.*, 2017), underscoring a significant health risk.

Multi locus sequence typing is used to investigate the genetic relatedness among *E. coli* strains. In the current study, 36 different STs were found, including a novel ST designated as ST15980. Each ST carried a variety of VAG combinations. A ST15980, which was identified for the first time in this study, carried genes promoting adhesion, aiding in evasion of the host immune system, and causing tissue damage. This includes *hlyF*, which encodes hemolysin and may be linked to increased production of outer membrane vesicles, facilitating the release of cytolethal

distending toxin and other harmful substances (Murase *et al.*, 2015). In addition, it had the *terC* gene, which encodes a subunit of the tellurite resistance protein complex, potentially enhancing its fitness and enabling it to bypass the host's primary immune response (Valková *et al.*, 2007). This strain also had a broader spectrum of resistance genes conferring resistance to different classes of antimicrobials, complicating treatment with a wide range of common antibiotics and posing a serious health problem. It also contained plasmids from incompatibility groups (*IncI1*, *IncY*) and other MGEs such as IS*26***,** IS*30***,** and IS*Ec38*, which often carry genes that confer resistance to various antibiotics and can be efficiently transferred between bacterial cells (Foley *et al.*, 2021). These factors collectively increase the strain's virulence and its resistance to immune defenses (**Paper 2** and **Paper 4**).

The ST131 strain, which is known for its high virulence and extensive resistance to antimicrobials that poses a substantial threat by causing severe infections such as urinary tract and bloodstream infections (Lukjancenko *et al.*, 2010), was also detected in this study (**Paper 4**). Studies have shown an increasing prevalence of the ST131 strains in various African countries. For example, a genomic epidemiology study at Queen Elizabeth Central Hospital and referral hospital in Lilongwe, Malawi, found ST131 in 14.9% and 32.8% of sequenced *E. coli* isolates, respectively (Musicha *et al.*, 2017; Tegha *et al.*, 2021). In South Africa, it was identified in 18% of *E. coli* from urinary tract infections in both inpatients and outpatients (Hoog, 2021). In addition, studies in Tanzania and Uganda found ST131 in 8.4% and 2.9% of isolates, respectively (Sserwadda *et al.*, 2023). Therefore, due attention should be given to preventing and controlling the spread of the ST131 strain, because of its increased prevalence and pandemic nature.

A significant level of genetic diversity was observed among *E. coli*, which exhibited 42 distinct serotypes (**Paper 2** and **Paper 4**). The most frequently identified serotype was H30, which showed increased resistance to different classes of antibiotics. Previous study showed that this serotype harbored a plasmid encoding the $bla_{\text{CTX-M-15}}$ gene, one of the most widespread and predominant ESBL-encoding genes (Bevan *et al.*, 2017). They also carried genes that determine resistance to aminoglycosides, quinolones, sulfonamides, trimethoprim, tetracyclines, macrolides, and amphenicols and different groups of plasmids and MGEs containing genes for various VAGs encoding protectins, iron acquisition, cytotoxins, or adhesion factors (Pitout and

Chen, 2023). The high diversity of ARGs and VAGs and their distribution among serotypes suggest multiple sources of resistant and virulent bacteria and the flow of these genes between different bacterial populations (Katale *et al.*, 2020).

In the present study, we identified a variety of VAGs encoding adhesins, toxins, protectins, and iron uptake factors in all tested *E. coli* isolates. Studies have shown that both commensal and pathogenic *E. coli* can share virulence factors that are crucial for surviving and colonizing specific ecological niches within the host, highlighting the adaptability and versatility of *E. coli* (Riley, 2020). Lindstedt *et al*. found genes related to extraintestinal *E. coli* in 93.5% of the *E. coli* isolates initially classified as intestinal pathogenic *E. coli* pathotype (Lindstedt *et al.*, 2018). *S*tudies have revealed that the intestinal microbiota serves as a reservoir for ExPEC strains, indicating the potential for these strains to cause both intestinal and extraintestinal diseases (Erjavec and Žgur-Bertok, 2015).In our study, we detected a wide range of VAGs, including *csgA*, *fimH*, *hlyE*, *iss*, *nlpI*, *terC*, and *traT*, which have all been associated with ExPEC, in *E. coli* isolates obtained from stool samples (**Paper 2** and **Paper 4**). Among the VAGs detected in our study, previous studies in Ethiopia (Dadi *et al.*, 2020) and Tunisia (Tarchouna *et al.*, 2013) found the *fimH*, *afa*, *hly*, and *cnf* genes in *E. coli* isolated from patients with urinary tract infections. In addition, a study conducted in Egypt detected the *csgA*, *fimH*, and *traT* genes in *E. coli* from stool, urine, and blood samples (El-Baky *et al.*, 2020). Moreover, the *fimH*, *vat*, *sitA*, *hlyF*, and *iutA* genes were identified in *E. coli* isolates from patients with suspected UTIs in Zimbabwe (Mbanga and Mudzana, 2014). These findings suggest that *E. coli* strains carrying genes associated with both intestinal and extraintestinal infections may originate from commensal strains by acquiring various virulence factors through horizontal gene transfer while residing in the intestine, enabling them to become virulent and potentially cause both types of infections (Adwan *et al.*, 2016; Nielsen *et al.*, 2018; Riley, 2020).

Antimicrobial resistance is a significant global health challenge in the 21st century. It has contributed to an increased occurrence of hard to treat infections (Ayukekbong *et al.*, 2017). In this study, 54.2% of *E. coli* isolates were resistant to at least one of the antimicrobial agents tested (**Paper 3**). Particularly, the isolates in the present study exhibited high resistance rates to ampicillin (52.7%), which is in line with other reports of *E. coli* obtained from outpatients in hospitals in Lagos State, as well as from animal handlers in abattoirs, poultry farmers and open

markets in Lagos, Nigeria (59.1%) (Adenipekun *et al.*, 2016), and from poultry farmers in Lusaka, Zambia (46.8%) (Mwansa *et al.*, 2023). In contrast, we found a lower rate of resistance compared to reports of pathogenic *E. coli* strains isolated from children in Wolaita Sodo, Southern Ethiopia (70.6%) (Wolde, Deneke, *et al.*, 2022), diarrheagenic *E. coli* isolates in Trans-Nzoia County, Kenya (83.9%) (Kebenei *et al.*, 2016), and *E. coli* isolated from diarrheic patients in public health centers in Eastern Cape, South Africa (88%) (Mkuhlu *et al.*, 2020)**.**

In addition, the *E. coli* isolates showed relatively low rate of resistance to trimethoprim-sulfamethoxazole (29.6%) in our study compared to the 67.6% reported by Wolde A. *et al*. in 2017, which involved *E. coli* from children under five years of age with diarrhea at Sodo Christian Hospital in Wolaita Sodo, southern Ethiopia (Wolde, Deneke, *et al.*, 2022), 95.7% reported by Kebenei KC. *et al*. in Trans-Nzoia County, Kenya (Kebenei *et al.*, 2016); 78% reported by Mkuhlu NA. *et al*. among diarrheic patients at a public health center in Eastern Cape, South Africa (Mkuhlu *et al.*, 2020), 61.5% reported by Adenipekun EO. *et al*. among outpatients at hospitals in Lagos State, as well as livestock farmers at abattoirs, poultry farmers, and open markets in Lagos, Nigeria (Adenipekun *et al.*, 2016), and 48.3% reported by Mwansa, M. *et al*. among poultry farmers in Lusaka, Zambia (Mwansa *et al.*, 2023) (**Paper 3**).

Our study also revealed resistance to levofloxacin (6.9%) and ciprofloxacin (7.3%), which is consistent with the findings of Chastel C. *et al*. in Madibou, Brazzaville (4%) (Chastel *et al.*, 2021) and Sina H. *et al*. in Benin (9.5%) (Sina *et al.*, 2022). However, may be due to the fact that fluoroquinolones are among the most frequently prescribed classes of antimicrobials in both human and veterinary medicine in Nigeria (Adesokan *et al.*, 2015), a high percentage (21.9%) of *E. coli* resistant to ciprofloxacin was reported in Nigeria among poultry workers, chickens, and their environment in poultry farms and markets (Aworh *et al.*, 2023). In addition, resistance to cephalosporin group antimicrobials aligns with the resistance rates observed for cefepime (1.6%) in *E. coli* isolated from outpatients in hospitals in Lagos State and from animal caretakers in abattoirs, poultry farms, and open markets in Nigeria (Adenipekun *et al.*, 2016), as well as the resistance rates for cefotaxime (8.6%) in *E. coli* isolated from poultry farmers in Lusaka, Zambia (Mwansa *et al.*, 2023), with some minor variations. However, high rates of resistance to this class of antibiotics have been found in previous studies conducted in Benin on *E. coli* isolated from children under five years of age in the pediatric clinic in the commune of Abomey-Calavi

(cefotaxime, 100%) (Sina *et al.*, 2022), and from Congolese students in Madibou, Brazzaville (ceftazidime, 65%) (Chastel *et al.*, 2021). This could be explained by the frequent prescription of these antimicrobials for treating infections caused by *Enterobacteriaceae*, which increases the selection pressure for resistant organisms in some developing countries (**Paper 3**).

In the current study, 19.2% of isolates were MDR (**Paper 3**), which is comparable to previous reports from Ethiopia on *E. coli* isolates from diarrheic patients at Selam Health Center in Addis Ababa (15.1%) (Negeri *et al.*, 2019) and from poultry farmers in Lusaka, Zambia (29.3%) (Mwansa *et al.*, 2023). However, previous studies conducted among hospitalized patients with a high likelihood of exposure to MDR organisms reported very high rates of MDR *E. coli* isolates: 78.2% in Ethiopia (Beyene *et al.*, 2022), 42.2% in Kenya (Kebenei *et al.*, 2016), and 69.6% in Lagos, Nigeria (Adenipekun *et al.*, 2016).

Furthermore, 8.5% of *E. coli* strains were found to be ESBL-producing in the current study (**Paper 3**). However, a higher rate of ESBL-producing *E. coli* was reported by Falgenhauer *et al*. for strains isolated from hospitalized children who had close contact with free-roaming chickens in the area and consumed chicken meat as part of their regular diet in Ghana. ESBL-producing bacteria are frequently isolated from these sources, and a genetic link between ESBL-producing *E. coli* in poultry and human populations has been demonstrated (Falgenhauer *et al.*, 2019). In the current study, a higher percentage of MDR (61.2%) and ESBL-producing *E. coli* (59.1%) was found among *E. coli* isolates from Hossana, where agriculture and animal husbandry are common practices in the community. This could be related to the imprudent use of antimicrobials in humans and agriculture, which is considered one of the most important factors contributing to the emergence and spread of resistant bacteria (Manyi-Loh *et al.*, 2018). The low socioeconomic status in Hossana, poor sanitation, and unhygienic conditions may also have contributed to the easy circulation of resistant organisms and resistant genetic markers. The inappropriate use of antimicrobials in agriculture can lead to selection pressure on microorganisms in food, water, and the environment, which can serve as a source of infection with MDR organisms for humans and animals (Gemeda *et al.*, 2020).

The fundamental reason why some bacteria are able to resist the effects of antibiotics is that they have specific genes that code for resistance mechanisms (Jian *et al.*, 2021). The present study

detected ARGs in 82.4% of the isolates tested (**Paper 2** and **Paper 4**), which is consistent with a previous report in which resistance genes were found in 84.9% of *E. coli* isolates from humans in the Central Zambia region (Mainda *et al.*, 2019). However, a lower rate (38%) of ARGs was detected in *E. coli* isolates obtained from human samples in a systematic review and meta-analysis conducted in South Africa (Ramatla *et al.*, 2023). The $bla_{TEM\text{-}1B}$ gene was dominant among the isolates, which is consistent with results found in studies involving children under five years of age with and without diarrhea in Ethiopia (Zenebe *et al.*, 2023), diarrheic patients in Ghana (Dela *et al.*, 2022), and a systematic review and meta-analysis of *E. coli* isolates from human, animal, and environmental samples (Ramatla *et al.*, 2023) (**Paper 4**).

A single strain of *E. coli* can carry ARGs that confer resistance to different classes of antibiotics in many cases (Galindo-Méndez, 2020). This ability significantly complicates the treatment of *E. coli* infections, as clinicians have fewer effective antibiotic options to choose from (Barlow, 2018). The current study found a high rate of multiple ARGs among the isolates tested. For example, all the isolates carrying aminoglycoside resistance genes were also confirmed to carry resistance genes for β-lactams, quinolones, sulfonamides, and trimethoprim. Many of the detected resistance genes, including those encoding aminoglycoside-modifying enzymes (AMEs), β-lactamases, and determinants of quinolone resistance, sulfonamides, trimethoprim, and tetracyclines resistance, are often located on MGEs (Rozwandowicz *et al.*, 2018; Snaith *et al.*, 2023; Tansirichaiya *et al.*, 2022). Furthermore, plasmids belonging to different incompatibility groups, and other MGEs, which have an important role in the transmission of ARGs, were simultaneously detected in the isolates in the current study. This observation suggests that the diverse MGEs may have the capacity to disseminate different antibiotic classes, further exacerbating the problem of multi-drug-resistant *E. coli* infections and the need to target plasmids to limit acquisition and transmission of antimicrobial resistance, as already reviewed by Vrancianu *et al*. (Vrancianu *et al.*, 2020) (**Paper 2** and **Paper 4**).

The analysis of MGEs showed that the isolates harbored different plasmids and other MGEs, which were simultaneously detected with ARGs and VAGs (**Paper 2** and **Paper 4**). This may indicate the potential for horizontal gene transfer, which can spread antibiotic resistance and virulence traits between bacterial populations (Evans *et al.*, 2020). This further exacerbates the issue of multi-antimicrobial-resistant *E. coli* infections and highlights the need to target plasmids

to limit the acquisition and transmission of antimicrobial resistance, as previously reviewed by Vrancianu *et al*. (Vrancianu *et al.*, 2020).

The identification and characterization of plasmid mobility remain unexplored. Approximately 56.9% of plasmids were found to be self-transferable, possessing key genetic elements such as *oriT*s, relaxases, T4CPs, and T4SSs. The majorities (75.9%) of them were ESBL-producing and third-generation cephalosporin resistant isolates (**Paper 4**). This high proportion of self-transferable plasmids indicates their substantial potential for self-transfer and dissemination within bacterial populations. Additionally, it was observed that 96.6% of the conjugative plasmids examined were found to carry acquired ARGs. This suggests that these conjugative plasmids have the capacity to potentially spread and disseminate ARGs among different bacterial species (Smillie *et al.*, 2010), which poses a significant concern in the context of antimicrobial resistance.

Limitations of the study

Limitations of the study include the lack of clinical data, reliance on self-reported behaviors, and the inability of the results to represent the true burden of the problem in the population because the study was health institution based. Secondly, due to resource constraints, it was not possible to include a large, representative sample for laboratory testing. This may have led to an under- or overestimation of the rate of antimicrobial resistance and the genetic characteristics of the E. coli isolates. Another limitation of this study was that it could not be conclusively determined whether they were plasmid-encoded or chromosomally localized. This technical limitation limits the understanding of the genetic mechanisms underlying the observed multidrug resistance.

6 Conclusion and recommendations

In conclusion, immunization status, sources of drinking water, number of people sharing a bedroom, contact with animal feces/food, frequency of cleaning water fetching materials, BMI level, and presence of a toilet, are potential predictors of diarrhea.

Four major pathotypes and one hybrid pathotype (STEC/NTEC2) were identified by WGS analysis, with EPEC being the predominant pathotype. The *E. coli* isolates also showed a broad spectrum of antimicrobial resistance. A significant prevalence of resistance to ampicillin and

trimethoprim-sulfamethoxazole was observed. However, all isolates tested were sensitive to carbapenems and amikacin, while all DEC isolates were also sensitive to eight antimicrobials, including carbapenems (ertapenem, imipenem and meropenem), aminoglycosides (amikacin, gentamicin and tobramycin), levofloxacin and piperacillin-tazobactam. 8.5% of *E. coli* isolates were phenotypically confirmed to produce ESBL. Of the total ESBL-producing *E. coli* isolates, 78.9% had the $bla_{CTX\text{-}M\text{-}15}$ gene. The most frequently identified ST was ST10 and ST15980 is a novel sequence type identified in this study. The most frequently identified serotype was H30. The genetically diverse resistant *E. coli* strains with a variety of VAGs and ARGs along with different MGEs are widely distributed in the study area, indicating a significant potential for gene exchange between isolates. This complicates infection control and treatment efforts. This study also showed that *E. coli* isolates carried a high proportion of self-transferable conjugative elements, indicating a significant potential for horizontal gene transfer and the spread of clinically important traits such as virulence and antibiotic resistance.

Recommendations

Efforts should be made to improve water safety, environmental and domestic hygiene of residential places, and increase uptake of child immunization to mitigate the impact of diarrhea. Enhanced surveillance covering a broad geographical area, with a representative number of samples in collaboration with various stakeholders and sectors, is recommended for the future to find real estimate of AMR and genetic diversity address to this growing public health challenge. In addition, the implementation of strategies to contain the infection and spread of this urgent health threat is essential. Furthermore, these findings highlight the urgent need for comprehensive strategies to combat antimicrobial resistance, alongside the prudent use of antibiotics to reduce the burden of antimicrobial resistance and ensure the long-term efficacy of available treatments. Further studies using advanced sequencing techniques would be required to conclusively determine whether ARGs were plasmid-encoded or chromosomally localized and gain a more comprehensive understanding of the antimicrobial resistance landscape in the region.

7 References

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Annexes

Annex I. English Version Participants' information sheet

Background information of the study

This study is designed to assess determinants of diarrhea, and conduct phenotypic and genotypic characterization of *E. coli* isolates obtained from stool samples of patients visiting primary healthcare facilities in Addis Ababa and Hossana, Ethiopia. Therefore, you are voluntarily requested to participate in the study after understanding the objectives of the study, the risk and discomfort, benefits and confidentiality. However, if you choose not to participate in the study, it will have no effect on the care you will be provided from the health facility and hence you still receive the same treatment as others without any prejudice.

Objectives of the study

The general objective of this study was to assess determinants of diarrhea, and to conduct phenotypic and genotypic characterization of *E. coli* isolates obtained from stool samples of patients visiting primary healthcare facilities in Addis Ababa and Hossana, Ethiopia.

Study participants

In this study, a total of 732 participants of different age of both sex will be involved at health facilities from the two study sites.

Samples required and examinations/Research

If you agree to participate in the study, you will provide stool samples for isolation and characterization of *E. coli*.

Risk and discomfort

We do not expect a definitive risk as a result of sample collection or participating in the study.

Benefits

Whether you participated or not you will get the routine health services rendered by health facility you visited. This means there is no direct benefit as a result of your participation in this study. However results from this study may benefit patients in the future.

Incentives

You will not be given any incentives by participating in the study.

Confidentiality

Information related to your name will be treated strictly confidential. Names and identifiers will be coded and deleted after data collection and therefore data will be treated anonymously for communication of results. Informed consent forms and questionnaires will be kept in a locked cabinet and will be destroyed after a year following termination of data collection. The results of your laboratory examination will not be shown to anyone except the research team and your doctor

Annex II. Amharic Version Participants' information sheet

ጥናቱን በተመለከተ ለተሳታፊዎች የሚሰጥ መግለጫ

ይህ ጥናት የተቅማጥ አጋላጭ ሁነቶች ፣ ኢሼሪሺያ ኮላይን መለየት እና መድኃኒቶችን የመቋቋም አቅማቸዉን አንድሁም መድኃኒቶችን አንድላመዱ የሚያደርጉ እና በሽታ እንድያመጡ የሚረዱ ጂኖችንለማጥናት ያቀደ ሲሆን የዚህጥናት አስፈላጊነቱ ታምኖበት በአዲስ አበባ ዩኒቨርሲቲ አክሊሉ ለማ ፓቶባዮሎጂ ተቋም የጥናትና ምርምር ስነምግባር ኮሚቴ ፈቃድ አግኝቷል:: ስለዚህ እርስዎ ቃለመጠይቅ ላይ በመሳተፍ እንዲሁም ናሙና በመስጠት በጥናቱ እንዲሳተፉ ፈቃድዎን እየጠየቅን በምርምሩ ለመሳተፍ ከመወሰንዎ በፊት የሚከተሉት ዝርዝር መረጃ ማለትም የጥናቱ ተሳታፊዎች የሚሰጡት ናሙና፣የሚደረገዉ ምርመራ በጥናቱ በመሳተፍዎ ሊከሰቱ የሚችሉ ችግሮች፣ ጥናቱ የሚሰጠዉጥቅም፣የሚስጥርአጠባበቅን በተመለከተ በማንበብ ወይም እንዲነበብሎት በማድረግና ጥያቄ ካልዎት በመጠየቅ በቂ ግንዛቤ እንዲወስዱ ይጠየቃሉ:: በዚህ ጥናት መሳተፍ ሙሉ በሙሉ ፈቃደኝነት ላይ የተመሰረተ ሲሆን ምንም አይነት ምክንያት መስጠት ሳያስፈልግዎት በጥናቱ የሚኖርዎትን ተሳትፎ በማንኛዉም ጊዜ ማቆም የችላሉ:: ባለመሳተፍዎ ምክንያት ከጤና ተቋም ልያገኙ የሚችሉትን የጤና አግልግሎት አሁንም ሆነ ወደፊት ያለ ምንም አድልዎ ይሰጥዎታል::

የጥናቱ አላማ

ጥናቱ በሚካሄድበት አካባቢ ለተቅማጥ በሽታ አጋላጭ ሁነቶችን፣ ኢሼሪሺያ ኮላይን መለየት እና መድኃኒቶችን የመቋቋም አቅማቸዉን አንድሁም መድኃኒቶችን አንድላመዱ የሚያደርጉ እና በሽታ እንድያመጡ የሚረዱ ጂኖችን ማጥናት ነዉ።

የጥናቱ ተሳታፊዎች

በዚህ ጥናት ተቅማጥ ያለባቸዉ እና የሌለባቸዉ በተለያየ የእድሜ ክልል ያሉ ሰዎች ይሰተፋሉ። ከጥናቱ ተሳታፊዎች የሚወሰድ ናሙና እና መረጃ በመስጠት በጥናቱ ለመሳተፍ ፈቃደኛከሆኑ የሰገራ ናሙና እንወስዳለን:: ይህም ናሙና ኢሼሪሺያ ኮላይ መኖር ያለመኖሩን ለማወቅ ይመረመራል:: በተጨማሪ ክብደትና ቁመት ይለካል::

በጥናቱ መሳተፍ ሊያስከትል የሚችላቸው ችግሮች

በጥናቱ በመሳተፍ እና ናሙና በመስጠትጋር በተያያዘ ሊከሰቱ የሚችሉ ችግሮች አይኖሩም ::

በጥናቱ መሳተፍ ሊያስገኝ የሚችለዉ ጥቅም

በጥናቱ ቢሳተፉም ባይሳተፉም ከጤና ተቋም የሚያገኙት የህክምና አገልግሎት እንደ ተጠበቀ ይሆናል፡: በጥናቱ በመሳተፍዎ የሚያገኙት ቀጥተኛ የሆነ ጥቅም አይኖርም::

ማበረታቻ

በጥናቱ እንዲሳተፉ ተብሎ ልዩ የገንዘብ ማበረታቻ አይደረግም ::

ምስጢር መጠበቅ

ከእርስዎ የተሰበሰበ ማንኛዉም መረጃ በምስጢር ይያዛል:: እርስዎን ገላጭ የሆኑ ስምን ጨምሮ በምስጢርቁጥር በመተካት የጥናቱ ዉጤት ይታተማል:: የስምምነትና የመጠየቅያ ቅፆች ጥናቱ ከተጠናቀቀ ከአንድ ዓመት በኃላ የሚቃጠል ሲሆን እስከዝያዉም ድረስ በሳጥን በመቆለፍ በምስጢር ይያዛል:: የላቦራቶሪ ዉጤትዎ ከሀክምዎና ከተመራማሪዎች ዉጭ ለሌላ ሰዉትላልፎ አይሰጥም::

Annex III. English Version Study participant consent form

Name of participant _____ age _____ sex_____

Code_____ study site and health facility_____

I confirm that I have been given adequate information about the research project "Determinants of diarrhea, phenotypic and genomic characterization of *Escherichia coli* isolates from diarrheic and non-diarrheic patients attending public primary health facilities in Addis Ababa and Hossana, Ethiopia" I, have been requested to provide stool sample and answer a questionnaire. The researchers informed me that there is no major risk associated with participating in the study or providing the requested samples. I have also understood that the results of laboratory diagnosis will be used for research purposes and the information related to myself/ my family will be kept strictly confidential. I am well informed that participation in the study is fully voluntary and I can withdraw anytime without giving any reason. Moreover, I am fully aware that non-participation in this project will not subject me to any health service denial from the health facility either now or in the times to come. I confirm that all the information provided to me is clear and has been conveyed by the language that I fully understand. Finally, I declare that I have been given enough time to deliberate before I agree to participate in the study.

Name of partcipant_____Signature_____Date_____

Name of data collector _____ signature_____ Date_____

Name and address of contact person if there are any concerns of problems

Deneke Wolde, ALIPB, AAU, Tele: +251916821776

Tadesse Eguale (Prof), ALIPB, AAU Tele: +251911435759

Annex IV. Amharic Version Study participant consent form

የጥናቱ ተሳታፊዎች የስምምነት ቅፅ

የጥናቱ ተሳታፊ ስም _____ እድሜ _____ ፆታ _____
የሚስጢር ቁጥር _____ የጥናቱቦታ / የጤና ተቋም _____
እኔ ስሜ ከዚህ በላይ የተገለፀዉ ግለሰብ ይህ ጥናት የተቅማጥ አጋላጭ ሁነቶች ፣ ኢሼሪሺያ ኮላይን መለየት እና መድኃኒቶችን የመቋቋም አቅማቸዉን አንድሁም መድኃኒቶችን አንድላመዱ የሚያደርጉ እና በሽታ እንድያመጡ የሚረዱ ጂኖችን ለማጥናት የተዘጋጀ ሲሆን በጥናቱ ለመሳተፍ ከመወሰኔ በፊት ሰለጥናቱ በቂ ዝርዝር መረጃ የተሰጠኝ መሆኑን አረጋግጣለሁ::

በዚሁም መሰረት የሰገራ ናሙና ለመስጠት ተጠይቄለሁ:: በጥናቱ በመሳተፍ እና ናሙና ከመስጠትጋር በተያያዘ ሊከሰቱ የሚችሉ ችግሮች እንደሌሉ በተመራማሪዎቹ ተገልፆልኛል:: በተጨማሪም የላቦራቶሪና የህክምና ዉጤት በሚስጢር እንደሚያዝና ለታቀደዉ ምርምርና ጥናት ብቻ እንደሚዉል ተገልፆልኛል :: በጥናቱ መሳተፍ ሙሉ በሙሉ በፍቃድኝነት ላይ የተመሰረተ እንደሆነና አስፈላጊ ከሆነም ምንም አይነት ምክንያት መስጠት ሳያስፈልግ በማንኛዉም ጊዜ የጥናቱ ተሳታፍነቴን ማቋረጥ እንደምችል ተረድቻለሁ:: ይህን በማድረጌም የህክምና አገልግሎት እንደማይቋረጥብኝ ተገልፆልኛል::

በመጨረሻም የተገለፀልኝ በቂ መረጃ ከተረዳሁ በኃላ በጥናቱ ለማሳተፍ መስማማቴን በፊርማዬ አረጋግጣለሁ::

የተሳታፊ ስም _____ፊርማ _____ቀን _____

የተሳታፊ ስምምንት የተቀበሉ ባለሞያ ስም _____ፊርማ_____ቀን _____

በስምምነት ወቅት የነበሩ እማኞች ሰምና ፊርማ

1._____

2._____

3._____

የምርምሩ ተጠሪ ስምና አድራሻ

ደነቀ ወልዴ

ስልክ 0916821776

አዲስ አበባ ዩኒቨርስቲ፣ አዲስ አበባ፣ ኢትዮጵያ

ታደሰ እጓለ (ፕ/ር)፣

አዲስ አበባ ዩኒቨርስቲ፣ አዲስ አበባ፣ ኢትዮጵያ

የመልክት ሳጥን ቁጥር- 1176 ስልክ 0911435759

Annex V. English version Questionnaire

Date: _____

Code_____ study site/ health facility_____ sub-city _____

SN	Variables	Responses	Skip
Part I. Socio-demographic characteristics			
101	Sex	0. Male 1. Female	
102	Age of respondents in year	_____years	
103	What is your relationship to child? if the patient is child	0. Father/mother 1. Grandfather/mother 2. Brother/sister 3. Uncle/ant 4. Other (specify)_____	
104	Marital status of respondents	0. Single 1. Married 2. Divorced 3. Separated 4. Widowed	
105	Residence sub-city		
106	Village or kebele		
107	Educational status (the highest level of education reached)	0. No formal education 1. Primary school (1-8) 2. Secondary school (9-12) 3. Above secondary school	
108	Occupation	0. Student 1. Government employee 2. NGO employee 3. Merchant 4. Other (specify)_____	
Part II. Socio-economic and Environmental variables			
201	What is the average monthly income of the entire household in birr	_____	
202	What is the number of rooms in your house?		
203	How many people live in your household?	_____	
204	Is there a person under the age of five?	0. No 1. Yes	If no skip to 206
205	If your response is yes for Q 204, How many are under the age of five?	_____	
206	What is the number of people sleeping in one room?	_____	
207	Is there animals living in your household?	0. No 1. Yes	If no skip to 212

208	If your response is yes for Q 207, type of animal(s): [check all that apply]	0. Cat 1. Dog 2. Cattle 3. Goat 4. sheep 5. Other (specify)_____	
209	If your response is yes for Q 207, where do they stay at night?	0. Inside dwelling 1. Outside dwelling 2. Both outside and inside	
210	If your response is yes for Q 207, Do your children have close contact with these animals? Like playing with them?	0. No 1. Yes	
211	If your response is yes for Q 207, Is there any occasion where when your child plays with animal feed, animal dung?	0. No 1. Yes	
212	What is the source of drinking water?	0. Tap water with private connection 1. Public tap water 2. Protected well/spring 3. Unprotected well/spring 4. Bottled water 5. Tanker truck 6. Other (specify)_____	
213	Do you do anything to your water to make it safer to drink?	0. No 1. Yes	
214	If your response is yes for Q 214, what do you usually do to the water to make it safer to drink?	0. Boil 1. Add liquid chlorine 2. Strain it through a cloth 3. Use a water filter 4. Let it stand and settle 5. Other (specify)_____	
215	How do you usually store your household drinking water?	0. Plastic jerrycan with cover 1. Plastic jerrycan without cover 2. Other (specify)_____	
216	How frequently do you clean your water fetching material?	_____	
217	How frequently do you clean your water storing material?	_____	
218	Do you have rack for drying dishes in home?	0. No 1. Yes	
219	How do you usually store prepared food?	0. Covered container or plate 1. Refrigerator 2. On fire or hot ash 3. Other (specify)_____	
220	What kind of toilet facility that you usually use?	0. No facilities or bush or field	

		1. Flush toilet 2. Ventilated improved pit (VIP) latrine 3. Pit latrine wit slab 4. Open pit latrine 5. Other (specify)_____ ___	
221	Do you share this facility with other households?	0. No 1. Yes	
222	If YES, how many people share this toilet facility?	_____ number of people	
223	How do you dispose the stools of your young children?	0. Child use toilet/latrine 1. Put in toilet 2. Put in drain or ditch 3. Thrown in garbage 4. Other (specify)_____	
224	How do you usually dispose of household rubbish?	0. Rubbish pit 1. Put in garden 2. Put in bush 3. Open burning 4. Other (specify)_____	
Part III. Clinical characteristics			
301	Do you currently have diarrhea?	0. No 1. Yes	
302	If your response for Q 301 is yes, What is the type of diarrhea?	0. Watery 1. Bloody	
303	If your response for Q 301 is yes, how long have you had diarrhea for?	_____days	
304	Currently, what kind of symptoms (complaints) do you have? NB: multiple choice is possible	0. Fever 1. Abdominal pain 2. Abdominal cramp 3. Loss of appetite 4. Tenusmus 5. Nausea 6. Vomiting 7. Other (specify) _____	
305	Do you have previous history of diarrhea during the last 3 months?	0. No 1. Yes	
306	Level of dehydration (using WHO criteria)	0. None 1. Mild 2. Moderate 3. Severe	
307	Do you have contact with diarrheic patients before this study?	0. No 1. Yes	
308	Does/do child/you have history of recurrent (≥2 episodes) diarrhea in last 3 months?	0. No 1. Yes	
309	Does/do child/you have history of persistent (≥14 days) diarrhea in last 3 months?	0. No 1. Yes	

310	Do you have known comorbidities	0. No 1. Yes	
311	What is your child immunization status according to national immunization program?	0. not vaccinated 1. Partially vaccinated 2. Fully vaccinated	
312	Height of the participants in cm	_____ cm	
313	Weight of the study participants in kg	_____ kg	
Part IV. Behavioral characteristics			
401	Do you wash your hands frequently?	0. No 1. Yes	
402	If your response is yes for Q 401, with what?	0. Soap and water 1. With water only 2. Other (specify)_____	
403	Please tell me about any time when you wash your hands during the day *(Do not read the responses below. Allow respondent to answer, then TICK the relevant items below*	0. When it looks dirty 1. After using toilet (urination or defecation) 2. After cleaning a child after defecation 3. Before preparing food 4. Before serving a meal 5. Before eating 6. Before feeding a child 7. When I am reminded to wash my hand 8. Other (specify)_____	
404	Do you have habit of consuming raw vegetable?	0. No 1. Yes	
405	Do you have habit of consuming raw milk?	0. No 1. Yes	
406	Do you have the habit of eating cooked food after its overnight storage?	0. No 1. Yes	
Part V Laboratory information			
501	What is the appearance of stool?	0. Watery 1. Bloody 2. Mucoid 3. Formed	
502	Was there growth of bacteria?	0. No 1. Yes	

Annex V. Amharic version Questionnaire

Code_____ study site/ health facility_____

ተቁ	መጠይቆች	መልስ	እለፍ
ክፍል I. ስነህዝብና ማህበራዊኩነቶችጋርየተያየዙጥያቄዎች			
101	ፆታ (አይተዉ ይሙሉ)	0. ወንድ 1. ሴት	
102	ዕድሜዎ ስንትነው? (በዓመት ይግለፁ)	_____አመት	
103	እርሶ ለህፃኑ/ኗ ምኑ/ኗ ነዎት? (ለህፃናት ብቻ)	0. አባት/እናት 1. አያት 2. ወንድም/እህት 3. አጎት/አክስት 4. ሌላ ካለ (ይጥቀሱ)_____	
104	የጋብቻ ሁኔታዎ ምን ይመስላል?	0. አላገበሁም 1. አግብቻለው 2. ተፋትቻለው 3. ተለያይተናል 4. ባሌ/ምስቴ ሞቶብኛል/ሞታብኛለች	
105	የመኖርያ ክፍለ ከተማ	_____	
106	የመኖርያ ቀበሌ	_____	
107	የትምህርት ሁኔታ (ከፍተኛ የትምህርት ደረጃዎንይግለፁ)	0. መደበኛ ት/ት አልተማርኩም 1. የመጀመሪያ ደረጃ ት/ት (1-8) 2. ሁለተኛ ደረጃ ት/ት (9-12) 3. ከሁለተኛ ደረጃ ት/ት በላይ	
108	የስራዎ ሁኔታ ምን ይመስላል?	0. ተማሪ 1. የመንግስት ሠራተኛ 2. መንግስታዊ ያልሆነ ድርጅት ሠራተኛ 3. ነጋዴ 4. ሌላ ካለ(ግለፁ)_____	
ክፍል II፡ ማኅበራዊ ኢኮኖሚያዊ እነዲሁም አካባቢያዊ ጥየቄዎች			
201	በአማካኝ የሙሉ ቤተሰብ ወራዊ ገቢ በብር ስንት ይሆናል?	_____ ብር	
202	የመኖሪያ ቤትዎ ስንት ክፍሎች አሉት?	_____	
203	በቤትዎ ዉስጥ በአጠቃላይ ስንት ሰዎች ይኖራሉ?	_____	
204	እድሜው ከ 5 አመት በታች የሆነ ሰው በቤትዎ አለ?	0. የለም 1. አዎን	If no skip to 206
205	ለጥያቄ ቁ 204 መልሰዎ አዎ ከሆነ ከ 5 አመት በታች የሆኑት ስንት ናቸዉ?	_____	
206	በአንድ ክፍል ዉስጥ ስንት ሰዉ ይተኛል?	_____	
207	በቤትዎ ዉስጥ የሚኖሩ እንስሳቶች አሉ?	0. የለም	If no

		1. አዎን	skip to 210
208	ለጥያቄ ቁ 207 መልስዎ አዎን ከሆነ የትኞቹ/ኛዉ እንስሳቶች?	0. ድመት 1. ዉሻ 2. ከብት 3. ፍየል 4. በግ 5. ሌላ ካለ(ግለፁ)_____	
209	ለጥያቄ ቁ 207 መልስዎ አዎን ከሆነ እንስሳቶቹ ማታ የት ያድራሉ?	0. መኖሪያ ቤት ዉስጥ 1. ከመኖሪያ ቤት ዉጪ 2. በቤት ዉስጥም ከቤት ዉጪም	
210	ለጥያቄ ቁ 207 መልስዎ አዎን ከሆነ ከህፃናት ጋር ንክኪ ይኖራቸዋል? ለምሳሌ ከእንስሳቶች ጋር ይጫወታሉ?	0. አይጫወቱም 1. አዎን	
211	ለጥያቄ ቁ 207 መልስዎ አዎን ከሆነ ህፃናት ከእንስሳቶች ምግብ ጋር ይጫወታሉ?	0. አይጫወቱም 1. አዎን	
212	የመጠጥ ዉሃ የሚያገኙት ከየት ነዉ?	0. የቧንቧ ዉሃ (የግል መስመር) 1. የቧንቧ ዉሃ (ቦኖ) 2. የታሸገ ዉሃ 3. የታንከር ዉሃ 4. ሌላ ካለ(ግለፁ)_____	
213	የመጠጥ ዉሃን ጤናማ ለማድረግ የሚያደርጉት ነገር ይኖራል?	0. የለም 1. አዎን	
214	ለጥያቄ ቁ 215 መልስዎ አዎን ከሆነ ዉሃን ጤናማ ለማድረግ ብዙ ጊዜ ምን ያደርጋሉ?	0. አፈላለሁ 1. ክሎሪን እጨምርበታለሁ 2. በንፁህ ጨርቅ አጣራለሁ 3. የዉሃ ማጣሪያ እጠቀማለሁ 4. አስቀምጬ እንድዘቅጥ አደርጋለሁ 5. ሌላ ካለ(ግለፁ)_____	
215	በቤትዎ የመጠጥ ዉሃ እንዴት ነዉ ሚያስቀምጡት?	0. ክዳን ባለዉ ጄሪካን ዉስጥ 1. ክዳን በሌለዉ ጄሪካን ዉስጥ 2. ሌላ ካለ(ግለፁ)_____	
216	የመጠጥ ዉሃ ምቀዱበትን ዕቃ በየስንት ጊዜ ያፀዳሉ?	_____	
217	የመጠጥ ዉሃ ምቀመጥትን ዕቃ በየስንት ጊዜ ያፀዳሉ?	_____	
218	የታጠቡት የመመገብያና ማብሰያ ዕቃዎች ማድረቅያ አልዎት	0. የለኝም 1. አዎን	
219	ለመመገቢያነት የተዘጋጁ ምግቦችን እንዴት ያስቀምጣሉ?	0. ክዳን ባለዉ ሳህን 1. ፍሪጅ ዉስጥ 2. በእንፋሎት ወይም ፊም እሳት ዉስጥ 3. ሌላ ካለ(ግለፁ)_____	
220	በአብዛኛው ጊዜ የምትጠቀሙት ምን አይነት መፀዳጃ ቤት ነዉ?	0. ባለ ከፍታ መቀመጫ የዉሃ ታጣቢ መፀዳጃ ቤት 1. መደበኛ የጉድጓድ ሽንትቤት	

		2. የተሻሻለ ባለአየር ማስተንፈሻ መፀዳጃ ቤት 3. በኮንክሪት ወለል የተሰራ በደበኛ የጉድጓድ መፀዳጃ ቤት 4. ክፍት ጉድጓድ 5. ሌላ ካለ ይጥቀሱ	
221	መፀዳጃ ቤቱን ከሌላ ቤተሰብ ጋር ይጋሩታል?	0. አልጋራም 1. አዎን	
222	ለጥያቄ ቁ 223 መልስዎ አዎን ከሆነ መፀዳጃ ቤቱን ስንት ሰዉ ይጠቀመዋል?	_____ ሰዉ	
223	የህፃኑን ሠገራ እንዴት ያስወግዱታል?	0. ህፃኑ ሽንት ቤት ይጠቀማል 1. ሽንት ቤት ዉስጥ እጥለዋለሁ 2. ቱቦ ዉስጥ እጥለዋለሁ 3. ቆሻሻ ማጠራቀሚያ ዉስጥ እጥለዋለሁ 4. ሌላ ካለ ይጥቀሱ_____	
224	የቤት ዉስጥ ቆሻሻዎችን እንዴት ያስወግዳሉ?	5. በቆሻሻ ጉድጓድ ዉስጥ 6. በአትክልት ስፈራ 7. ወደ ጫካ ወረዉራለሁ 8. አቃጥላለሁ 9. ሌላ ካለ ይጥቀሱ_____	
ክፍል III. ጤና ነክ ጉዳዮች			
301	አሁን ተቅማጥ አለብዎት?	0. የለብኝም 1. አዎን	
302	ለጥያቄ ቁ 301 መልስዎ አዎን ከሆነ ተቅማጡ ምን ዓይነት ነዉ?	0. ዉሃ የሚመስል 1. ደም የተቀላቀለበት	
303	ለጥያቄ ቁ 301 መልስዎ አዎን ከሆነ ከጀመረዎት ምን ያህል ቀናትሆነዎት?	_____ቀናት	
304	አሁን ላይ ያለብዎት ምልክት/ቶች ምን ምን ናቸዉ? ማሳሰቢያ፡ ከአንድ በላይ ምላሽልሰጡይቻላሉ	0. ትኩሳት 1. የሆድ ህመም 2. የሆድ ቁርጠት 3. የምግብ ፍላጎት ማነስ 4. ማስማጥ 5. ማቅለሽለሽ 6. ትውከት 7. ሌላ ካለ ይጥቀሱ_____	
305	ከዚህ በፊት ተቅማጥ ይዘዎት ያውቃል?	0. አያዉቅም 1. አዎን	
306	ዉሃአነስነት ስሜት ደረጃው (በዓለም ጤና ድርጅት መለክያ መሠረት)	0. ከፍተኛ 1. መጠነኛ 2. በትንሹ 3. የለም	
307	በቅርቡ ተቅማጥ ካለበት ሰው ጋር ንክኪ ነበረዎት?	0. አልነበረኝም	

		1. አዎን	
308	ልጅዎት/እርስዎ በዚህ 3 ወር ተቅማጥ በተደጋጋሚ (2≥ጊዜ) ተመላልሶበታል?	0. በጭራሽ 1. አዎን	
309	ልጅዎት/እርስዎ በዚህ 3 ወር ቶሎ በማይለቅ (≥14 ቀናት ምቆይ) ዓይነት ተጠቅቶዋል?	0. በጭራሽ 1. አዎን	
310	በህክምና የታወቀ ተጓዳኝ በሽታ አለብዎት?	0. የለብኝም 1. አዎን	
311	የሆድ ቀዶ ጥገና ተደርጎሎት የውቃል?	0. አያውቅም 1. አዎን	
312	የክትባት ሁኔታ ምን ይመስላል? (ለህፃናት ብቻ)	0. ያልተከተበ 1. በከፊል ክትባት የወሰደ 2. ሙሉለሙሉ ያልተከተበ	
313	የተሳታፊዉ ቁመት በሴሜ	_____በሴሜ	
314	የተሳታፊዉ ክብደት በኪግ	_____ በኪግ	
ክፍል IV. ባህርይ ተያያዝ ጉዳዮች			
401	እጅዎን በየጊዜ በአግባቡ ይታጠባሉ?	0. አልታጠብም 1. አዎን	
403	ለጥያቄ ቁ 401 መልስዎ አዎን ከሆነ በምንድነው የሚየፀዱት?	0. በሳሙናና ውሃ 1. በዉሃ ብቻ 2. ሌላ ካለ ይጥቀሱ	
404	እባክዎ እጅዎትን መቼ መቼ እንደምታጠቡ ይግለፁ (ምርጫዎቹን አያንብቡላቸዉ. የሚመልሱትን ብቻ ምልክት የድርጉባቸዉ)	0. ቆሸሾ ካየሁ 1. ከመፀዳጃ ቤት መልስ 2. ልጅ ካፀደሁ በኃላ 3. ምግብ ከማዘጋጀቴ በፊት 4. ምግብ ከማቅረቤ በፊት 5. ምግብ ከመብላቴ በፊት 6. ልጅ ከመመገቤ በፊት 7. ሰዎች እንድታጠብ ስነግሩኝ 8. ሌላ ካለ ይጥቀሱ_____	
405	ያልበሰለ(ጥሬ) አትክልቶችን የመብላት ልምድ አለክ/ሽ?	0. የለም 1. አዎን	
406	ያደረ ምግብ ለልጅዎ ይሰጣሉ	0. የለም 1. አዎን	
ክፍል V. የላቦራቶሪ መረጃ			
501	የተሰበሰበዉ የሰገራ ናሙና ምን ይመስላል?	1. ዉሃ አይነት 2. ደም የተቀላቀለበት 3. ዝልግልግ ዓይነት 4. ደረቅያለ	
502	ሰገራዉ ያደገ ባክቴሪያ ነበር?	1. አልነበረም 2. አዎን	

Paper 1

Determinants of diarrheal diseases among patients attending public health facilities in Addis Ababa and Hossana, Ethiopia: A matched case control study

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Abstract

Background: The incidence of diarrhea varies greatly between and within countries due to differences in socioeconomic, environmental, and behavioral factors. The aim of this study was to assess the determinants of diarrheal diseases among patients attending public health facilities in Addis Ababa and Hossana, Ethiopia.

Methods: Age matched health facility-based case-control study design was employed to recruit study participants and collect data from December 2021 to September 2022. A structured questionnaire was used to collect socio-demographic data and other risk factors from study participants. Conditional logistic regression was used to identify the independent predictor variables. Adjusted odds ratio with its corresponding 95%CI was used to measure the strength of the associations and statistical significance is reported whenever p-value less than 0.05.

Results: Being partially vaccinated (AOR: 2.70; 95%CI: 1.2, 5.9), use of tap water for drinking (AOR: 2.20; 95%CI: 1.1, 4.4) and use of protected well/spring water for drinking (AOR: 13.90; 95%CI: 3.7, 51.5), overcrowded sleeping arrangement (AOR: 1.50; 95%CI: 1.2, 1.8), contact with animal feces/food (AOR: 15.10; 95%CI: 4.2, 53.6), use of cleaning water fetching material sometimes (AOR: 2.40; 95%CI: 1.2, 4.5) and use of cleaning water fetching material rarely (AOR: 3.03; 95%CI: 1.2, 7.4), and use of open pit latrine (AOR: 5.61; 95%CI: 1.5, 21.0) were significantly associated with increased odds of having diarrhea. Higher value of BMI (AOR: 0.75; 95%CI: 0.7, 0.8) is significantly associated with not having diarrhea.

Conclusion: The occurrence of diarrheal diseases was influenced by various factors, including the vaccination status of children and unhygienic living conditions. Therefore, timely vaccinations, access to safe drinking water, proper hygiene practices, and improved sanitation are essential for combating diarrheal diseases and safeguarding public health.

Keywords: Determinants, Diarrhea, patients, Addis Ababa, Hossana, Ethiopia

Introduction

Diarrheal disease is a global health problem that affects people in all age categories and both sexes [1]. In the general population, diarrhea is the third largest cause of morbidity and the sixth largest cause of mortality [2,3], and it accounts for 4.3% of the total global disease burden [4]. Approximately 1.6 million deaths occur each year globally due to diarrheal diseases with the highest burden occurring in developing countries [4]. In Ethiopia, diarrheal diseases were among the top leading causes of high disease burden both at the national and regional levels, and 76.4 deaths from diarrheal illness was reported in 2019 for every 100,000 people [5]. A systematic subnational analysis from the global burden of disease study indicated that in 2019, Addis Ababa had 105,243.37 cases and 24.42 deaths from diarrheal illnesses per 100,000 populations, while the Southern Nations, Nationalities, and Peoples' Region, which formerly included the Hadiya Zone, had 126,151.96 cases and 83.72 deaths per 100,000 population [6].

The intricate interplay between socioeconomic, environmental, and behavioural variables determines the occurrence of diarrheal diseases [7,8]. Poor housing conditions including overcrowding, dirty floors, inadequate food storage, and cohabitation with domestic animals are also associated with diarrheal diseases [9]. The majority of diarrheal disease is attributable to unsafe drinking water supply, inadequate sanitation, and poor hygiene [10]. More than 2 billion people worldwide lack access to basic sanitation [11,12], and Ethiopia has the lowest access to safe drinking water and sanitation among sub-Saharan African countries [13,14]. According to the 2019 mini-Ethiopia Demographic and Health Surveys (EDHS), access to basic sanitation services in Ethiopia is only 7.5% [15]. In 2023, access to basic sanitation and hand-washing facilities in Hossana was reported at 35.1% and 16.8%, respectively. [16] The majority of the population in Addis Ababa lives in polluted environments, exposing them to water and sanitation-related diseases [17]. A study conducted in Addis Ababa revealed that 53% of surface water sources and 62% of water source catchments are classified as having very high-risk and high-risk contamination levels, respectively [18].

The risk factors for this disease vary with age, the pathogens involved, the season in which the infection is acquired, and the local environment [19]. Previous studies have been conducted in a single geographic area that may not reflect the broader population and the majority of previous studies in Ethiopia focused on identifying risk factors in children

younger than five years [20–22]. This study was conducted in two geographic locations with varying socioeconomic and demographic characteristics in Ethiopia (i.e., Addis Ababa and Hossana) among all age groups to provide a comprehensive understanding of the determinants of diarrheal diseases in patients attending public health facilities.

Methods

Study setting and study period

This study was conducted in Addis Ababa city and Hossana town from December 2021 to September 2022. Addis Ababa is the capital of Ethiopia, home for 23.8% of all urban dwellers in Ethiopia, and has an estimated density of 5936.2 persons per square kilometer. The majority of the populations of Addis Ababa live in a polluted environment, exposed to water and sanitation-related diseases [17]. There are 13 government owned hospitals and 98 health centers in the city.

The other study area is Hossana town which is located in Hadiya zone in central Ethiopia region. The town is 230 km far from the capital Addis Ababa to South West. The zone is one of the most densely populated regions in Ethiopia. The population density of Hadiya zone is 415 persons per square kilometers. The total population of Hossana town is estimated at 75,963. The town has one government owned comprehensive specialized hospital, one private owned general hospital, three public health centers (primary healthcare facilities) and eight urban health extension workers offices. Figure 1 shows the map of the study area.

Selection of health facilities for the current study

A multi-stage sampling method was used to select study participants in Addis Ababa. First, four sub-cities from Addis Ababa city administrative (which is 40% of the total) were selected. Then, 13 health centers (3 from Addis ketema, 4 from Arada, 4 from Kirkos, and 2 from Gulale sub-cities) were randomly selected based on the number of health centers available in the sub-cities. Finally, study participants from the health facilities were recruited proportionally according to patient flow in the randomly selected health facilities. In Hossana all three public health centers were included and participants were recruited proportionally according to patient flow like Addis Ababa.

Study design and study participants

A health institution-based matched case control study design with 1:1 ratio was employed. Case and control were matched by age (maximum of three years of age difference). Persons of any age who had diarrhea and attended health facilities selected for the current study were considered as cases. Patients with no symptom of diarrhea or gastrointestinal complaint for two weeks before enrolment in the study attending the same health facility were recruited as a control. Diarrhea was defined as a passage of three or more loose or liquid stools per day according to WHO guideline [23]. Consecutive cases of diarrheic patients of any age and sex group who visited the health facilities during the data collection time were included. Comparison groups were selected from the same health facilities that do not have symptoms of diarrhea, who visited the facilities for other health purposes during the study period, and for each case was age match control. Controls were recruited within the same week after the recruitment of each diarrhea case. Any participants who had a sibling recruited as a case or control were excluded from the study. Potential controls who had diarrhea in the previous 7 days before the initiation of the study were also excluded.

Sample size estimation and Sampling procedure

The sample size was calculated by using Stata version 14.0 by considering 95% confidence level, power 90%, case to control ratio of 1:1, OR=2.68, design effect=2 and proportion of controls with exposure 35% The odds ratio and proportion for control are taken from solid waste disposal category of a study conducted in Ethiopia by Mosisa *et al*., in 2021 [21] because it gave us the largest sample size amongst other studies conducted in Ethiopia. After adding a 10% non-response rate, the final sample size for cases and controls in Addis Ababa was 488 (244 cases and 244 controls). The same sample size, without considering the design effect, was used for Hossana, resulting in a final sample size of 244 (122 cases and 122 controls). Overall, the total sample size used for assessing determinants of diarrhea was 732 (366 cases and 366 controls).

Sampling procedures of study participants

The total sample size for Addis Ababa was distributed to health facilities proportional to their patient flow. Consecutive cases of diarrheic patients of any age and sex group who visit the health facilities during the data collection time were included. Comparison groups were

selected from the same health facilities that do not have symptoms of diarrhea, who visited the facilities for other health purposes during the study period, and for each case was age match control. Nurses who were work in a triage in each healthcare facility recruited the cases and controls for the study.

Data collection methods and instruments

Demographic data

Data on socio-demographic, clinical data and other risk factors were collected through face-to-face interview using a structured questionnaire. The questionnaire was prepared in English, translated into Amharic, and finally, translated back to English to check its consistency.

Anthropometric measurements

Weight and height of study participants were measured at enrollment to determine the influence of anthropometry on the occurrence of diarrheal diseases. The standard procedure and measuring scale was utilized to determine the height. The height of infant age 6–23 months was measured in recumbent position while height of the other study participants was measured in standing position. The study participants were requested to takeoff their shoes before taking height measurement. Height measurement was taken to the nearest of 0.1cm. The calibrated beam balance was employed to determine the weight of the participants, and prior to each measurement, the scale was verified to ensure it was set to zero. Weight was measured to the nearest 10g by removing heavy cloths. For a child who cannot stand alone, the mother was weighed together with a child and then without the child. The difference between the two measurements was taken as the child's weight.

Data quality and management

Quality of the data was assured by using standardization of anthropometric measurements, providing adequate training for data collectors and supervision during the field work.

Statistical analysis

Data analysis was done by Stata version 14.0. Continuous variables were described based on their distribution, either using the mean (standard deviation) or median (interquartile ranges),

and categorical variables were reported as a number and percentage. The Shapiro-Wilk test was used to evaluate the normality of the quantitative variable data, and the variance inflation factor (VIF) test was used to check multi-collinearity. Conditional logistic regression was used to identify independent predictors after checking all the preliminary assumptions of the model. Variables with p-value less than 0.25 in a bivariable analysis were identified as candidate for multivariable model. In multivariable analysis, variables with p-value $\leq$ 0.05 were reported as being statistically significant and AOR with 95% CI was reported as measures of the strength of the associations.

Operational definitions

Diarrhea: is defined as having three or more loose or watery stool in a 24 hour period

Completely vaccinated: children were those who received all childhood vaccines according to Ethiopia national immunization program

Partially vaccinated: children who have received one or more immunizations according to the National Immunization Schedule, but have not completed all of them

Frequency of cleaning water fetching devices

Frequently: cleaning fetching materials four to six times per week

Sometimes: cleaning fetching materials one to three times per week

Rarely: not cleaning fetching materials at least once a week

Results

Socio-demographic and socio-economic characteristics

A total of 732 participants (366 cases and 366 controls) were included in the current study. The median (IQR) age was 18.0 (7.0-30.0) years for cases and 20.0 (8.0-30.0) years for controls, and significant proportions were from the 0-14 years age group (i.e 42.9% of the cases and 41.5% of the controls). The median (IQR) income of cases' and controls' household was 5000 (3500-7071) and 6000 (4000-8000) Ethiopian birr, respectively. The mean (±SD) number of rooms in the house of the cases and controls was 2.16 (±1.1) and 2.40 (±1.1), respectively. Regarding household family size, 66.4% of cases and 68.1% of controls had ≥4 members. On average, 4.14 (±1.5) persons live in the house of the cases and 4.25 (±1.5) persons live in the house of controls. On average 2.48 (sd=1.1) persons in the house of cases and 2.44 (sd=1.1) persons in the house of controls share a single sleeping room (Table 1).

Environmental and Behavioral characteristics

The presence of domestic animals in the households was reported by 26.2% of cases and by 18.4% of controls in Addis Ababa, and 38.5% of cases and 22.1% of controls in Hossana. Of the total animals reported, cats accounted for 70.3% of cases and 65.3% of controls. Similarly, dogs were reported by 42.3% of cases and 34.7% of controls.

In Addis Ababa, only 1.2% of cases and 0.4% of control reported using protected well or spring water for drinking. In Hossana, 23.0% of cases and 7.4% of controls reported using protected well or spring water as a source of drinking water. Overall, 18.0% of cases and 18.9% of controls treat their drinking water at home using various methods. More than half (56.5%) of cases and 36.9% of controls in Hossana used either an open pit latrine or a bush for defecation, while only 3.7% of cases and 1.6% of controls in Addis Ababa used these facilities for defecation. The average number of people sharing a toilet was 19.3 persons in the case group and 11.5 persons in the control group. The environmental and behavioral characteristics of the study participants are summarized in Table 2.

Determinants of diarrheal disease among patients attending public health facilities

Multivariable binary logistic regression result showed use of tap water and use of protected well/spring water for drinking, child being partially vaccinated, a higher number of people sleeping in one room, contact with animal feces/feed, frequency of cleaning water fetching materials, increase in BMI level, and unavailability of toilet facility or use of open pit latrine as independent determinants of diarrhea (Table 3)

Discussion

This study was conducted to assess the determinants of diarrheal disease among patients attending public health facilities in Addis Ababa and Hossana. Preventable factors that include drinking water source, children's immunization status, overcrowded sleeping places, having contact with animal feces/food, cleaning of water fetching materials, unavailability of a toilet or use of an open pit, and BMI are significantly associated with likelihood of having diarrheal disease.

Although diarrheal diseases can be prevented by the provision of clean water, hundreds of millions of people in developing countries do not have access to clean water [24]. People in Ethiopia use both tap water and protected wells/springs as sources of drinking water, which are significantly associated with diarrhea case in the current study. Patients using tap water or protected well or spring water for drinking were more likely to have diarrhea, than patients using bottled water. This finding is consistent with a community-based comparative study among children under five years of age in Jawi district, Awi Zone Ethiopia [25], a study conducted among children under five years of age in Efoulan health district in Cameroon [26], and in a household in Mopani district in South Africa [27]. A study among children under five years of age in three districts in central Tanzania [28] showed a protective effect of drinking tap water against diarrhea. This may be related to the absence of a regular drinking water quality control system in Ethiopia, which highlights the risk of water contamination with a range of pathogenic microorganisms that cause diarrheal diseases. In addition, In most cases, water collection points are located some distance from the house, requiring water to be fetched and transported from the source and then stored in the household. As a result, unhygienic practices lead to water contamination during collection, transport, storage, and pickup. Moreover, the frequency of washing water fetching material was another important factor in determining diarrhea occurrence. This study found that patients who occasionally

clean their water fetching materials had a 2.40 times greater risk of developing diarrhea compared to patients who frequently wash their water fetching materials. Likewise, the odds of developing diarrhea was 3.03 times higher among patients who rarely clean their water fetching materials compared to patients who frequently wash their water fetching materials. This is in agreement with the study conducted in under-five children in Bosaso district, Puntland-Somalia [29].

Sanitation is considered as a primary barrier to infection because it excludes disease-causing microorganisms from the environment [30]. Unhygienic practices could contribute to over 23% of diarrheal cases [31]. There is significant association in the current study between use of an open pit latrine and diarrheal disease, showing that patients who used an open pit latrine were 5.61 times more likely to have diarrhea compared to patients who used a flush toilet. which is consistent with other studies in Benishangul Gumuz Regional State of Ethiopia [32]; Dakahlia in Egypt [33]; Accra Metropolitan Area in Ghana [34]; and slum areas of Douala in Cameroon [35]. The possible explanation might be the likelihood of fecal contamination in the environment, facilitating the spread of pathogens responsible for diarrheal diseases. Moreover, poor sanitation practices allow human waste to enter drinking water supplies, contaminating nearby water sources and exposing the people to pathogenic microorganisms.

Adequate space is a fundamental element to support the maintenance of clean indoor air and reduce the risk of disease transmission. One of the factors associated with diarrheal disease in the current study is number of people sharing a bedroom. As the number of people sharing bedrooms increased by one, the odds of developing diarrhea increased by 50% which is in lines with the findings of previous studies in suburbs of Johannesburg, South Africa [36], in Salvador in northeastern Brazil [37], and in multisite study conducted in 8 countries [38]. The reason for this may be crowding creates an optimal environment for transmission of diarrheal pathogens and increases exposure to other risk factors associated with diarrhea.

Most human diarrheal pathogens are zoonotic, and pathogen transmission can occur through contaminated food, water, the environment, and direct contact with animals. In low- and middle-income countries, animals that harbor pathogens that can infect humans live in close proximity to them in domestic settings [39]. These conditions increase the potential for fecal contamination from animals in the home environment and subsequent zoonotic transmission of enteric pathogens carried by these food-producing animals. Animals in particular have

been implicated as a source of fecal contamination of soil [40]. Closely related pathogens have been isolated from humans and animals, suggesting possible cross-infection between humans and domestic animals [41]. These pathogens are excreted in the feces of animals. Consequently, animal feces may contribute to the incidence of diarrheal disease in humans by introducing zoonotic pathogens that cause the disease or by increasing the transmission of pathogens found in both animals and humans [42]. Exposure of children to animal feces/feed was associated with an increased risk of diarrheal disease in this study. Compared with their counterparts, children exposed to animal feces/feed had a 15.1-fold higher risk of developing diarrheal disease. Similar to this finding, a systematic review by Penakalapati *et al* [43] also showed that exposure to animal feces was associated with diarrheal disease. In addition, a concordant result was obtained in a case-control study matched for age, sex, and place of residence done in children younger than five years in western Kenya [44]. This is possibly because enteric pathogens are often shed in the animal feces; contact of children with animal feces can lead to fecal-oral transmission of these pathogens through fecal contamination of fingers [45].

The relationship between diarrhea and malnutrition is bidirectional. Malnutrition leads to increased frequency and duration of diarrheal illness. This study found an association between the level of BMI and diarrheal disease in patients attending public health facilities. A 1 kg/m^2 increase in BMI decreased the likelihood of developing diarrhea by 25%. This is consistent with previous studies in children under five years of age, which found that malnourished people were more likely to have diarrhea in West Africa [46] and Namibia (45). Malnutrition can predispose to infection because of several physiologic vulnerabilities, deficiency of essential nutrients [47].

Childhood immunization status is identified as one of the factors associated with diarrheal disease in patients attending public health facilities. The odds of partially vaccinated children compared to fully vaccinated children to have diarrhea is about three times. This finding is consistent with previous studies in children under five years of age in different regions of Ethiopia [48–50], Nyarugenge district in Rwanda [51], Bankura slums in West Bengal [52], and Epworth Township in Harare in Zimbabwe [53]. This may be because vaccination significantly reduces morbidity and mortality from serious infections that disproportionately affect children [54]. Accordingly, vaccines developed targeting the most common etiologies of diarrheal disease contributed significantly to the decline in diarrheal disease [55]. The limitations of the study include reliance on self-reported behavior and the inability of the

results to represent the true burden of the problem in the population because the study was health institution based.

In conclusion, immunization status, sources of drinking water, number of people sharing a bedroom, contact with animal feces/food, frequency of cleaning water fetching materials, BMI level, and presence of a toilet, are potential predictors of diarrhea. Therefore, efforts should be made to encourage and ensure that children receive timely vaccinations, as well as to improve water safety and environmental and domestic hygiene in residential areas to decrease the incidence of diarrhea and mitigate the impact of diarrhea.

List of Abbreviations

AOR: Adjusted Odds Ratio, BMI: Body Mass Index, IQR: Interquartile Range, S: Standard Deviation, WHO: World Health Organization

Ethical approval and consent to participate

Ethical approval was obtained from the institutional review board of ALIPB, AAU. Permission to conduct the study was obtained from the Addis Ababa Health Bureau and the Haddiya Zone Health Department. Verbal consent or assent was obtained from each study participant. Patients were informed that they had the right to withdraw from the study at any time and that participation in the study was completely voluntary. All information obtained from participants during the study was kept confidential.

Availability of data and materials

Data will be available from the corresponding author upon a reasonable request.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceptualization DW and TE, methodology, DW, GM, AM software, DW, GM, KSK, validation, TE, final analysis, DW, TE, GM, HA, KSK, AM, resources, TE, data curation, DW, GAT, FRS, WH, Writing-original draft preparation, DW, writing-review and editing, DW, TE, GM, HA, AFH, GAT, KSK, FRS, WH, AM, visualization, DW, TE, Supervision,

TE, GM, AFH, AM, Project administration, TE, funding acquisition, TE. All authors have revised and approved the final manuscript.

Acknowledgements

The authors would like to thank Addis Ababa University for partially supporting this study through thematic research project. We would like to thank our study participants for their willingness to be part of the study and for providing the required data.

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Paper 2

International Journal of
Molecular Sciences

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Article

Diarrheagenic *Escherichia coli* in Stool Specimens Collected from Patients Attending Primary Healthcare Facilities in Ethiopia: Whole-Genome Sequencing-Based Molecular Characterization

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Abstract: The diarrheagenic *Escherichia coli* (DEC) is the major cause of diarrheal diseases in Africa, including Ethiopia. However, the genetic diversity of *E. coli* pathotypes found in Ethiopia has not been studied well. This study aimed to characterize potential DEC belonging to enteropathogenic (EPEC), Shiga toxin-producing (STEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), and enteroinvasive (EIEC) *E. coli* pathotypes from stool specimens of patients attending primary healthcare units (n = 260) in Addis Ababa and Hossana using whole-genome sequencing. Real-time PCR assays were used to identify DEC isolates belonging to EPEC, STEC, EAEC, ETEC, and EIEC pathotypes, which were then subjected to whole-genome sequencing on the Illumina platform. Twenty-four whole-genome nucleotide sequences of DEC strains with good enough quality were analyzed for virulence-associated genes (VAGs), antibiotic resistance genes (ARGs), phylogenetic groups, serogroups, and sequence types. The majority (62.5%) of DEC isolates belonged to the phylogenetic group B1. The identified DEC isolates belonged to 21 different serogroups and 17 different sequence types. All tested DEC isolates carried multiple VAGs and ARGs. The findings highlight the high diversity in the population structure of the studied DEC isolates, which is important for designing targeted interventions to reduce the diarrheal burden in Ethiopia.

Keywords: diarrheagenic *Escherichia coli*; whole-genome sequencing; phylogeny; virulence-associated genes; antibiotic resistance genes; Ethiopia

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Citation: Wolde, D.; Eguale, T.; Medhin, G.; Haile, A.F.; Alemayehu, H.; Mihret, A.; Pirs, M.; Strašek Smrdel, K.; Avberšek, J.; Kušar, D.; et al. Diarrheagenic *Escherichia coli* in Stool Specimens Collected from Patients Attending Primary Healthcare Facilities in Ethiopia: Whole-Genome Sequencing-Based Molecular Characterization. *Int. J. Mol. Sci.* **2024**, *25*, 10251. https://doi.org/10.3390/ijms251910251

Academic Editor: Alberto Rizzo

Received: 15 July 2024
Revised: 19 September 2024
Accepted: 19 September 2024
Published: 24 September 2024

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1. Introduction

Escherichia coli is a genetically heterogeneous bacterium known to survive in various niches, including the gastrointestinal tract of humans and warm-blooded animals [1]. It is also one of the bacteria that poses a significant threat to human health due to its increasing resistance to different groups of antibiotics using a variety of mechanisms [2]. According

to the World Health Organization (WHO), the resistance of *E. coli* to common antibiotics has reached alarming level in many parts of the world [3]. Furthermore, *E. coli* is known to serve as a reservoir for various antibiotic resistance genes (ARGs) and is capable of horizontally transferring these genes to other pathogenic and commensal organisms. Therefore, understanding the antimicrobial susceptibility of *E. coli* and genetic markers associated with resistance may provide an indication of the burden of antimicrobial resistance in other Gram-negative organisms circulating in a given community [4]. Moreover, antimicrobial resistance in *E. coli* strains is rapidly increasing in Ethiopia [5]. Multidrug resistance was reported in 62–88% of *E. coli* isolates in this country [6–8].

Some strains of *E. coli* harbor virulence factors that affect a broad spectrum of cellular functions and lead to a variety of intestinal and extraintestinal infections, including diarrheal diseases, neonatal meningitis, septicemia, and urinary tract infections [9]. Diarrheagenic *E. coli* (DEC) strains have been classified by their virulent characteristics into six different pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC) [10]. These pathotypes carry one or more specific virulence genes that are not present in commensal *E. coli* [11]. The genes encoding virulence factors in *E. coli* are likely acquired through horizontal gene transfer from other bacterial species through plasmids, integrons, bacteriophages, pathogenicity islands, and transposons [12]. The most prominent and well-characterized pathotypes known to cause diarrhea are EPEC, ETEC, EAEC, STEC, and EIEC [13]. However, the pathogenicity and epidemiological importance of DAEC isolates in causing diarrheal disease has been the subject of ongoing debate and controversy [14]. DEC pathotypes are responsible for about 30–40% of acute diarrhea episodes in children [15]. They also play a considerable role in causing diarrhea in adults [16]. They are widespread worldwide, with prevalence varying by geographic region [17] and the incidence of different pathotypes in different age groups [18,19]. The variability in distribution of DEC is also observed in Ethiopia. For example, a study conducted in central Ethiopia and Wolaita Sodo, southern Ethiopia, found a high incidence of the EAEC strain [6,20]. In contrast, a study conducted in Bahir Dar town in northwestern Ethiopia showed a high prevalence of ETEC in children and calves [21]. On the other hand, a study conducted in the northwestern region of Ethiopia reported a high prevalence of the STEC strain from abattoir workers hands, carcass, cattle feces, and other environmental samples [22].

Phylogenetic grouping and sequence typing are used to categorize different strains of *E. coli* based on their genetic relatedness. *E. coli* can be assigned into different phylogroups (e.g., A, B1, B2, C ...) based on the detection of the gene encoding the ankyrin repeat protein A (*arpA)*, a gene required for heme transport *(chuA)* and a gene that encodes a putative transcriptional regulator *(yjaA)* and the DNA fragment TSPE4.C2 [23]. Multilocus sequence typing is the genotyping method based on the sequences of specific loci of seven housekeeping genes that are highly conserved and present in all *E. coli* strains. Isolates are assigned into different sequence types (STs) based on the combination of different alleles of the seven housekeeping genes for each strain [24].

Genome analysis of the DEC strains can provide a comprehensive overview of the distribution of different DEC pathotypes. This analysis can reveal the repertoire of virulence-associated genes (VAGs) and ARGs present in DEC isolates. Furthermore, whole-genome sequence-based phylogenetic analysis can help clarify the evolutionary relationship and genetic relatedness between the different DEC strains [25]. This information can then be utilized for public health investigations, such as tracing the source and transmission patterns of DEC strains, identifying the emergence and spread of new, potentially more virulent or antibiotic-resistant DEC strains, and monitoring the evolution and dissemination of DEC subtypes across different geographic regions and populations. However, there is a lack of comprehensive genomic data on the diversity of *E. coli*, as well as the repertoires of ARGs, VAGs, STs, and serotypes. Therefore, the objective of this study was to characterize DEC isolates (VAGs, ARGs, STs, serotypes, and phylogenetic groups) of the most prominent

pathotypes (EPEC, STEC, EAEC, ETEC, and EIEC) obtained from stool samples of patients visiting primary healthcare facilities in Addis Ababa and Hossana, Ethiopia, using whole-genome sequencing and bioinformatic tools.

2. Results

2.1. Whole-Genome Sequencing of DEC Isolates

All 30 DEC isolates detected by real-time PCR were subjected to whole-genome sequencing (WGS). Based on the preliminary quality assessment criteria, which included a depth of coverage > 30×, N50 > 15 kbp, and <500 contigs in the assembly, whole-genome sequence data of only 25 isolates passed and were hence further used for bioinformatics analysis. The whole-genome sequence analysis revealed that one out of the 25 isolates did not harbor any DEC specific genes, so whole-genome sequence data from this isolate were excluded from further analysis. The predominant DEC pathotype was the EPEC strain (10/24; 41.7%), followed by the EAEC (6/24; 25%) and ETEC strains (5/24; 20.8%). Three (12.5%) of *E. coli* isolates belonged to STEC pathotype. No *E. coli* isolates belonged to EIEC pathotype. All STEC isolates were detected in stool from non-diarrheic patients; however, the other pathotypes were detected from both diarrheic and non-diarrheic patients. Of the 24 DEC isolates, 14 (58.3%) were isolated from stool specimens of diarrheic patients. Overall, the DEC isolates were detected in equal frequency in both Addis Ababa and Hossana. A high proportion of DEC isolates (45.8%) was detected in the age group 20–45 years. However, there was no significant difference in the distribution of DEC among different age groups (Fisher exact $p = 0.873$). The prevalence of DEC isolates was slightly higher in females (58.3%) compared to males (41.7%) (Table 1).

Table 1. Distribution among study sites and patient's characteristics of DEC isolates.

Characteristics	Number of DEC Isolates (%)			
	EPEC (*n* = 10)	EAEC (*n* = 6)	ETEC (*n* = 5)	STEC (*n* = 3)
Study site				
Addis Ababa (*n* = 12)	4 (16.7)	3 (12.5)	2 (8.3)	3 (12.5)
Hossana (*n* = 12)	6 (25)	3 (12.5)	3 (12.5)	0
Sex				
Male (*n* = 10)	6 (25.0)	3 (12.5)	0	1 (4.2)
Female (*n* = 14)	4 (16.7)	3 (12.5)	5 (20.8)	2 (8.3)
Participants				
Diarrheic (*n* = 14)	7 (29.2)	3 (12.5)	4 (16.7)	0
Non-diarrheic (*n* = 10)	3 (12.5)	3 (12.5)	1 (4.2)	3 (12.5)
Age group				
0–4 (*n* = 3)	2 (8.3)	0	1 (4.2)	0
5–9 (*n* = 2)	1 (4.2)	1 (8.3)	0	0
10–14 (*n* = 3)	2 (8.3)	0	0	1 (4.2)
15–19 (*n* = 2)	0	1 (4.2)	1 (4.2)	0
20–45 (*n* = 11)	5 (20.8)	3 (12.5)	2 (8.3)	1 (4.2)
46–65 (*n* = 3)	0	1 (4.2)	1 (4.2)	1 (4.2)

According to the whole-genome sequence analysis, the Shiga toxin 1 encoding gene (*stx1)*, with its subtypes *stx1c* and *stx1a*, was detected in two isolates, while the Shiga toxin 2 encoding gene (*stx2)* variant *stx2c* was detected in one isolate. Six different subtypes of intimin (*eaeA)* genes (*eae-a01-α*, *eae-b01a-β*, *eae-e02-ε*, *eae-e08-π*, *eae-g02-θ*, and *eae-e06-η*) were detected in EPEC isolates. Furthermore, two variants of heat-labile enterotoxins (*eltIAB-8* and *eltIAB-11*) and one variant of heat-stable enterotoxin (*estah-STa3*), specific to ETEC, were detected (Table 2).

Table 2. Features of DEC isolates in terms of phylogroup, sequence types (STs), serotypes, and pathotype-specific virulence gene distribution.

Pathotype as Determined by WGS	Phylogroup	ST	Serotype	Pathotype Specific Virulence Genes
EPEC	A	10	O90:H40	*eae-g02-θ*
	A	10	O90:H40	*eae-g02-θ*
	A	382	H5	*eae-e08-π*
	B1	40	O21:H21	*eae-b01a-β*
	B1	517	O111:H19	*eae-e02-ε*
	B1	517	H19	*eae-e02-ε*
	B1	616	H21	*eae-e06-η*
	B1	4038	O93:H28	*eae-e06-η*
	B2	2346	O142:H34	*eae-a01-α*
	E	8508	O136:H49	*eae-g02-θ*
EAEC	A	10	O65:H12	*aggR*
	A	34	O99:H33	*aggR*
	B1	295	O171:H5	*aggR*
	B1	678	O104:H4	*aggR*
	B1	1136	O59:H19	*aggR*
	B1	1136	O167:H19	*aggR*
ETEC	A	10	O159:H21	*estah-STa3*
	A	1564	H21	*estah-STa3*
	B1	155	O58:H51	*eltIAB-8*
	B1	314	O112ab:H21	*eltIAB-11*
	B1	314	O112ab:H21	*eltIAB-11*
STEC	B1	101	O117:H12	*stx1* (*stx1a*)
	B1	155	H25	*stx2* (*stx2c*)
	B1	737	O81:H21	*stx1* (*stx1c*)

2.2. Serotypes, Phylogenetic Groups, and Multilocus Sequence Typing of DEC Isolates

The DEC isolates (n = 24) confirmed by WGS belonged to 21 different serotypes. Five isolates (20.8%) belonging to STEC, EPEC, and ETEC pathotypes were defined as 'rough' (lacking expression of the O antigen). Serotyping results showed that the EPEC isolates belonged to nine different serotypes (Tables 2 and S1).

Phylogenomic analysis revealed considerable diversity among the 24 WGS confirmed DEC isolates. Most of the isolates (n = 15; 62.5%) belonged to phylogenetic group B1, seven (29.2%) to group A, and the remaining isolates to phylogroups B2 (n = 1; 4.2%) and E (n = 1; 4.2%) (Figure 1).

Phylogroup B1 was equally detected in Hossana and Addis Ababa, while four isolates from Addis Ababa and three isolates from Hossana were detected in phylogroup A. Phylogroup B2 was detected only among isolates from Hossana. Phylogroups A and B1 were detected in all age groups except in the age group 5–9 years. 'Rough' serotypes were identified as phylogroup A and B1 (Table S1).

The 24 WGS-confirmed DEC isolates were assigned to 17 different STs, of which the dominant DEC pathotype (EPEC) was detected in isolates belonging to 8 different STs and 9 different serotypes. The most frequent ST was the ST10, representing four (16.7%) of the DEC isolates (Table 2).

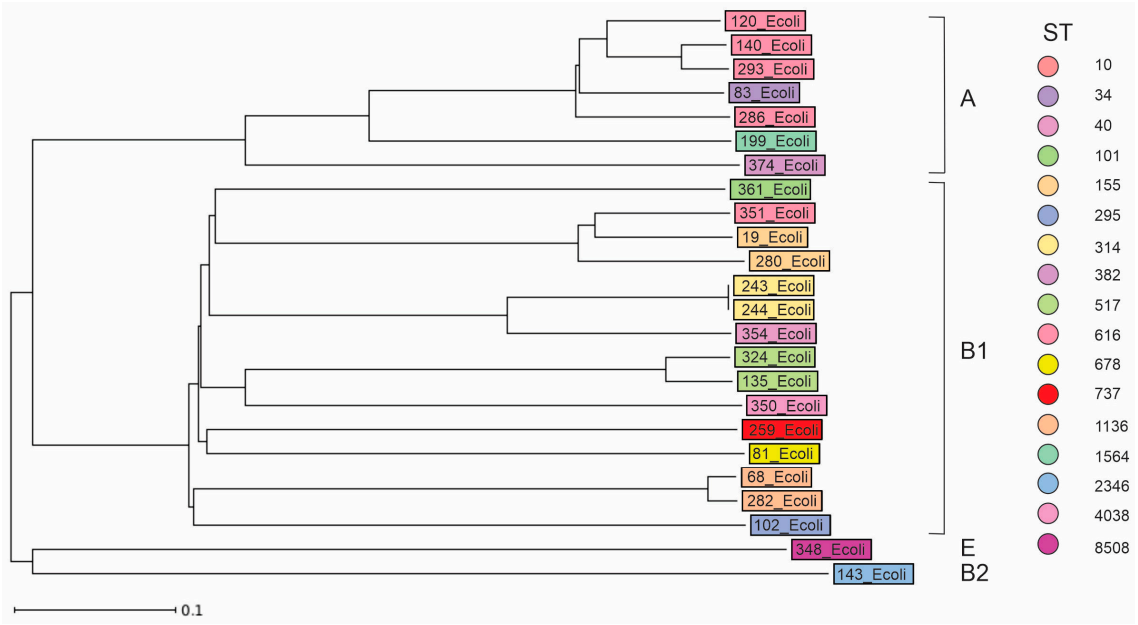

Figure 1. Phylogenetic tree of 24 WGS-confirmed DEC isolates. Different sequence types (STs) are color marked. The phylogenetic groups of the isolates are also given.

2.3. Virulence-Associated Genes of DEC Isolates

The VAGs profiles of each *E. coli* isolates are summarized in Table S1. Among 24 WGS-confirmed DEC isolates, 117 VAGs (involved in iron acquisition, adherence, and toxin production) were identified, of which 42 VAGs were detected only in a single pathotype. All DEC isolates tested had 14 to 37 VAGs. Virulence-associated genes were detected more frequently in the EPEC strain compared to other pathotypes. Genes involved in toxin production, including Shiga toxin-encoding genes (*stx1* or *stx2*) and enterotoxin-encoding genes, such as *astA*, *eltIAB-8*, *eltIAB-11*, *estah-STa3*, *estY2*, *pet*, *pic*, *sat* and *senB* were detected. In addition, bacteriocins and microcins encoding genes, namely *cba*, *cea*, *cia*, *cib*, *cma*, *colE2*, *colE5*, and *cva*, were detected in various DEC isolates. Other VAGs were also identified, including *ehxA*, encoding enterohemolysin, and *cdt-IIIB*, which is a variant of cytolethal distending toxin IIIB. In addition, phages associated with the *stx* genes were identified in STEC isolates using PHASTEST. This showed that the *stx1* gene was carried by a complete, intact phage in isolate 259, while the *stx2* gene was associated with an incomplete phage in isolate 280. In the third STEC isolate, isolate 361 possessing *stx1* gene, no phage sequences in the contig harboring the *stx1* gene were detected, although the contig was long enough. Furthermore, the cytotoxic necrotizing factor 2 (*cnf2*), which is associated with cell death or tissue damage, which is a defining virulence factor of necrotoxigenic *E. coli* (NTEC2), was detected in the isolate 361. This suggests that this isolate represents a hybrid pathotype, containing virulence factors associated with both NTEC and STEC strains (Tables 3 and S1).

Table 3. Distribution of VAGs among different DEC isolates as determined by WGS.

Virulence-Associated Genes and Their Encoded Proteins/Functions			Prevalence in Each Pathotypes N (%), N = 24			
			EPEC (*n* = 10)	EAEC (*n* = 6)	ETEC (*n* = 5)	STEC (*n* = 3)
Adhesins	*aap*	Dispersin, antiaggregation protein	0	6 (25)	0	0
	afaA	Transcriptional regulator	1 (4.2)	0	0	0
	afaC	Outer membrane usher protein	1 (4.2)	0	0	0
	afaD	Afimbrial adhesion	1 (4.2)	3 (12.5)	0	0
	agg3A	AAF/III major fimbrial subunit	0	3 (12.5)	0	0
	agg3B	AAF/III minor adhesin	0	3 (12.5)	0	0
	agg3C	Usher, AAF/III assembly unit	0	3 (12.5)	0	0

Table 3. *Cont.*

Virulence-Associated Genes and Their Encoded Proteins/Functions			Prevalence in Each Pathotypes N (%), N = 24			
			EPEC (*n* = 10)	EAEC (*n* = 6)	ETEC (*n* = 5)	STEC (*n* = 3)
Adhesins	*agg3D*	Chaperone, AAF/III assembly unit	0	3 (12.5)	0	0
	agg4A	AAF/IV major fimbrial subunit	0	2 (8.3)	0	0
	agg4C	Usher, AAF/IV assembly unit	0	2 (8.3)	0	0
	aggA	AAF/I major fimbrial subunit	0	1 (4.2)	0	0
	aggC	Usher, AAF/I assembly unit	0	1 (4.2)	0	0
	aggD	Chaperone, AAF/I assembly unit	0	1 (4.2)	0	0
	aggR	AraC transcriptional activator	0	6 (25)	0	0
	cif	Type III secreted effector	4 (16.7)	0	0	0
	csgA	Curli major subunit CsgA	9 (37.5)	6 (25)	5 (20.8)	2 (8.3)
	cswR	CS12 Transcriptional activator	0	0	2 (8.3)	0
	eaeA	Intimin	10 (41.7)	0	0	0
	efa1	EHEC factor for adherence; lymphostatin Efa1-LifA	1 (4.2)	0	0	0
	etpB	EtpB Nonfimbrial adhesin/TPS transporter	0	0	1 (4.2)	0
	faeC	F4 (K88) Minor fimbrial subunit	0	0	0	1 (4.2)
	fdeC	Intimin-like adhesin FdeC	5 (20.8)	2 (8.3)	3 (12.5)	3 (12.5)
	fimH	Type 1 fimbriae	8 (33.3)	5 (20.8)	4 (16.7)	3 (12.5)
	hra	Heat-resistant agglutinin	0	2 (8.3)	1 (4.2)	0
	iha	Adherence protein	0	2 (8.3)	0	3 (12.5)
	lpfA	Long polar fimbriae	4 (16.7)	4 (16.7)	3 (12.5)	3 (12.5)
	nlpI	Lipoprotein NlpI precursor	10 (41.7)	6 (25)	5 (20.8)	3 (12.5)
	perA	EPEC adherence factor	1 (4.2)	0	0	0
	tir	Translocated intimin receptor protein	9 (37.5)	0	0	0
	yehA	Outer membrane lipoprotein, YHD fimbrial cluster	6 (25)	3 (12.5)	4 (16.7)	2 (8.3)
	yehB	Usher, YHD fimbrial cluster	8 (33.3)	6 (25)	5 (20.8)	3 (12.5)
	yehC	Chaperone, YHD fimbrial cluster	8 (33.3)	6 (25)	5 (20.8)	3 (12.5)
	yehD	Major pilin subunit, YHD fimbrial cluster	8 (33.3)	5 (20.8)	5 (20.8)	3 (12.5)
	yfcV	Fimbrial protein	1 (4.2)	0	0	0
Protectins	*aaiC*	Type VI secretion protein	0	3 (12.5)	0	0
	aar	AggR-activated regulator	0	4 (16.7)	0	0
	aatA	Dispersin transporter protein	0	6 (25)	0	0
	aamR:FN554766	Not defined	0	1 (4.2)	0	0
	anr	AraC negative regulator	3 (12.5)	5 (20.8)	1 (4.2)	0
	aslA	Arylsulfatase-like protein	1 (4.2)	2 (8.3)	0	0
	capU	Hexosyltransferase homolog	2 (8.3)	3 (12.5)	1 (4.2)	0
	cba	Colicin B	0	0	0	1 (4.2)
	cea	Colicin E1	0	1 (4.2)	0	0
	cia	Colicin Ia	1 (4.2)	0	0	1 (4.2)
	cib	Colicin Ib	0	0	2 (8.3)	0
	cma	Colicin M	0	0	0	1 (4.2)
	colE2	Colicin E2	0	0	0	1 (4.2)
	colE5	Colicin E5 lysis protein Lys	0	2 (8.3)	0	0
	cvaC	Microcin C	1 (4.2)	0	0	0
	eilA	*Salmonella* HilA homolog	1 (4.2)	0	0	0
	espA	Type III secretions system	10 (41.7)	0	0	0
	espB	Secreted protein B	1 (4.2)	0	0	0
	espC	*Escherichia coli* enterotoxin EspC	1 (4.2)	0	0	0
	espF	EPEC secreted protein F; Type III secretion system	4 (16.7)	0	0	0
	espI	Serine protease autotransporters of *Enterobacteriaceae* (SPATE) EspI	0	1 (4.2)	0	1 (4.2)
	espJ	Prophage-encoded type III secretion system effector	5 (20.8)	0	0	0
	espP	Extracellular serine protease plasmid-encoded	0	0	0	1 (4.2)
	espY2	Non-LEE-encoded type III secreted effector	1 (4.2)	0	0	0
	etpC	EtpC Glycotransferase	2 (8.3)	0	0	0
	etpD	Type II secretion protein EtpD	1 (4.2)	0	1 (4.2)	0
	etsC	Putative type I secretion outer membrane protein	1 (4.2)	0	1 (4.2)	0
	gad	Glutamate decarboxylase	3 (12.5)	2 (8.3)	2 (8.3)	0
	hha	Hemolysin expression modulator Hha (previous *rmoA*)	5 (20.8)	1 (4.2)	0	0
	iss	Increased serum survival	4 (16.7)	2 (8.3)	1 (4.2)	2 (8.3)

Table 3. *Cont.*

Virulence-Associated Genes and Their Encoded Proteins/Functions			Prevalence in Each Pathotypes N (%), N = 24			
			EPEC (*n* = 10)	EAEC (*n* = 6)	ETEC (*n* = 5)	STEC (*n* = 3)
	kpsE	Capsule polysaccharide export inner-membrane protein	0	0	0	1 (4.2)
	mchB	Precursor of microcin H47	0	1 (4.2)	0	1 (4.2)
	mchC	MchC protein	0	1 (4.2)	0	1 (4.2)
	mchF	ABC transporter protein MchF	1 (4.2)	1 (4.2)	0	1 (4.2)
	mcmA	Microcin M	0	1 (4.2)	0	1 (4.2)
	neuC	Polysialic acid capsule biosynthesis protein	0	2 (8.3)	0	0
	nleB	Non-LEE encoded effector B	6 (25)	0	0	0
	ompT	Outer membrane protease (protein protease 7)	3 (12.5)	0	1 (4.2)	2 (8.3)
	ORF3	Isoprenoid Biosynthesis	0	6 (25)	0	0
	ORF4	Putative isopentenyl-diphosphate delta-isomerase	0	6 (25)	0	0
	shiA	Homologue of the *Shigella flexneri* SHI-2 pathogenicity island gene *shiA*	0	2 (8.3)	1 (4.2)	0
	shiB	Homologue of the *Shigella flexneri* SHI-2 pathogenicity island gene *shiB*	1 (4.2)	3 (12.5)	0	1 (4.2)
	tia	Tia invasion determinant	0	0	0	1 (4.2)
	traJ	Protein TraJ (Positive regulator of conjugal transfer operon)	4 (16.7)	3 (12.5)	3 (12.5)	0
	traT	Outer membrane protein complement resistance	3 (12.5)	3 (12.5)	3 (12.5)	3 (12.5)
	tsh	Serine protease autotransporter of *Enterobacteriaceae* (SPATE)-Immunoglobulin A1 protease-Temperature-sensitive hemagglutinin	1 (4.2)	0	0	0
Iron uptake	*aalF*	CS23 Minor structural subunit	0	0	0	1 (4.2)
	chuA	Outer membrane hemin receptor	2 (8.3)	0	0	0
	fyuA	Yersiniabactin siderophore receptor	0	3 (12.5)	0	1 (4.2)
	ireA	Iron-regulated outer membrane protein IreA	0	2 (8.3)	0	0
	iroN	Salmochelin siderophore receptor protein	1 (4.2)	0	0	0
	irp2	Yersiniabactin non-ribosomal peptide synthetase	0	2 (8.3)	0	1 (4.2)
	iucC	Aerobactin synthetase	1 (4.2)	4 (16.7)	0	1 (4.2)
	iutA	Ferric aerobactin receptor	1 (4.2)	4 (16.7)	0	1 (4.2)
	sitA	Iron transport protein	1 (4.2)	2 (8.3)	1 (4.2)	0
	terC	Tellurium ion resistance protein	9 (37.5)	6 (25)	5 (20.8)	3 (12.5)
Toxins	*astA*	Heat-stable enterotoxin EAST-1	1 (4.2)	4 (16.7)	4 (16.7)	0
	cdt-IIIB	Cytolethal distending toxin III subunit B	0	0	0	1 (4.2)
	cnf2	Cytotoxic necrotizing factor 2	0	0	0	1 (4.2)
	ehxA	Enterohaemolysin	0	0	0	1 (4.2)
	eltIAB-8	Heat-labile enterotoxin LTIh-8	0	0	3 (12.5)	0
	eltIAB-11	Heat-labile enterotoxin LTIh-11				
	estah-STa3	Heat-stable enterotoxin STa3 human variant	0	0	2 (8.3)	0
	hlyA	Hemolysin A	1 (4.2)	0	0	0
	hlyE	Avian *E. coli* haemolysin	8 (33.3)	6 (25)	4 (16.7)	3 (12.5)
	hlyF	Hemolysin F	1 (4.2)	0	1 (4.2)	0
	pet	Autotransporter enterotoxin	1 (4.2)	0	0	0
	pic	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) Pic	0	2 (8.3)	0	0
	sat	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) Sat	2 (8.3)	2 (8.3)	0	0
	senB	Plasmid-encoded enterotoxin	2 (8.3)	0	0	0
	sigA	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) *Shigella* IgA-like protease homologue	0	3 (12.5)	0	0
	stx1	Shiga toxin 1	0	0	0	2 (8.3)
	stx2	Shiga toxin 2	0	0	0	1 (4.2)
	subA	Subtilase toxin subunit	0	0	0	1 (4.2)
	toxB	Toxin B	1 (4.2)	0	0	0
	vat	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) Vat	0	0	0	1 (4.2)

Numerous VAGs were identified in all phylogroups, with the genes being more common in phylogroup B1 strains than in strains from other groups. Phylogroup A and B1 shared 52 similar virulence-associated genes. Additionally, seventeen different virulence-associated genes were detected in phylogroup B2 strain. Two virulence-associated genes (*eilA* and *espY2*) were exclusively detected in phylogroup E. Eight virulence-associated genes such as *hlyE*, *nlpI*, *lpfA*, *terC*, *yehA*, *yehB*, *yehC*, and *yehD* were identified in the entire phylogroup B1. The *toxB* and *espC* genes were detected only in DEC isolate of phylogroup B2. Five virulence-associated genes, namely, *nlpI*, *terC*, *yehB*, *yehC*, and *yehD*, were confirmed to be shared by all four phylogroups (Tables 4 and S1).

Table 4. Distribution of VAGs among different *E. coli* phylogroups as determined by WGS.

Virulence-Associated Genes and Their Encoded Proteins/Functions			Prevalence in Each Phylogenetic Group N (%), N = 24			
			A (*n* = 7)	B1 (*n* = 15)	B2 (*n* = 1)	E (*n* = 1)
Adhesins	*aap*	Dispersin, antiaggregation protein	2 (8.3)	4 (16.7)	0	0
	afaA	Transcriptional regulator	1 (4.2)	0	0	0
	afaC	Outer membrane usher protein	1 (4.2)	0	0	0
	afaD	Afimbrial adhesion	2 (8.3)	2 (8.3)	0	0
	agg3A	AAF/III major fimbrial subunit	1 (4.2)	2 (8.3)	0	0
	agg3B	AAF/III minor adhesin	1 (4.2)	2 (8.3)	0	0
	agg3C	Usher, AAF/III assembly unit	1 (4.2)	2 (8.3)	0	0
	agg3D	Chaperone, AAF/III assembly unit	1 (4.2)	2 (8.3)	0	0
	agg4A	AAF/IV major fimbrial subunit	0	2 (8.3)	0	0
	agg4C	Usher, AAF/IV assembly unit	0	2 (8.3)	0	0
	aggA	AAF/I major fimbrial subunit	1 (4.2)	0	0	0
	aggC	Usher, AAF/I assembly unit	1 (4.2)	0	0	0
	aggD	Chaperone, AAF/I assembly unit	1 (4.2)	0	0	0
	aggR	AraC transcriptional activator	2 (8.3)	4 (16.7)	0	0
	cif	Type III secreted effector	2 (8.3)	1 (4.2)	0	1 (4.2)
	csgA	Curli major subunit CsgA	7 (29.2)	15 (62.5)	1 (4.2)	1 (4.2)
	cswR	CS12 Transcriptional activator	0	2 (8.3)	0	0
	eaeA	Intimin	3 (12.5)	4 (16.7)	1 (4.2)	1 (4.2)
	efa1	EHEC factor for adherence; lymphostatin Efa1-LifA	0	1 (4.2)	0	0
	etpB	EtpB nonfimbrial adhesin/TPS transporter	1 (4.2)	0	0	0
	faeC	F4 (K88) Minor fimbrial subunit	0	1 (4.2)	0	0
	fdeC	Intimin-like adhesin FdeC	0	12 (50)	0	1 (4.2)
	fimH	Type 1 fimbriae	6 (25)	13 (54.2)	0	1 (4.2)
	hra	Heat-resistant agglutinin	1 (4.2)	2 (8.3)	0	0
	iha	Adherence protein	0	5 (20.8)	0	0
	lpfA	Long polar fimbriae	0	14 (58.3)	0	0
	nlpI	Lipoprotein NlpI precursor	6 (25)	12 (50)	1 (4.2)	1 (4.2)
	perA	EPEC adherence factor	0	0	1 (4.2)	0
	tir	Translocated intimin receptor protein	3 (12.5)	5 (20.8)	0	1 (4.2)
	yehA	Outer membrane lipoprotein, YHD fimbrial cluster	7 (29.2)	7 (29.2)	1 (4.2)	0
	yehB	Usher, YHD fimbrial cluster	5 (20.8)	15 (62.5)	1 (4.2)	1 (4.2)
	yehC	Chaperone, YHD fimbrial cluster	5 (20.8)	15 (62.5)	1 (4.2)	1 (4.2)
	yehD	Major pilin subunit, YHD fimbrial cluster	5 (20.8)	14 (58.3)	1 (4.2)	1 (4.2)
	yfcV	Fimbrial protein	0	0	1 (4.2)	1 (4.2)
Protectins	*aaiC*	Type VI secretion protein	1 (4.2)	1 (4.2)	0	0
	aar	AggR-activated regulator	1 (4.2)	1 (4.2)	0	0
	aatA	Dispersin transporter protein	1 (4.2)	2 (8.3)	0	0
	aamR:FN554766	Not defined	0	1 (4.2)	0	0
	anr	AraC negative regulator	4 (16.7)	2 (8.3)	0	0
	aslA	Arylsulfatase-like protein	1 (4.2)	0	1 (4.2)	0
	capU	Hexosyltransferase homolog	1 (4.2)	3 (12.5)	0	0
	cba	Colicin B	0	1 (4.2)	0	0
	cea	Colicin E1	1 (4.2)	0	0	0
	cia	Colicin Ia	1 (4.2)	1 (4.2)	0	0
	cib	Colicin Ib	2 (8.3)	0	0	0
	cma	Colicin M	0	1 (4.2)	0	0
	colE2	Colicin E2	0	1 (4.2)	0	0
	colE5	Colicin E5 lysis protein Lys	0	2 (8.3)	0	0

Table 4. *Cont.*

Virulence-Associated Genes and Their Encoded Proteins/Functions			Prevalence in Each Phylogenetic Group N (%), N = 24			
			A (*n* = 7)	B1 (*n* = 15)	B2 (*n* = 1)	E (*n* = 1)
	cvaC	Microcin C	1 (4.2)	0	0	0
	eilA	*Salmonella* HilA homolog	0	0	0	1 (4.2)
	espA	Type III secretions system	3 (12.5)	5 (20.8)	1 (4.2)	1 (4.2)
	espB	Secreted protein B	0	1 (4.2)	0	0
	espC	*Escherichia coli* enterotoxin EspC	0	0	1 (4.2)	0
	espF	EPEC secreted protein F; Type III secretion system	1 (4.2)	3 (12.5)	0	0
	espI	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) EspI	1 (4.2)	1 (4.2)	0	0
	espJ	Prophage-encoded type III secretion system effector	0	3 (12.5)	1 (4.2)	1 (4.2)
	espP	Extracellular serine protease plasmid-encoded	0	1 (4.2)	0	0
	espY2	Non-LEE-encoded type III secreted effector	0	0	0	1 (4.2)
	etpC	EtpC Glycotransferase	1 (4.2)	0	0	0
	etpD	Type II secretion protein EtpD	1 (4.2)	1 (4.2)	0	0
	etsC	Putative type I secretion outer membrane protein	1 (4.2)	0	0	0
	gad	Glutamate decarboxylase	2 (8.3)	4 (16.7)	0	0
	hha	Hemolysin expression modulator Hha (previous *rmoA*)	1 (4.2)	3 (12.5)	0	1 (4.2)
	iss	Increased serum survival	3 (12.5)	5 (20.8)	0	0
Protectins	*kpsE*	Capsule polysaccharide export inner-membrane protein	0	1 (4.2)	0	0
	mchB	Precursor of microcin H47	0	2 (8.3)	0	0
	mchC	MchC protein	0	2 (8.3)	0	0
	mchF	ABC transporter protein MchF	1 (4.2)	2 (8.3)	0	0
	mcmA	Microcin M	0	2 (8.3)	0	0
	neuC	Polysialic acid capsule biosynthesis protein	0	1 (4.2)	0	0
	nleB	Non-LEE encoded effector B	3 (12.5)	2 (8.3)	0	1 (4.2)
	ompT	Outer membrane protease (protein protease 7)	1 (4.2)	3 (12.5)	1 (4.2)	0
	ORF3	Isoprenoid Biosynthesis	2 (8.3)	4 (16.7)	0	0
	ORF4	Putative isopentenyl-diphosphate delta-isomerase	2 (8.3)	4 (16.7)		0
	shiA	Homologue of the *Shigella flexneri* SHI-2 pathogenicity island gene *shiA*	2 (8.3)	1 (4.2)	0	0
	shiB	Homologue of the *Shigella flexneri* SHI-2 pathogenicity island gene *shiB*	1 (4.2)	4 (16.7)	0	0
	tia	Tia invasion determinant	0	1 (4.2)	0	0
	traJ	Protein TraJ (Positive regulator of conjugal transfer operon)	4 (16.7)	3 (12.5)	1 (4.2)	0
	traT	Outer membrane protein complement resistance	3 (12.5)	6 (25)	0	0
	tsh	Serine protease autotransporter of *Enterobacteriaceae* (SPATE)-Immunoglobulin A1 protease-Temperature-sensitive hemagglutinin	1 (4.2)	0	0	0
	aalF	CS23 Minor structural subunit	0	1 (4.2)	0	0
	chuA	Outer membrane hemin receptor	0	0	1 (4.2)	1 (4.2)
	fyuA	Yersiniabactin siderophore receptor	2 (8.3)	2 (8.3)	0	0
	ireA	Iron-regulated outer membrane protein IreA	0	1 (4.2)	0	0
	iroN	Salmochelin siderophore receptor protein	1 (4.2)	0	0	0
Iron uptake	*irp2*	Yersiniabactin non-ribosomal peptide synthetase	1 (4.2)	2 (8.3)	0	0
	iucC	Aerobactin synthetase	1 (4.2)	4 (16.7)	0	0
	iutA	Ferric aerobactin receptor	1 (4.2)	4 (16.7)	0	0
	sitA	Iron transport protein	1 (4.2)	1 (4.2)	0	0
	terC	Tellurium ion resistance protein	7 (29.2)	15 (62.5)	1 (4.2)	1 (4.2)
	astA	Heat-stable enterotoxin EAST-1	3 (12.5)	4 (16.7)	0	0
	cdt-IIIB	Cytolethal distending toxin III subunit B	0	1 (4.2)	0	0
	cnf2	Cytotoxic necrotizing factor 2	0	1 (4.2)	0	0
Toxins	*ehxA*	Enterohaemolysin	0	1 (4.2)	0	0
	eltIAB-8	Heat-labile enterotoxin LTIh-8	0	1 (4.2)	0	0
	eltIAB-11	Heat-labile enterotoxin LTIh-11	0	2 (8.3)	0	0

Table 4. *Cont.*

Virulence-Associated Genes and Their Encoded Proteins/Functions			Prevalence in Each Phylogenetic Group N (%), N = 24			
			A (*n* = 7)	B1 (*n* = 15)	B2 (*n* = 1)	E (*n* = 1)
Toxins	*estah-STa3*	Heat-stable enterotoxin STa3 human variant	2 (8.3)	0	0	0
	hlyA	Hemolysin A	0	1 (4.2)	0	0
	hlyE	Avian *E. coli* haemolysin	6 (25)	15 (62.5)	0	1 (4.2)
	hlyF	Hemolysin F	1 (4.2)	0	0	0
	pet	Autotransporter enterotoxin	0	1 (4.2)	0	0
	pic	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) Pic	1 (4.2)	1 (4.2)	0	0
	sat	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) Sat	1 (4.2)	3 (12.5)	0	0
	senB	Plasmid-encoded enterotoxin	0	2 (8.3)	0	0
	sigA	Serine protease autotransporter of *Enterobacteriaceae* (SPATE), *Shigella* IgA-like protease homologue	0	2 (8.3)	0	0
	stx1	Shiga toxin 1	0	2 (8.3)	0	0
	stx2	Shiga toxin 2	0	1 (4.2)	0	0
	subA	Subtilase toxin subunit	0	1 (4.2)	0	0
	toxB	Toxin B	0	0	1 (4.2)	0
	vat	Serine protease autotransporter of *Enterobacteriaceae* (SPATE) Vat	0	1 (4.2)	0	0

2.4. Antimicrobial Resistance Genes in DEC Isolates

In the phenotypic antimicrobial susceptibility testing, 18 out of the total 30 DEC isolates (60%) showed resistance to at least one of the tested antimicrobial agents (Wolde et al., 2024). A total of 23 different ARGs conferring resistance to β-lactams, aminoglycosides, quinolones, sulfonamides, trimethoprim, the macrolide/lincosamide/streptogramin (MLS) group, and tetracycline were detected in 18 (75%) DEC isolates out of 24 isolates subjected to whole-genome sequencing (Table 5). The most common ARG was $bla_{\text{TEM-1B}}$, which was found in 12 DEC isolates. The results indicated that the EPEC and ETEC isolates carried multiple ARGs conferring resistance to a range of antimicrobials. In contrast, the STEC isolates were not resistant to any of the tested antibiotics and did not harbor any of the ARGs.

Table 5. Antimicrobial resistance genes among DEC isolates.

Antimicrobial Class	Antimicrobial Resistance Gene (ARG)	Prevalence N (%)	Conferring Resistance to	Found in DEC Pathotype
β-lactams	$bla_{\text{TEM-1B}}$	12 (50)	amoxicillin, ampicillin, piperacillin, ticarcillin, cephalothin	EPEC, EAEC, ETEC
	$bla_{\text{TEM-122}}$	1 (4.2)	amoxicillin, amoxicillin + clavulanic acid, ampicillin, ampicillin + clavulanic acid, piperacillin, piperacillin + tazobactam, ticarcillin, ticarcillin + clavulanic acid	ETEC
	$bla_{\text{TEM-163}}$	1 (4.2)	amoxicillin, amoxicillin + clavulanic acid, ampicillin, ampicillin + clavulanic acid, piperacillin, piperacillin + tazobactam, ticarcillin, ticarcillin + clavulanic acid	EPEC
	$bla_{\text{CTX-M-3}}$	1 (4.2)	amoxicillin, ampicillin, cefepime, cefotaxime, ceftazidime, piperacillin, aztreonam, ticarcillin, ceftriaxone	EPEC
	$bla_{\text{CTX-M-15}}$	2 (8.3)	amoxicillin, ampicillin, cefepime, cefotaxime, ceftazidime, piperacillin, aztreonam, ticarcillin, ceftriaxone	ETEC
Sulfonamides	*sul1*	2 (8.3)	sulfamethoxazole	EPEC
	sul2	8 (33.3)	sulfamethoxazole	EPEC, EAEC, ETEC
Aminoglycosides	*aph(6)-Id*	6 (25)	streptomycin	EPEC, ETEC
	aph(3″)-Ib	6 (25)	streptomycin	EPEC, ETEC
	aac(3)-IIa	1 (4.2)	gentamicin, tobramycin	EPEC
	aadA1	2 (8.3)	streptomycin, spectinomycin	EAEC, ETEC
	aadA5	1 (4.2)	streptomycin, spectinomycin	EPEC
	aadA24	1 (4.2)	streptomycin, spectinomycin	EPEC
Tetracyclines	*tet*(A)	4 (16.7)	tetracycline, doxycycline	EPEC
	tet(B)	3 (12.5)	tetracycline, doxycycline, minocycline	EPEC, ETEC
Quinolones	*qnrS1*	2 (8.3)	ciprofloxacin	ETEC

Table 5. *Cont.*

Antimicrobial Class	Antimicrobial Resistance Gene (ARG)	Prevalence N (%)	Conferring Resistance to	Found in DEC Pathotype
Trimethoprim	*dfrA1*	2 (8.3)	trimethoprim	EAEC, ETEC
	dfrA7	1 (4.2)	trimethoprim	EPEC
	dfrA8	3 (12.5)	trimethoprim	EPEC, EAEC, ETEC
	dfrA14	1 (4.2)	trimethoprim	EPEC
	dfrA15	1 (4.2)	trimethoprim	ETEC
	dfrA17	1 (4.2)	trimethoprim	EPEC
Macrolide/lincosamide/streptogramin (MLS) group	*mph*(A)	6 (25)	erythromycin, azithromycin, spiramycin, telithromycin	EPEC, EAEC, ETEC
	erm(B)	1 (4.2)	lincomycin, clindamycin, erythromycin, quinupristin, pristinamycin IA, virginiamycin S	EAEC

2.5. Plasmids and Other Mobile Genetic Elements

Eighty-two different mobile genetic elements were identified in *E. coli* isolates in this study. Of these, 22 were plasmids, 50 were insertion sequence elements (ISs), 9 were transposons (three-unit transposons and six composite transposons), and 1 was a miniature inverted repeat (*MITEEc1*). The majority of MGEs were detected in the EPEC strain, followed by the EAEC strain. Twenty-three MGEs (9 plasmids, 10 ISs, and 4 transposons) were identified only in the EPEC isolates. Twenty-two (91.7%) of the DEC isolates were confirmed to harbor one or more plasmids. The *IncF* plasmid with different replicon types and replicon variants, including *IncFII*, *IncFII(29)*, *IncFIB(AP001918)*, *IncFII(pRSB107)*, *IncFIB(pB171)*, *IncFII(pHN7A8)*, *IncFII(pCoo)*, *IncFII(pSE11)*, *IncFIC(FII)*, *IncFIA(HI1)*, *IncFIA*, and *IncFIB(K)* was the most abundant plasmid in *E. coli* isolates. Additionally, *Col* plasmid groups such as *Col(MG828)*, *Col156*, *ColRNAI*, *Col(BS512)*, and *ColpVC* were also detected in DEC isolates. Fifteen (68.2%) of the total plasmids were identified in EPEC isolates, while only five (22.7%) were identified in STEC isolates (Figure 2).

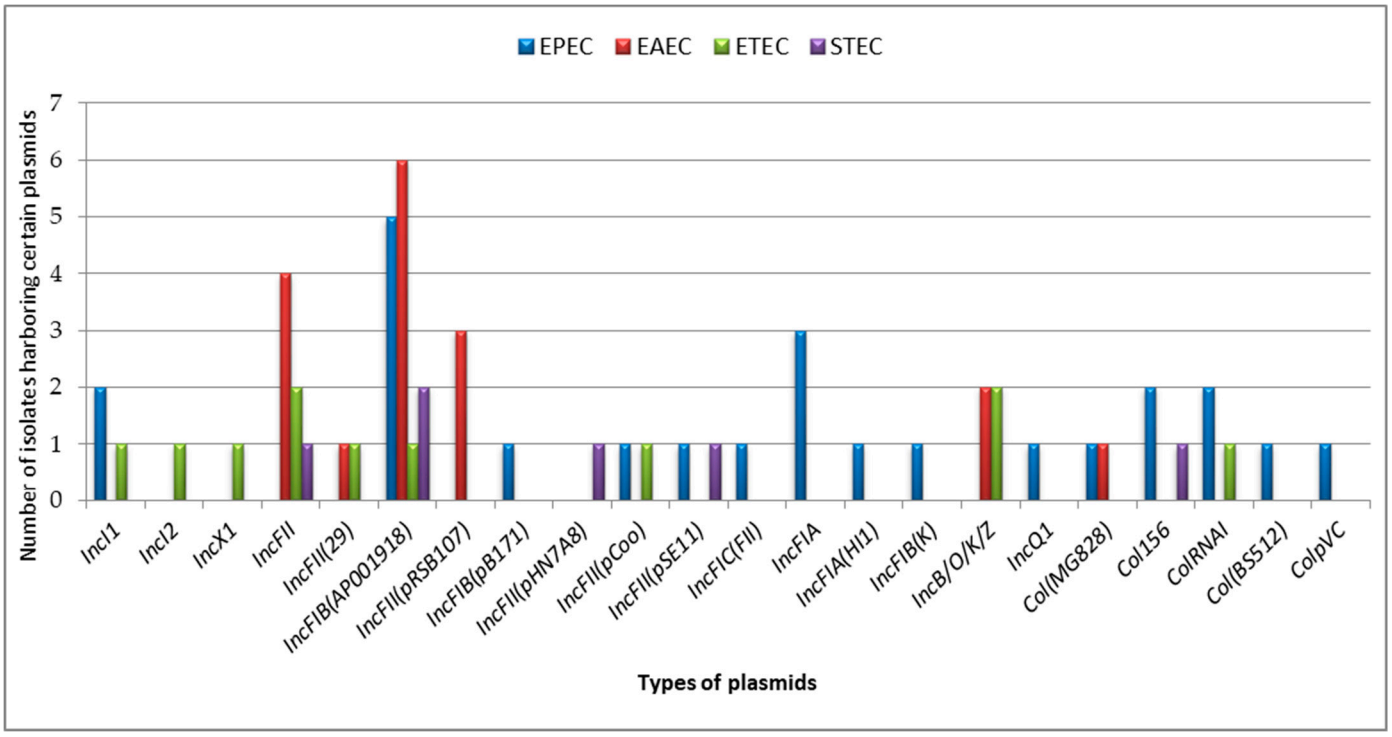

Figure 2. Frequency of plasmid types in pathotypes of studied DEC isolates.

In the current study, we found that all DEC isolates harbored *MITEEc1*, 66.7% harbored *IS609*, and 62.5% harbored *ISEc1*. Transposons such as *Tn2*, *Tn7*, and *Tn6024* were identified in 16.7% of the DEC isolates. Thirty-seven (61.7%) of the MGEs, apart from plasmids, were detected in the EPEC strain, while 29 (48.3%) were detected in the ETEC strain.

On average, all the DEC isolates carried 7 MGEs, excluding plasmids, with some carrying as many as 16. *MITEEc1* and 11 IS elements, including *IS100*, *IS30*, *IS609*, *IS629*, *ISEc1*, *ISEc13*, *ISEc31*, *ISEc38*, *ISEc39*, *ISSfl8*, and *ISSfl10*, were detected in the STEC isolates (Figure 3).

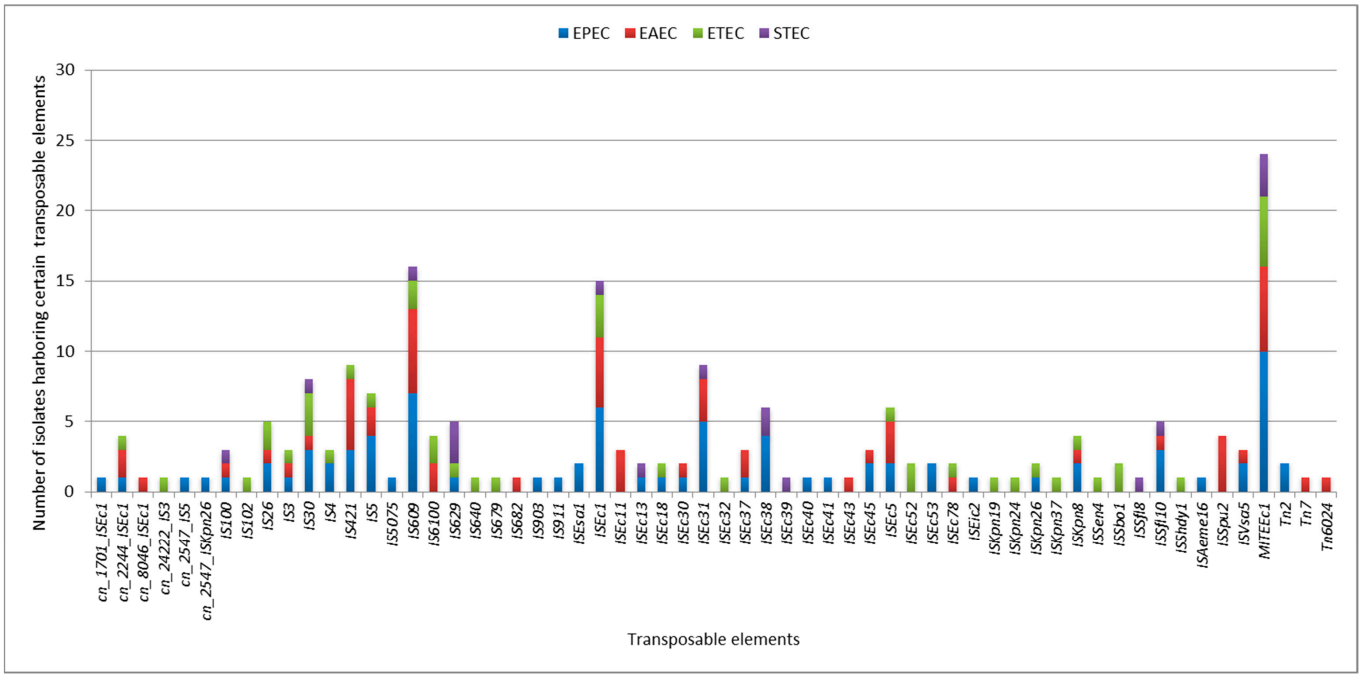

Figure 3. Frequency of transposable elements in pathotypes of studied DEC isolates.

3. Discussion

The DEC strains exhibited genetic diversity both within and between different pathotypes [26]. The overall rate of DEC isolation in this study was 11.5%, which is comparable to previous reports in diarrheic children under the age of five years and in tracked human contacts in rural and urban areas in Eastern Ethiopia (10.3%) [27], among diarrheic children in Abuja, Nigeria (12.8%) [28], as well as in children with diarrhea in Burkina Faso (7.4%) [29]. However, a higher number of DEC isolates were reported in diarrheic patients of all age groups in Kwali, Nigeria (27%) [30], and in Tunisia among children and adults with and without diarrhea (48.2%) [31]. Similarly, high rates of DEC isolates were detected in children with and without diarrhea in central Ethiopia (38.2%) [20], in Nigeria (73.8%) [32], and in Gabon (68.5%) [33], in children under five years with diarrhea and food animals in Kenya (23.0%) [34] and in Mozambique (48.6%) [35].

Phylogenetic group analysis of unknown *E. coli* strains is crucial for understanding the population structure and diversity of *E. coli* strains [36]. The present study showed a difference in phylogroup structure, with phylogroup B1 being predominant (62.5%), which is consistent with a study conducted in southwestern Nigeria among *E. coli* isolates from human where phylogroup B1 was predominant [37]. In contrast, a study conducted among diarrheic children and adults in Cote d'Ivoire showed the predominance of phylogroup A (53%) [38]. In addition two studies on *E. coli* isolated from different clinical sources in Egypt from patients aged 10 to 65 years and symptomatic UTIs (urinary tract infections) and diarrheic patients showed the predominance of phylogroup A (53%) [39,40].

E. coli strains belonging to phylogroup A and B1 are commonly considered as commensal organisms [41]. However, this study found that *E. coli* strains in these groups contained

a high number of VAGs that enable them to cause infection in the intestinal tract, which may have been acquired through horizontal gene transfer [42], representing a significant health concern. This is consistent with the finding of a systematic review and meta-analysis of human commensal *E. coli* from different regions of the world that showed dominance of phylogroup A and B1 [36].

This is the first study in Ethiopia to determine the population structure of DEC isolates using WGS, providing a comprehensive understanding of the genetic diversity of DEC isolates. The DEC isolates in our study revealed high genetic diversity in the population structure of DEC pathotypes. This is in agreement with a study conducted in Malawi using *E. coli* collected from 2012 to 2018 [43]. Seventeen different STs were identified in our study, with the dominant EPEC pathotype in this study belonging to eight different STs and nine different serotypes, indicating a broad distribution of genes encoding the EPEC strain across the different STs and serotypes of *E. coli*. Each ST contained a variety of VAG combinations. This poses a challenge in reducing the transmission of infections associated with DEC pathotypes. The most frequent ST was ST10. In a study conducted on EAEC isolates from children with and without diarrhea in Nigeria, ST10 was the most prevalent [44]. Our study, however, indicated that only one EAEC was in ST10.

Both commensal and pathogenic *E. coli* can share virulence factors that are essential to survive and colonize specific ecological niches in the host, highlighting the versatility of *E. coli* [45]. Our analysis revealed that all *E. coli* isolates tested carried a variety of VAGs encoding adhesins, toxins, protectins, and iron uptake factors. Genes related to extra-intestinal *E. coli* pathotypes, such as *csgA*, *fimH*, *hlyE*, *iss*, *nlpI*, *terC* and *traT*, were distributed across all pathotype groups. Of these, the genes *csgA*, *fimH*, and *traT* were detected in *E. coli* from stool, urine, and blood samples in Egypt [46]. The *fimH*, *afa*, *hly*, and *cnf* genes were also detected in *E. coli* isolated from patients with urinary tract infections in Addis Ababa, Ethiopia [47], and Tunisia [48]. In addition, the *fimH*, *vat*, *sitA*, *hlyF*, and *iutA* genes were detected in uropathogenic *E. coli* isolated from patients with suspected UTIs in Zimbabwe [49]. This suggests that *E. coli* strains carrying genes responsible for the development of both intestinal and extraintestinal infections may be derived from commensal strains through the acquisition of different virulence factors via horizontal gene transfer during their presence in the intestine, enabling them to become virulent and theoretically cause both intestinal and extraintestinal infections [45,50,51].

Hybrid pathotypes have diverse and potentially enhanced pathogenicity potential. The identification of such hybrid strains is an important public health concern [52]. In the current study, an NTEC/STEC hybrid pathotype was detected. This pathotype carried the *cnf*2 gene, which encodes the cytotoxin necrotizing factor 2 and the *stx1* gene, which encodes for the Shiga toxin. Cytotoxic necrotizing factor and Shiga toxin are cyclomodulins known to modulate cellular differentiation, apoptosis, and proliferation [53]. The *cnf*2 gene, which is known to encode a lethal and dermonecrotic toxin, is located on pVir-like conjugative plasmids [54]. The other cyclomodulin encoding gene detected in this isolate, the *cdt-IIIB* gene is also known to be located on pVir-like conjugative plasmids. This cyclomodulin induces DNA damage and cell cycle arrest, leading to cytotoxicity and facilitating the entry of other toxins [55]. The isolate also carried a serine protease encoded by *espP*, which has proteolytic activity and can cleave human coagulation factor V [56]. The synergistic effect of several toxins could increase the ability of the isolate to cause more severe disease. This pathotype can be considered the first to be reported from Ethiopia. This is an indication that appropriate prevention and control measures need to be developed and implemented to control this type of virulent strains as they may pose a potential health threat to the population.

The most widely used class of antibiotics in humans for the treatment of pathogenic *E. coli* is the β-lactams [57]. However, in this study, a high frequency of DEC isolates resistant not only to β-lactam but also aminoglycosides, quinolones, macrolides, sulfonamides, trimethoprim, and tetracycline antibiotics were found. The $bla_{\text{TEM-1B}}$ gene was the dominant resistance gene among the DEC in our study. Similar results were found in a study in children under five years of age with and without diarrhea in Ethiopia [58], diarrheic

patients in Ghana [59], and a systematic review and meta-analysis of *E. coli* isolates from human, animal, and environmental samples [60].

Several VAGs and ARGs are located on MGEs. The analysis of MGEs showed DEC isolates harbored different plasmids and other MGEs, which might indicate potentials for horizontal gene transfer, which can spread antibiotic resistance and virulence traits between bacterial populations [61].

STEC is a zoonotic pathogen and poses a major public health challenge due to its high pathogenicity for humans. It causes diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) in humans worldwide [62]. In the current study, 12.5% of DEC isolates belonged to the STEC group. However, the underestimation or misclassification of STEC strains into different pathotypes could occur due to the loss of Stx-converting bacteriophages [63]. Importantly, all STEC isolates were sensitive to all antimicrobials tested and did not carry ARGs. This finding may be due to the fact that STEC infections are not usually treated with aggressive antimicrobial therapy [64,65]. Despite the relatively low proportion of STEC strains among DEC isolates and the absence of antimicrobial resistance, the development of appropriate risk mitigation strategies, such as implementing strict hygiene and sanitation practices in food production, processing, and handling, as well as improving drinking water safety and environmental sanitation, is essential considering the significant impact that STEC infections can have on public health, as STEC infection is acquired via the fecal–oral route through contaminated food and water or direct contact with STEC-carrying animals [66]. The development of rapid and accurate detection methods for STEC strains is of great importance for improving prognosis and reducing mortality and associated complications [67].

The results of this study showed high diversity in the population structure of DEC isolates in terms of phylogroup, serotypes, sequence types, and VAGs. Serotypes that have higher potential to cause severe disease were also identified in this study.

4. Materials and Methods

4.1. Bacterial Isolates

A total of 260 *E. coli* isolates obtained from stool of (139 diarrheic and 121 non-diarrheic) patients of all age groups attending primary health care facilities in Addis Ababa and Hossana were used for the present investigation as a continuation of a previous study. As described in our previous study, *E. coli* was isolated from stool sample pre-enriched in buffered peptone water (BPW) and incubated overnight at 37 °C. Then, the enriched sample was streaked on eosin methylene blue agar (EMB), and colonies exhibiting a metallic green sheen were further examined using biochemical tests and confirmed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry [68].

4.2. DNA Extraction and PCR Detection of E. coli Pathotypes Genes

Genomic DNA was extracted using STARMag 96 × 4 Universal Cartridge Kit (Seegene Inc, Walnut Creek, CA, USA) on the automated system Seegene STARlet (Seegene) according to manufacturer's instructions. Multiplex real-time PCR was used to determine the presence of EPEC (*eaeA*), STEC (*stx1/stx2*), EAEC (*aggR*), and ETEC (*lt/st*) genes. The PCR reaction was prepared instantly on the same automated system Seegene STARlet (Seegene) according to the manufacturer's instructions (Allplex™ GI-Bacteria (II) Assay, Seegene). Briefly, the 25 µL of PCR reaction contained 5 µL 5× MuDT Oligo Mix (amplification and detection reagent), 10 µL of RNase-free water, 5 µL of premix with DNA polymerase, Uracil-DNA glycosylase (UDG), and Buffer containing dNTPs and 5 µL of DNA template. As a negative control, 5 µL of RNase-free water was used, whereas 5 µL of GI-B(II) PC was used as a positive control. The amplification and detection procedures were carried out on a CFX96™ Real-time PCR Detection System (CFX Manager™ Software-IVD v1.6) with steps: 20 min at 50 °C, 15 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 1 min at 60 °C and 30 s at 72 °C [69]. The determination of enteroinvasive *E. coli* (EIEC) was performed by real-time PCR, namely, by detecting the invasion plasmid antigen H (*ipaH*) gene. The

primers and probe targeting the *ipaH* gene were previously described by Wang et al. (2010). In brief, the 10 µL PCR reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 1× assay mix (mixture of 500 nM of both PCR primers and 200 nM of TaqMan probe labeled with FAM dye), and 1 µL of DNA template. PCR amplification (2 min at 50 °C, 10 min at 95 °C, followed by 35 cycles of 15 s at 95 °C and 1 min at 60 °C) and detection were performed using the QuantStudio 5 real-time PCR system (Applied Biosystems, USA) [70].

4.3. Whole-Genome Sequencing, Raw Data Pre-Processing, De Novo Assembly and Quality Control

WGS was performed for all DEC isolates identified by GI-Bacteria (II) Assay and *ipaH* PCR. Genomic libraries were prepared using Illumina DNA Prep (Illumina, San Diego, CA, USA). Isolates were sequenced on the NextSeq 2000 system (Illumina) using 2 × 150 bp paired-end reads chemistry [71]. Trimmomatic was used to trim raw reads from adapter sequences and low quality reads [72]. The quality of both raw and trimmed reads was assessed using FastQC v0.11.9 [73]. Species identification was confirmed using KmerFinder v3.0.2 based on trimmed reads. Assembly of trimmed reads into contigs was done with SPAdes v3.15.3 [74] using the default Kmer values and the "-isolate" parameters. Quast v5.2.0 was used for quality assessment of the assemblies [75]. Assemblies with N50 < 15 Kb and >500 contigs were excluded. To determine coverage of assembly, fastq files were converted into .bam using samtools. Subsequently, »samtools depth« was used to obtain base coverages, which were then averaged and reported.

4.4. Phylogenetic Groups, In Silico Multilocus Sequence Typing, and In Silico Serotyping

Phylogenetic groups of DEC isolates were determined using ClermonTyping 2.0.9 [76] available at http://clermontyping.iame-research.center/ (accessed on 5 June 2024). STs were determined by MLST based on the seven gene Achtman scheme using the website https://pubmlst.org/ (accessed on 5 June 2024) [77]. The assembled genomes were compared using a curated *E. coli* core genome MLST scheme available in SeqSphere+ (version 10.0.2, Ridom, https://www.ridom.de, accessed on 2 September 2024). A phylogenetic tree was constructed using the neighbor joining algorithm with the software's default parameters [78]. Target genes (188 in total) that were not found in all of the 24 samples were excluded from the analysis. The detection of DEC VAGs was performed using VirulenceFinder 2.0 available at the www.genomicepidemiology.org (accessed on 15 June 2024) with the default parameters (90% identity over 60% minimum Query/high-scoring segment pair length) [79]. Gene prediction was confirmed if the assembled sequence had at least 97% nucleotide match and 100% coverage with genes in the curated *Escherichia coli* database. SerotypeFinder 2.0 available at the www.genomicepidemiology.org (accessed on 15 June 2024) was used to identify the serotypes of *E. coli* with the default parameters (85% identity over 60% minimum Template/HSP length) [80]. Mobile genetic elements were identified using MobileElementFinder version 1.0 with MGEdb v1.0.2, available at https://cge.food.dtu.dk/services/MobileElementFinder/ (accessed on 26 August 2024) [81]. The *stx*-carrying prophages from whole-genome sequenced STEC isolates were identified by using PHASTEST (phage search tool with enhanced sequence translation) web-based tool (https://phastest.ca/) (accessed on 3 September 2024) [82].

4.5. Statistical Analysis

Descriptive analyses were performed using Microsoft Excel. Frequency and percentages were used to summarize the variables. The Fisher exact test was used to assess association between distribution of DEC and different variables using the Stata 14.0. A p-value of < 0.05 was considered statistically significant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms251910251/s1.

Author Contributions: Conceptualization, D.W., M.P., J.A. and M.S.E.; methodology, D.W., K.S.S., J.A., M.P., T.C.K. and D.K.; software, D.W., G.M., T.C.K., T.J., A.S. and M.S.E.; validation, T.E. and M.S.E.; formal analysis, D.W., K.S.S., T.C.K. and M.S.E.; investigation, D.W., M.P., K.S.S., J.A., D.K., T.C.K., T.J. and A.S.; resources, M.P., J.A., A.S. and M.S.E.; data curation, D.W., A.F.H., H.A., A.M., K.S.S., J.A., D.K., T.C.K., T.J., A.S. and M.S.E.; writing—original draft preparation, D.W.; writing—review and editing, D.W., T.E., G.M., A.F.H., H.A., A.M., M.P., K.S.S., J.A., D.K., T.C.K., T.J., A.S. and M.S.E.; supervision, T.E., A.F.H., G.M, A.M. and M.S.E.; project administration, M.S.E.; funding acquisition, M.S.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially supported by the Addis Ababa University through the thematic research project and by the Slovenian Research and Innovation Agency (research core funding no. P4-0092 'Animal health, environment and food safety'; no. P1-0198 'Molecular biology of microorganisms; no. P3-0083 'Host-parasite relationship', and the APC was funded by P1-0198). The study was partially supported by the project SLOSEQ Consolidation and Integration of Whole Genome Sequencing (WGS) into Routine Surveillance in Slovenia (101112671), co-funded by the European Union. D.W. was a grant recipient from the Swedish International Development Cooperation Agency.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of ALIPB, AAU (protocol code ALIPB IRB/66/2013/21 and date of approval 19 August 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The generated sequencing raw data and assembled genomes were submitted to SRA—Sequence Read Archive (accession number: PRJNA1105046, URL https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1105046, accessed on 10 May 2024).

Acknowledgments: The authors would like to thank Marko Kolenc from the Institute of Microbiology and Immunology, University of Ljubljana, and Tom Koritnik from National Laboratory of Health, Environment and Food, Ljubljana, Slovenia, for their support in conducting PCR in determination of DEC and sequencing isolates, respectively.

Conflicts of Interest: The authors declare no conflicts of interest.

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Paper 3

antibiotics

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Article

Antimicrobial Susceptibility and Characterization of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Isolated from Stools of Primary Healthcare Patients in Ethiopia

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Abstract: Antimicrobial resistance of *Escherichia coli* is a growing problem in both developed and developing countries. This study aimed to investigate the phenotypic antimicrobial resistance of *E. coli* isolates (n = 260) isolated from the stool specimen of patients attending public health facilities in Addis Ababa and Hossana. This study also aimed to characterize phenotypically confirmed extended-spectrum beta-lactamase (ESBL)-producing *E. coli* isolates (n = 22) using whole-genome sequencing. Resistance to 18 different antimicrobials was assessed using the disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. The highest resistance rate among the *E. coli* isolates was found for ampicillin (52.7%), followed by trimethoprim-sulfamethoxazole (29.6%). Of all isolates, 50 (19.2%) were multidrug-resistant and 22 (8.5%) were ESBL producers. ESBL genes were detected in 94.7% of the sequenced *E. coli* isolates, and multiple β-lactamase genes were detected in 57.9% of the isolates. The predominant ESBL gene identified was $bla_{\text{CTX-M-15}}$ (78.9%). The $bla_{\text{TEM-1B}}$ gene was detected in combination with other ESBL genes in 57.9% of the isolates, while only one of the sequenced isolates contained the $bla_{\text{TEM-1B}}$ gene alone. The $bla_{\text{CTX-M-3}}$ gene was detected in three isolates. The genes $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ as well as $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-169}}$ were confirmed to coexist in 52.6% and 10.5% of the sequenced *E. coli* isolates, respectively. In addition, $bla_{\text{OXA-1}}$ was identified together with $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ in one isolate, and in one isolate, $bla_{\text{TEM-169}}$ together with $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ was found. The results obtained show that measures need to be taken to reduce the spread of drug resistance and ensure the long-term use of available antimicrobials.

Keywords: *Escherichia coli*; antimicrobial susceptibility; ESBL; Ethiopia; whole-genome sequencing

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Citation: Wolde, D.; Eguale, T.; Alemayehu, H.; Medhin, G.; Haile, A.F.; Pirs, M.; Strašek Smrdel, K.; Avberšek, J.; Kušar, D.; Cerar Kišek, T.; et al. Antimicrobial Susceptibility and Characterization of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Isolated from Stools of Primary Healthcare Patients in Ethiopia. *Antibiotics* **2024**, *13*, 93. https://doi.org/10.3390/antibiotics13010093

Academic Editor: Juhee Ahn

Received: 22 December 2023
Revised: 12 January 2024
Accepted: 16 January 2024
Published: 18 January 2024

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1. Introduction

Antimicrobial resistance (AMR) is a serious public health problem. It is rapidly becoming one of the major health problems that could seriously affect the functioning of health systems [1]. Every year, increasing numbers of multidrug-resistant (MDR) bacterial strains cause life-threatening infections and the deaths of thousands of people [2]. The

World Health Organization's (WHO) global report on AMR has shown that bacterial resistance to common antimicrobials has reached alarming levels in many parts of the world, suggesting that many of the available treatments for common infections are becoming ineffective in some areas [3]. In 2019, an estimated 4.95 million deaths associated with bacterial AMR strains were reported [4]. Six pathogens were responsible for 73.4% of deaths attributable to bacterial AMR strains. The most prevalent pathogen among the six was *Escherichia coli* [4]. *E. coli* is one of the most important human pathogens known to cause a wide range of intestinal and extra-intestinal infections. AMR in *E. coli* is reported worldwide, and the increasing resistance rates in *E. coli* is a growing problem in both developed and developing countries [5]. *E. coli* and other members of the *Enterobacteriaceae* family are on the WHO list of antibiotic-resistant "priority pathogens" that pose the greatest threat to human health [6]. The antimicrobial resistance of *E. coli* in developing countries, including Ethiopia, is a major burden for patients and healthcare systems. Studies conducted in Ethiopia showed high rates of MDR *E. coli* [7]. More than 50% of *E. coli* isolates are resistant to commonly used antimicrobials (including third-generation cephalosporins) [8].

The most common mechanism by which bacteria acquire resistance to β-lactam antibiotics is through the expression of β-lactamases. Currently, over 1150 chromosomal, plasmid- and transposon-mediated β-lactamase genes are known [9]. Critical β-lactamases are enzymes whose genes are encoded on mobile genetic elements that are transferable from strain to strain and between bacterial species. The three major categories of β-lactamases are plasmid-mediated extended-spectrum β-lactamases (ESBLs), AmpC cephalosporinases and carbapenemases [10]. *E. coli* is one of the predominant ESBL-producing *Enterobacteriaceae* strains [11]. ESBL production in *E. coli* is usually associated with resistance genes encoded by bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ [12]. Currently, more than 187 variants of TEM types, more than 141 variants of SHV, and 119 variants of CTX-M have been identified [9]. The number of ESBL-producing *E. coli* has increased enormously worldwide, and infections associated with these strains are a major problem in clinical practice due to the limited therapeutic options for their treatment [13]. The prevalence of ESBL-producing pathogens in Ethiopia is alarmingly high [14]. The prevalence of ESBL-producing bacteria varies between 41.2% and 59.0% [15–17]. As carbapenems and other alternative antimicrobials are expensive and difficult to find in developing countries, infections caused by ESBL-producing bacteria often result in a high mortality rate [16]. According to Tufa et al., the mortality rate for infections caused by ESBL-producing bacteria is 86% [16]. Understanding the burden of AMR is critical to making informed decisions, particularly with regard to antimicrobial stewardship, and could help to develop guidelines for the empirical treatment of *E. coli*. In addition, *E. coli* is known to serve as a reservoir for several antimicrobial resistance genes and is capable of horizontally transferring these genes to other pathogenic and commensal organisms. Therefore, understanding the antimicrobial susceptibility of *E. coli* may provide an indication of the burden of antimicrobial resistance in other Gram-negative organisms circulating in a given community [5]. However, there are limited data on the antimicrobial susceptibility patterns and genomic characteristics of ESBL-producing *E. coli* in Ethiopia, although *E. coli* resistance to common antimicrobial agents used for treatment continues to increase. The aim of this study was to evaluate the phenotypic antimicrobial susceptibility of *E. coli* from patients attending public health centers in Addis Ababa city and Hossana town, Ethiopia, and to bioinformatically characterize ESBL-producing isolates based on the whole-genome sequences obtained.

2. Results

2.1. Study Participants

Table 1 lists the sociodemographic characteristics of the patients from whom *E. coli* isolates were obtained. The median income and age of participants with an interquartile range are 106.38 (70.92–153.37) USD and 22 (9–32) years, respectively.

Table 1. Sociodemographic characteristics of the study participants.

Characteristics	Response Category	Number (%)
Location of the health facilities		
	Addis Ababa	147 (56.5)
	Hossana	113 (43.5)
Sex		
	Female	110 (42.3)
	Male	150 (57.7)
Age group		
	0–4 years	27 (10.4)
	5–9 years	43 (16.5)
	10–14 years	31 (11.9)
	15–19 years	21 (8.1)
	20–45 years	121 (46.5)
	46–65 years	16 (6.2)
	>65 year	1 (0.4)
Marital status		
	Single	164 (63.1)
	Married	95 (36.5)
	Divorced	1 (0.4)

2.2. Antimicrobial Susceptibility of E. coli Isolates

The antimicrobial susceptibility of the studied *E. coli* isolates is summarized in Tables 2 and S1. One hundred and nineteen (45.8%) *E. coli* isolates were susceptible to all antimicrobials tested: seventy-one (48.3%) from Addis Ababa and forty-eight (42.5%) from Hossana. The highest resistance rates among the *E. coli* isolates were found for ampicillin and trimethoprim-sulfamethoxazole, followed by amoxicillin-clavulanic acid, cefuroxime, ceftriaxone, and cefotaxime. A very high percentage (86.9%) of *E. coli* strains were sensitive to increased exposure to cefuroxime. The *E. coli* isolates in the patients from Hossana town showed a higher resistance rate to all other antimicrobials, except for ampicillin and amoxicillin-clavulanic acid. All isolates were susceptible to carbapenems (ertapenem, imipenem, and meropenem) and amikacin. In addition, all *E. coli* isolates from Addis Ababa were susceptible to tobramycin, in contrast to 2.7% of tobramycin-resistant isolates from Hossana.

Chi-square analysis revealed a statistically significant difference in antimicrobial susceptibility patterns in Addis Ababa and Hossana ($p < 0.05$). Resistance to aztreonam ($p = 0.045$), ciprofloxacin ($p = 0.023$), cefotaxime ($p = 0.033$), cefepime ($p = 0.041$), and levofloxacin ($p = 0.020$) was significantly higher in Hossana than in Addis Ababa (Table 2).

2.3. Antimicrobial Resistance Patterns of E. coli Isolates

The antimicrobial resistance patterns observed in the *E. coli* tested are summarized in Table 3. Resistance to one or more antimicrobials was detected in 54.2% of the isolates. Of the resistant isolates, 24.1% were resistant to a single agent, such as ampicillin and trimethoprim-sulfamethoxazole. MDR was detected in 50 (19.2%) isolates. Resistance to six or more antimicrobials was detected in 27 (10.4%) isolates: 16 from Hossana and 11 from Addis Ababa. Thirteen (5.0%) isolates were resistant to all cephalosporins tested. The results of this study showed that the proportion of MDR in *E. coli* was significantly higher in isolates from Hossana than in isolates from Addis Ababa ($X^2 = 5.27$, $p = 0.022$). Nearly 62% of MDR isolates were from Hossana town. Males and those in the age group between 20 and 45 years contributed to 59.2% and 44.9% of the MDR *E. coli* isolates, respectively. Of the MDR isolates, 100.0% were resistant to ampicillin, 75.5% were resistant to trimethoprim-sulfamethoxazole, 71.4% were resistant to ceftriaxone, and 69.4% were resistant to cefotaxime. The isolates had widely varying resistance patterns. A total of 33 resistance patterns were observed among the *E. coli* isolates: 20 in Addis Ababa, 25 in Hossana, and 12 common to both. The most frequently detected MDR pattern was AM, AMC, SXT, followed by AM, CIP, LVX, SXT (Table 3).

Table 2. Antimicrobial susceptibility of studied *E. coli* isolates.

Antibiotic	Antibiotic Class	S (Susceptible, Standard Dosing Regimen)			I (Susceptible, Increased Exposure)			R (Resistant)		
		Total Isolates (*n* = 260)	Addis Ababa Strains (*n* = 147)	Hossana Strains (*n* = 113)	Total Strains (*n* = 260)	Addis Ababa Strains (*n* = 147)	Hossana Strains (*n* = 113)	Total Strains (*n* = 260)	Addis Ababa Strains (*n* = 147)	Hossana Strains (*n* =113)
		n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
AM	Penicillin	123 (47.3)	73 (49.7)	50 (44.2)	0	0	0	137 (52.7)	74 (50.3)	63 (54.3)
AMC	β-lactam + inhibitors	223 (85.8)	123 (83.7)	100 (88.5)	0	0	0	37 (14.2)	24 (16.3)	13 (11.5)
TZP	Antipseudomonal penicillin + β-lactam inhibitors	257 (98.8)	145 (98.6)	112 (99.1)	0	0	0	3 (1.2)	2 (1.4)	1 (0.9)
CXMp	Non-extended spectrum cephalosporins	0	0	0	226 (86.9)	133 (90.5)	93 (82.3)	34 (13.1)	14 (9.5)	20 (17.7)
CTX	Extended spectrum cephalosporins	226 (86.9)	133 (90.5)	93 (82.3)	1 (0.4)	1 (0.7)	0	33 (12.7)	13 (8.8)	20 (17.7) *
CRO		226 (86.9)	133 (90.5)	93 (82.3)	0	0	0	34 (13.1)	14 (9.5)	20 (17.7)
CAZ		232 (89.2)	137 (93.2)	95 (84.1)	6 (2.3)	1 (0.7)	5 (4.4)	22 (8.5)	9 (6.1)	13 (11.5)
FEP		234 (90.0)	138 (93.9)	96 (85.0)	8 (3.1)	3 (2.0)	5 (4.4)	18 (6.9)	6 (4.1)	12 (10.6) *
ETP	Carbapenems	260 (100)	147 (100)	113 (100)	0	0	0	0	0	0
IPM		260 (100)	147 (100)	113 (100)	0	0	0	0	0	0
MEM		260 (100)	147 (100)	113 (100)	0	0	0	0	0	0
ATM	Monobactams	227 (87.3)	133 (90.5)	94 (83.2)	13 (5.0)	7 (4.8)	6 (5.3)	20 (7.7)	7 (4.8)	13 (11.5) *
AN	Aminoglycosides	260 (100)	147 (100)	113 (100)	0	0	0	0	0	0
GM		255 (98.1)	146 (99.3)	109 (96.5)	0	0	0	5 (1.9)	1 (0.7)	4 (3.5)
NN		257 (98.8)	147 (100)	110 (97.3)	0	0	0	3 (1.2)	0	3 (2.7)
CIP	Fluoroquinolones	238 (91.5)	140 (95.2)	98 (86.7)	3 (1.2)	1 (0.7)	2 (1.8)	19 (7.3)	6 (4.1)	13 (11.5) *
LVX		240 (92.3)	140 (95.2)	100 (88.5)	2 (0.8)	2 (1.4)	0	18 (6.9)	5 (3.4)	13 (11.5) *
SXT	Folate pathway inhibitors	183 (70.4)	109 (74.1)	74 (65.5)	0	0	0	77 (29.6)	38 (25.9)	39 (34.5)

AM—ampicillin, AMC—amoxicillin-clavulanic acid, AN—amikacin, ATM—aztreonam, CAZ—ceftazidime, CIP—ciprofloxacin, CRO—ceftriaxone, CTX—cefotaxime, CXMp—cefuroxime-parenteral, ETP—ertapenem, FEP—cefepime, GM—gentamicin, IPM—imipenem, LVX—levofloxacin, MEM—meropenem, NN—tobramycin, SXT—trimethoprim-sulfamethoxazole, TZP—piperacillin-tazobactam, and * indicates statistically significant resistance ($p < 0.05$).

Table 3. Antimicrobial resistance patterns of *E. coli* isolates.

Antimicrobial Resistance Pattern	Resistant Isolates (*n* = 141) Addis Ababa (*n* = 77); Hossana (*n* = 64)				ESBL-Producing Isolates (*n* = 22) Addis Ababa (*n* = 9); Hossana (*n* = 13)		
	No. of Antimicrobial Groups in Resistance Pattern	No. of Isolates with This Resistance Pattern *n* (%)	No. of Isolates with This Resistance Pattern from Addis Ababa *n* (%)	No. of Isolates with This Resistance Pattern from Hossana *n* (%)	No. of ESBL-Producing Isolates with This Resistance Pattern *n* (%)	No. of ESBL Isolates with This Resistance Pattern from Addis Ababa *n* (%)	No. of ESBL Isolates with This Resistance Pattern from Hossana *n* (%)
AM	1	30 (21.3)	16 (20.8)	14 (21.9)	0	0	0
SXT	1	4 (2.8)	2 (2.6)	2 (3.1)	0	0	0
AM, SXT	2	37 (26.2)	22 (28.6)	15 (23.4)	0	0	0
AM, AMC	2	20 (14.2)	17 (22.1)	3 (4.7)	0	0	0
AM, AMC, TZP	3	1 (0.7)	1 (1.3)	0	0	0	0
AM, AMC, SXT	3	7 (5.0)	3 (3.9)	4 (6.3)	0	0	0
AM, CIP, LVX, SXT	3	5 (3.5)	1 (1.3)	4 (6.3)	0	0	0
AM, SXT, CXMp, CRO	4	1 (0.7)	1 (1.3)	0	1 (4.5)	1 (11.1)	0
AM, AMC, GM, SXT	4	1 (0.7)	0	1 (1.6)	0	0	0
AM, ATM, SXT, CAZ	4	1 (0.7)	1 (1.3)	0	0	0	0
AM, GM, CXMp, CTX, CRO	4	1 (0.7)	0	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, SXT, CXMp, CTX, CRO	4	4 (2.8)	2 (2.6)	2 (3.1)	2 (9.1)	1 (11.1)	1 (7.7)
AM, CIP, GM, LVX, NN	3	1 (0.7)	0	1 (1.6)	0	0	0
AM, CXMp, CTX, CRO, FEP	3	1 (0.7)	0	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, SXT, GM, CTX, CXMp, CRO	5	1 (0.7)	1 (1.3)	0	1 (4.5)	1 (11.1)	0
AM, AMC, CXMp, CTX, CRO, FEP	4	2 (1.4)	1 (1.3)	1 (1.6)	2 (9.1)	1 (11.1)	1 (7.7)
AM, ATM, CXMp, CTX, CRO, CAZ	4	2 (1.4)	1 (1.3)	1 (1.6)	0	0	0
AM, SXT, CXMp, CTX, CRO, CAZ	4	2 (1.4)	1 (1.3)	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, CIP, LVX, CXMp, CTX, CRO	4	1 (0.7)	1 (1.3)	0	1 (4.5)	1 (11.1)	0
AM, SXT, CXMp, CTX, CRO, CAZ, FEP	4	1 (0.7)	1 (1.3)	0	0	0	0
AM, ATM, SXT, CXMp, CTX, CRO, CAZ	5	2 (1.4)	0	2 (3.2)	2 (9.1)	0	2 (15.4)
AM, ATM, CXMp, CTX, CRO, CAZ, FEP	4	1 (0.7)	0	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, ATM, SXT, CXMp, CTX, CRO, CAZ, FEP	5	3 (2.1)	1 (1.3)	2 (3.2)	2 (9.1)	1 (11.1)	1 (7.7)
AM, ATM, CIP, LVX, CXMp, CTX, CRO, FEP	5	1 (0.7)	0	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, AMC, SXT, CIP, LVX, CXMp, CTX, CRO	6	1 (0.7)	0	1 (1.6)	0	0	0

Table 3. *Cont.*

Antimicrobial Resistance Pattern	Resistant Isolates (*n* = 141) Addis Ababa (*n* = 77); Hossana (*n* = 64)				ESBL-Producing Isolates (*n* = 22) Addis Ababa (*n* = 9); Hossana (*n* = 13)		
	No. of Antimicrobial Groups in Resistance Pattern	No. of Isolates with This Resistance Pattern *n* (%)	No. of Isolates with This Resistance Pattern from Addis Ababa *n* (%)	No. of Isolates with This Resistance Pattern from Hossana *n* (%)	No. of ESBL-Producing Isolates with This Resistance Pattern *n* (%)	No. of ESBL Isolates with This Resistance Pattern from Addis Ababa *n* (%)	No. of ESBL Isolates with This Resistance Pattern from Hossana *n* (%)
AM, ATM, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	5	1 (0.7)	0	1 (1.6)	0	0	0
AM, AMC, TZP, ATM, CXMp, CTX, CRO, CAZ, FEP	6	1 (0.7)	1 (1.3)	0	1 (4.5)	1 (11.1)	0
AM, AMC, ATM, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	6	1 (0.7)	0	1 (1.6)	0	0	0
AM, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	6	3 (2.1)	2 (2.6)	1 (1.6)	3 (13.6)	2 (22.2)	1 (7.7)
AM, AMC, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ	7	1 (0.7)	1 (1.3)	0	0	0	0
AM, AMC, TZP, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	8	1 (0.7)	0	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, AMC, ATM, SXT, CIP, LVX, NN, CXMp, CTX, CRO, CAZ, FEP	8	1 (0.7)	0	1 (1.6)	1 (4.5)	0	1 (7.7)
AM, AMC, ATM, SXT, CIP, LVX, GM, NN, CXMp, CTX, CRO, CAZ, FEP	8	1 (0.7)	0	1 (1.6)	0	0	0

AM—ampicillin, AMC—amoxicillin-clavulanic acid, ATM—aztreonam, CAZ—ceftazidime, CIP—ciprofloxacin, CRO—ceftriaxone, CTX—cefotaxime, CXMp—cefuroxime-parenteral, FEP—cefepime, GM—gentamicin, LVX—levofloxacin, NN—tobramycin, SXT—trimethoprim-sulfamethoxazole, and TZP—piperacillin-tazobactam.

2.4. ESBL-Producing *E. coli* Isolates

This study found that 22 (8.5%) of the strains were ESBL producers. ESBL-producing *E. coli* showed 100% resistance to ampicillin, ceftriaxone, and cefuroxime and 95.5% resistance to cefotaxime. In addition, ESBL-producing strains showed 22.7–63.6% resistance to amoxicillin-clavulanic acid, levofloxacin, ciprofloxacin, aztreonam, ceftazidime, cefepime, and trimethoprim-sulfamethoxazole. ESBL-producing *E. coli* showed the lowest resistance to piperacillin-tazobactam (9.1%), gentamicin (9.1%), and tobramycin (4.5%). Of the ESBL-producing *E. coli* strains, 59.1% were from the Hossana site. However, there was no significant difference in the distribution of ESBL-producing *E. coli* between the strain from Addis Ababa and Hossana (X^2 = 2.39, p = 0.122). Sixteen resistance patterns were observed among the ESBL-producing *E. coli* strains for the tested antimicrobials (Table 3).

2.5. Whole-Genome Sequencing Analysis of ESBL-Producing *E. coli* Isolates

Of the 22 phenotypically confirmed ESBL-producing *E. coli* isolates, 19 were subjected to whole-genome sequencing on the Illumina platform to identify the ESBL gene profile (unfortunately, three strains could not be revived). All assemblies passed the QC threshold of N50 > 15 Kb and <500 contigs. At least one known ESBL gene was detected in 94.7% of the sequenced isolates, and 57.9% harbored two or more β-lactamase genes. The predominant ESBL gene identified was *bla*$_{\text{CTX-M-15}}$ (78.9%). The *bla*$_{\text{TEM-1B}}$ gene was detected in combination with other ESBL genes in 57.9% of the isolates, while only one of the sequenced isolates contained the *bla*$_{\text{TEM-1B}}$ gene alone. The *bla*$_{\text{CTX-M-3}}$ gene was detected in three isolates. In this study, the coexistence of *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B,}}$ as well as of *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-169,}}$ was confirmed in 52.6% and 10.5% of the sequenced *E. coli* isolates, respectively. In addition, *bla*$_{\text{OXA-1}}$ was identified together with *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$ in one isolate, and *bla*$_{\text{TEM-169}}$ was confirmed together with *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$ in one isolate. Eight (80.0%) of the ten ESBL-producing *E. coli* isolates from Hossana harbored one or two β-lactamase genes in addition to *bla*$_{\text{CTX-M-15}}$ (Table 4).

Table 4. Phenotypic resistance pattern and associated genetic markers among ESBL-producing *E. coli* isolates.

Strain Designation	Pattern of ESBL Phenotype	Type of β-Lactamase Genes Detected	Origin of Isolate
13	AM, ATM, SXT, CXMp, CTX, CRO, CAZ, FEP	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Hossana
39	AM, SXT, CXMp, CRO	*bla*$_{\text{CTX-M-15}}$	Addis Ababa
63	AM, SXT, CXMp, CTX, CRO	*bla*$_{\text{CTX-M-15}}$	Addis Ababa
69	AM, CIP, LVX, CXMp, CTX, CRO	*bla*$_{\text{CTX-M-15}}$	Addis Ababa
75	AM, AMC, TZP, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-169}}$	Hossana
85	AM, AMC, CXMp, CTX, CRO, FEP	*bla*$_{\text{CTX-M-3}}$	Addis Ababa
86	AM, ATM, SXT, CIP, LVX, CXMp CTX, CRO, CAZ, FEP	*bla*$_{\text{CTX-M-15}}$	Addis Ababa
92	AM, SXT, CXMp, CTX, CRO, CAZ	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Addis Ababa
197	AM, ATM, SXT, CXMp, CTX, CRO, CAZ	#	Hossana
200	AM, AMC, TZP, ATM, CXMp CTX, CRO, CAZ, FEP	#	Addis Ababa
205	AM, ATM, SXT, CIP, LVX, CXMp CTX, CRO, CAZ, FEP	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Addis Ababa
232	AM, GM, CXMp, CTX, CRO	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Hossana
244	AM, ATM, SXT, CXMp, CTX, CRO, CAZ	*bla*$_{\text{TEM-1B}}$	Hossana
260	AM, ATM, SXT, CXMp, CTX, CRO, CAZ, FEP	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Hossana
268	AM, ATM, SXT, CIP, LVX, CXMp, CTX, CRO, CAZ, FEP	#	Hossana
289	AM, GM, SXT, CXMp, CTX, CRO	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Addis Ababa
302	AM, SXT, CXMp, CTX, CRO	*bla*$_{\text{TEM-1B}}$, *bla*$_{\text{CTX-M-15}}$	Hossana

Table 4. *Cont.*

Strain Designation	Pattern of ESBL Phenotype	Type of β-Lactamase Genes Detected	Origin of Isolate
303	AM, ATM, CXMp, CTX, CRO, CAZ, FEP	$bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$	Hossana
306	AM, AMC, ATM, SXT, CIP, LVX, NN, CXMp, CTX, CRO, CAZ, FEP	$bla_{\text{OXA-1}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{CTX-M-15}}$	Hossana
316	AM, ATM, CIP, LVX, CXMp, CTX, CRO, FEP	$bla_{\text{TEM-169}}$, $bla_{\text{CTX-M-15}}$	Hossana
324	AM, AMC, CXMp, CTX, CRO, FEP	$bla_{\text{CTX-M-3}}$	Hossana
340	AM, CXMp, CTX, CRO, FEP	$bla_{\text{CTX-M-3}}$	Hossana

Strain was not sequenced as it could not be revived.

3. Discussion

Antimicrobial resistance is one of the greatest threats to humanity in the 21st century [18]. This study investigated the antibiotic resistance profiles of *E. coli* isolated from the stools of patients and characterized ESBL-producing *E. coli* isolates. Unfortunately, we were not able to obtain any information about the clinical situation or diagnosis of patients, which is a limitation of the study.

3.1. Antimicrobial Resistance of Studied E. coli Isolates

This study found that 54.1% of *E. coli* strains were resistant to at least one or more of the antimicrobial agents tested. This is comparable to previous reports of pathogenic *E. coli* strains isolated from children in Wolaita Sodo, southern Ethiopia [19], and a meta-analysis in Ethiopia [7], where 61.7% and 45.4% of strains were resistant to at least one antimicrobial agent, respectively.

Ampicillin and trimethoprim-sulfamethoxazole are widely used antimicrobial agents for the treatment of infections in humans and animals. The isolates from the present study had high resistance to ampicillin (52.7%), which is comparable to other studies on *E. coli* obtained from outpatients in hospitals in Lagos State, from animal handlers in abattoirs, poultry farmers, and open markets in Lagos, Nigeria (59.1%) [20], and from poultry farmers in Lusaka, Zambia (46.8%) [21]. However, it is lower compared to previous reports of pathogenic *E. coli* strains isolated from children in Wolaita Sodo, southern Ethiopia (70.6%) [19], diarrheagenic *E. coli* isolates in Trans-Nzoia County, Kenya (83.9%) [22], and *E. coli* isolated from diarrheic patients in public health centers in Eastern Cape, South Africa (88%) [23]. In addition, our study showed a high percentage (29.6%) of *E. coli* isolates resistant to trimethoprim-sulfamethoxazole, which is lower than the resistance rate from previous studies on *E. coli* from children under five years of age with diarrhea at Sodo Christian Hospital in Wolaita Sodo, southern Ethiopia (67.6%) [19], in patients at Kitale County Referral Hospital in Trans-Nzoia County, Kenya (95.7%) [22], in diarrheic patients at a public health center in Eastern Cape, South Africa (78%) [23], in outpatients at hospitals in Lagos State and among livestock farmers at abattoirs, poultry farmers, and open markets in Lagos, Nigeria (61.5%) [20], and among poultry farmers in Lusaka, Zambia (48.3%) [21]. This could be due to the fact that the genes responsible for ampicillin and trimethoprim-sulfamethoxazole resistance probably co-occur due to the same mobile genetic elements [24,25]. As a result, resistance to multiple antimicrobials develops simultaneously, leading to a high level of resistance.

Relatively low proportions of *E. coli* isolates in the current study were resistant to amoxicillin-clavulanic acid (14.2%). However, in previous studies, a high percentage of resistance to amoxicillin-clavulanic acid was found in *E. coli* from diarrheic patients in Ethiopia (64.4%) [26] and in *E. coli* from different clinical samples from patients in different teaching hospitals in Sudan (50.4%) [27]. This may be due to the fact that amoxicillin-clavulanic acid is the most commonly used antibiotic for self-medication and is widely prescribed by healthcare providers. It is inexpensive and is considered first-line therapy in many low- and middle-income countries [28–30].

In this study, resistance to levofloxacin was detected in 6.9% of isolates and to ciprofloxacin in 7.3% of isolates. This is comparable to the rate of resistance to ciprofloxacin reported in a study on *E. coli* from Congolese students in Madibou, Brazzaville (4%) [31], and children under five years of age in a study in the pediatric clinic of the commune of Abomey-Calavi, Benin (9.5%) [32]. In contrast, a study conducted in Nigeria found a high percentage of resistance to ciprofloxacin in humans (21.9%), among poultry workers, chickens, and their environment in poultry farms/markets [33]. This could be due to the fact that fluoroquinolones are one of the most commonly prescribed antimicrobial classes in human and veterinary medicine in Nigeria, which could be responsible for the selection pressure that favors the development of quinolone resistance in *E. coli* isolates [34].

Aztreonam is among the last-resort antibiotics currently available for the treatment of these infections [35]. In the present study, 7.7% of the *E. coli* isolates were resistant to aztreonam. In contrast, in a study conducted in the pediatric clinic of the commune of Abomey-Calavi in Benin, children under five years of age showed 100% resistance to aztreonam [32]. This indicates a serious threat to human health as very few effective antibiotics are available for the clinical treatment of infections caused by aztreonam-resistant strains.

The resistance of *E. coli* to gentamicin (1.9%) in this study was consistent with previous findings in Eastern Cape, South Africa, from diarrheic patients attending a public health center (2%) [23]. In addition, resistance to tobramycin in this study was 1.2%, which was lower than the resistance rate found in an Egyptian study on *E. coli* strains isolated from children with diarrhea from Assiut Children's Hospital (68%) [36]. Interestingly, no resistance to carbapenems (ertapenem, meropenem, and imipenem) and amikacin was detected in the current study. In contrast, resistance to imipenem (1%) and amikacin (3%) was detected in *E. coli* from diarrheic patients attending a public health center in Eastern Cape, South Africa [23]. The most important factors contributing to the emergence of resistance to these antibiotics could be the availability and use of these antimicrobials in the treatment of infectious diseases in South Africa.

Cephalosporins are one of the limited available therapies for the treatment of severe bacterial infections in humans. The third, fourth, and fifth-generation cephalosporins are classified by the WHO as "critically important antimicrobials" in human medicine [37]. However, they are among the most frequently prescribed drugs for the treatment of infections caused by *Enterobacteriaceae*, which increases the selection pressure for resistant organisms. Resistance rates to cefepime (1.6%) in *E. coli* isolated from outpatients in Lagos State hospitals (Nigeria) and from animal caretakers in abattoirs, poultry farms, and open markets (1.6%) [20], and resistance rates to cefotaxime in Lusaka, Zambia (8.6%) in *E. coli* isolated from poultry farmers [21], were almost as low as in this study. However, high rates of resistance to this class of antibiotics were found in previous studies conducted in Benin on *E. coli* isolated from children under five years of age in the pediatric clinic in the commune of Abomey-Calavi (cefotaxime, 100%) [32], and *E. coli* isolated from Congolese students in Madibou, Brazzaville (ceftazidime, 65%) [31].

3.2. MDR and ESBL-Producing E. coli among Studied Isolates

ESBL-producing *E. coli* are a major cause of antimicrobial-resistant infections and pose a major threat worldwide as they can cause infections that are difficult to treat in animals and humans [38]. The overall percentage of ESBL-producing *E. coli* strains in this study was 8.5%. This result is lower than previous reports of ESBL-producing *E. coli* in children hospitalized in Ghana (61%) [39]. This could be due to differences in antimicrobial use among the regions or the study population. All children who participated in the Ghanaian study had contact with free-roaming chickens in town, and chicken meat was part of their regular diet. ESBL-producing bacteria are commonly isolated from poultry [39]. The genetic linkage of ESBL-producing *E. coli* between poultry and human populations was also demonstrated in the same study, which could indicate a potential risk of transmission of ESBL-producing bacteria between poultry products and humans and increase the incidence rate in the study population [39].

The MDR rate in the current study was 19.2%, of *E. coli*, which is comparable to the result of a previous study in Ethiopia on *E. coli* isolates from diarrheic patients at Selam Health Center, Addis Ababa (15.1%) [26] and *E. coli* from poultry farmers in Lusaka, Zambia (29.3%) [21]. The result of the current study is very low compared to a meta-analysis of resistance patterns of Gram-negative bacterial pathogens in Ethiopia (78.2%) [40] and other studies on *E. coli* isolated from the stool of hospitalized patients in Kenya [22] and community outpatients and animal caregivers in Lagos, Nigeria (69.6%) [20]. The difference might be related to the study population, sample size, and geographical location. Unlike the current study, where patients are those who come from the community, other studies were conducted among hospitalized patients with a high chance of being exposed to MDR organisms.

A higher percentage of MDR (61.2%) and ESBL-producing *E. coli* (59.1%) in the current study was obtained among the Hossana isolates, in which agricultural and animal husbandry are the common practices of the community. This could be related to the imprudent use of antimicrobials in humans and agriculture, which is considered to be one of the most important factors contributing to the emergence and spread of resistant bacteria [41]. The low socioeconomic status in Hossana, poor sanitation, and unhygienic conditions may also have contributed to the easy circulation of resistant organisms and resistant genetic markers. The inappropriate use of antimicrobials in agriculture can lead to selection pressure on microorganisms in food, water, and the environment, which can serve as a source of infection with MDR organisms for humans and animals [42].

The $bla_{\text{CTX-M-15}}$ gene is the most widely distributed gene encoding ESBLs associated with human infections [43]. It is mainly found in international high-risk clones [44]. This gene was the dominant genotype among the ESBL-positive isolates in our study. This is in line with the report from Ethiopia, where a study investigated the genome-based epidemiology of ESBL-producing *E. coli* among patients seeking medical care at a tertiary hospital in Jimma [45] and a study on the fecal carriage of ESBL-producing *Enterobacteriaceae* in young children in Tanzania [46]. In addition, the predominance of $bla_{\text{CTX-M-15}}$ in *E. coli* was also found in bacteriemic patients in two teaching hospitals in Bamako, Mali [47]. These higher rates of the $bla_{\text{CTX-M-15}}$ gene may be associated with incompatibility group FII conjugative plasmids which carry this gene and play a great role in the spread and acquisition of resistance genes in *E. coli* [48]. In contrast to the present study, bla_{TEM} was found to be the predominant β-lactamase gene in a study with children under five years of age in Ethiopia [49] and in diarrheic patients in Ghana [50]. Several variants of the $bla_{\text{CTX-M}}$ and bla_{TEM} genes have been identified on both plasmids and chromosomes [9,51]. ESBL genes located on plasmids spread rapidly between and within bacterial species, whereas ESBL genes integrated into chromosomes are mostly stable and persist without antibiotic selection pressure [51].

E. coli-encoding $bla_{\text{CTX-M-15}}$ is often characterized as multidrug-resistant and a co-producer of OXA-1 or TEM-1B [52,53]. The co-existence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ was observed in 52.6% of *E. coli* with ESBL genes in this study. In agreement with the current study, the coexistence of these genes in *E. coli* isolates was found in a study in Burkina Faso in ESBL-producing *Enterobacteriaceae* among clinical isolates [54] and in Cameroon in ESBLs in *Enterobacteriaceae* isolates in the community [55]. The *E. coli* isolates in our study, which possess $bla_{\text{CTX-M-15}}$ genes, also encode $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-169}}$ β-lactamase genes. This finding is consistent with a previous study from Pakistan that investigated the frequency of resistance to third-generation cephalosporin and the distribution of key genetic determinants of ESBL-producing clinical isolates. This study showed the co-existence of $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1,}}$ and $bla_{\text{TEM-1}}$ in 7% of ESBL-producing *E. coli* [56]. The result showed a high rate of co-existence of β-lactamase genes in isolates from Hossana compared to isolates from Addis Ababa, suggesting a high rate of co-selection of plasmids with different resistance genes due to the overuse of antimicrobials in Hossana.

4. Conclusions

Resistance to ampicillin and trimethoprim-sulfamethoxazole was high in the current study, and none of the isolates were resistant to carbapenems and amikacin. A high rate of ESBL-producing *E. coli* strains was detected, most of which were encoded by $bla_{\text{CTX-M-15}}$ and a combination of other ESBL genes in *E. coli*. Overall, MDR- and ESBL-producing isolates were frequently detected among the isolates from Hossana, indicating a high level of irrational use of antimicrobials, leading to high selection pressure. These findings suggest that prudent use of antimicrobials is advisable to reduce the burden of drug resistance and ensure the long-term use of available antimicrobials.

5. Materials and Methods

5.1. Study Design and Sample Collection

A health institution-based cross-sectional study was conducted in Addis Ababa city and Hossana town. Addis Ababa is the capital of Ethiopia, and it has an estimated population of 5,461,000. The city is home to 23.8% of all urban residents in Ethiopia, and it has an estimated density of 5936 per square kilometer. The majority of the population, especially the urban poor, lives in a slum area of Addis Ababa, and they are exposed to water and sanitation-related diseases [57]. The other study area, Hossana town, is located in the Hadiya zone, 230 km southwest of Addis Ababa, with a total estimated population of 75,963. Hadiya Zone is one of the central zones in the central Ethiopia region. The zone is one of the most densely populated areas in Ethiopia. The population density of the zone is 415 people per square kilometer.

Seventeen public health centers (primary healthcare units) in Ethiopia (thirteen randomly selected health centers from Addis Ababa city and all four health centers from Hossana town) were included in this study. This study included patients of all ages who visited the health centers at the time of data collection and had not taken any antimicrobial agents in the fifteen days prior to the study. Patients who met these inclusion criteria were informed of the purpose and procedures of the study and gave their informed consent before providing a fresh stool sample in a dry, sterile container with Cary–Blair transport medium. A total of 260 samples were collected from December 2021 to September 2022. Each stool sample was carefully labeled with a unique identifier and placed in a cool box (4 °C) with cold packs for transport to the microbiology laboratory for isolation of *E. coli*. Unfortunately, we were not provided with any information about the clinical situation and the diagnosis of patients.

5.2. Isolation and Identification of E. coli

For the isolation of *E. coli*, approximately 1 g of stool sample was pre-enriched in 9 mL of buffered peptone water (BPW) (Becton-Dickinson, Sparks, MD, USA) and incubated overnight at 37 °C. It was then streaked on eosin methylene blue agar (EMB) (Oxoid, Basingstoke, UK), and the plates were incubated at 37 °C for 24 h. Colonies that exhibited a metallic green sheen were further examined with biochemical tests (indole, citrate, and urease). A reference strain, *E. coli* ATCC 25922, was used as a positive control for biochemical analysis. After confirmation, *E. coli* isolates (only one from each stool sample) were frozen in a nutrient broth containing 20% glycerol and stored. A total of 260 strains were randomly selected using a computer-generated random sampling technique and exported on slant agars to Slovenia for further analysis, where each strain was streaked on MacConkey agar (BD MacConkey II Agar, Becton Dickinson, Germany) for revitalization. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Microflex LT with regularly updated BruckerMS library, Brucker Daltonics, Bremen, Germany) was used to confirm *E. coli* before antimicrobial susceptibility testing.

5.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

guidelines [58]. The following antimicrobial discs were used: ampicillin (Am 10 µg), amoxicillin-clavulanic acid (AMC 20/10 µg), piperacillin-tazobactam (TZP 30/6 µg), cefuroxime (CXMP 30 µg), cefotaxime (CTX 5 µg), ceftriaxone (CRO 30 µg), ceftazidime (CAZ 10 µg), cefepime (FEP 30 µg), ertapenem (ETP 10 µg), meropenem (MEM 10 µg), imipenem (IPM 10 µg), gentamicin (GM 10 µg), amikacin (AN 30 µg), ciprofloxacin (CIP 5 µg), levofloxacin (LVX 5 µg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75 µg), aztreonam (ATM 30 µg), and tobramycin (NN 10 µg). The results were interpreted according to EUCAST guidelines [59]. Multidrug resistance (MDR) was defined as resistance to at least one agent in three or more antimicrobial groups [60]. Extended-spectrum β-lactamase (ESBL) production among the strains was phenotypically determined using the double disk diffusion method suggested by the EUCAST [61]. The presence of ESBL was confirmed by a synergy test between a disk with amoxicillin-clavulanic acid and cephalosporins (cefotaxime and ceftazidime).

5.4. DNA Extraction and Whole-Genome Sequencing

Whole-genome sequencing was performed for 21 phenotypically confirmed ESBL-producing *E. coli* isolates. Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) [62] or STARMag 96 × 4 Universal Cartridge Kit (Seegene Inc. Walnut Creek, CA, USA) [63]. Genomic libraries were prepared using the Illumina DNA Prep (Illumina, San Diego, CA, USA). Isolates were sequenced on the NextSeq 2000 system (Illumina) using 2 × 149 bp paired-end reads chemistry [64].

5.4.1. Raw Data Pre-Processing

Trimmomatic was used to trim raw reads from adapter sequences and low-quality reads [65]. The quality of both raw and trimmed reads was assessed using FastQC v0.11.9 [66]. Species identification was confirmed using KmerFinder v3.0.2 based on trimmed reads.

5.4.2. De Novo Assembly and QC

The assembly of trimmed reads into contigs was carried out with SPAdes v3.15.3 [67] using the default K-Kmer values and the "--isolate" parameters. Quast v5.2.0 was used for the quality assessment of the assemblies [68]. To determine the coverage of the assembly, fastq files were converted into .bam using samtools. Subsequently, «samtools depth» was used to obtain base coverages, which were then averaged and reported.

5.4.3. AMR Identification

Resistance genes were detected with ResFinder 4.1 [69] with the default parameters (80% identity over 60% of the length of the target gene), using assembled sequences as input. Gene prediction was confirmed if the assembled sequence had at least 97% nucleotide match and 100% coverage with genes in the curated *Escherichia coli* database.

5.5. Statistical Analysis

Descriptive statistical analysis was performed using WHONET software version 2022, and a chi-square test was used to investigate the association between different variables using SPSS version 25.0. A p-value of <0.05 was considered an indicator of statistically significant association.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13010093/s1, Table S1: Results of antimicrobial susceptibility testing of 260 *Escherichia coli* isolates.

Author Contributions: Conceptualization, D.W., T.E. and M.S.E.; methodology, D.W., T.E., G.M., M.P., T.C.K. and M.S.E.; software, D.W., G.M., M.P., T.C.K., T.J. and A.S.; validation, T.E. and M.S.E.; formal analysis, D.W., G.M. and M.P.; investigation, D.W., H.A., A.F.H., M.P., K.S.S., J.A., D.K., T.C.K. and T.J.; resources, T.E., H.A., M.P., K.S.S., J.A., A.S. and M.S.E.; data curation, D.W., M.P., J.A., A.S. and M.S.E.; writing—original draft preparation, D.W., T.C.K. and T.J.; writing—review and editing, D.W., T.E., H.A., G.M., A.F.H., M.P., K.S.S., J.A., D.K., T.C.K., T.J., A.S. and M.S.E.; visualization, D.W., M.P. and M.S.E.; supervision, T.E., G.M., A.F.H. and M.S.E.; project administration, T.E. and M.S.E.; funding acquisition, T.E. and M.S.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially supported by Addis Ababa University through the thematic research project and by the Slovenian Research and Innovation Agency (research core funding no. P4-0092 'Animal health, environment and food safety'; no. P1-0198 'Molecular biology of microorganisms'; no. P3-0083 'Host-parasite relationship'; and the APC was funded by P1-0198). D.W. was a grant recipient from the Swedish International Development Cooperation Agency.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of ALIPB, AAU (protocol code ALIPB IRB/66/2013/21 and date of approval 19 August 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The generated sequencing raw data and assembled genomes were submitted to SRA—Sequence Read Archive (accession number: PRJNA1055930), URL https://www.ncbi.nlm.nih.gov/bioproject/1055930 (accessed on 17 January 2024).

Acknowledgments: The authors would like to thank the staff of the Institute of Microbiology and Immunology, University of Ljubljana, especially Nataša Krošelj, Brina Kovačič, Ivana Bohinc, Veronika Katarina Juren, Nuša Koblar, and Marko Kolenc for their support throughout the laboratory work. We also thank Eyosias Sileshi and Azeb Teklu for their full support in the isolation of the bacteria at ALIPB.

Conflicts of Interest: The authors declare no conflicts of interest.

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Paper 4

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Article

Genomic Characterization of Extended-Spectrum β-Lactamase-Producing and Third-Generation Cephalosporin-Resistant *Escherichia coli* Isolated from Stools of Primary Healthcare Patients in Ethiopia

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Abstract: The global spread of antimicrobial resistance genes (ARGs) in *Escherichia coli* is a major public health concern. The aim of this study was to investigate the genomic characteristics of extended-spectrum β-lactamase (ESBL)-producing and third-generation cephalosporin-resistant *E. coli* from a previously obtained collection of 260 *E. coli* isolates from fecal samples of patients attending primary healthcare facilities in Addis Ababa and Hossana, Ethiopia. A total of 29 *E. coli* isolates (19 phenotypically confirmed ESBL-producing and 10 third-generation cephalosporin-resistant isolates) were used. Whole-genome sequencing (NextSeq 2000 system, Illumina) and bioinformatic analysis (using online available tools) were performed to identify ARGs, virulence-associated genes (VAGs), mobile genetic elements (MGEs), serotypes, sequence types (STs), phylogeny and conjugative elements harbored by these isolates. A total of 7 phylogenetic groups, 22 STs, including ST131, and 23 serotypes with different VAGs were identified. A total of 31 different acquired ARGs and 10 chromosomal mutations in quinolone resistance-determining regions (QRDRs) were detected. The isolates harbored diverse types of MGEs, with *IncF* plasmids being the most prevalent (66.7%). Genetic determinants associated with conjugative transfer were identified in 75.9% of the *E. coli* isolates studied. In conclusion, the isolates exhibited considerable genetic diversity and showed a high potential for transferability of ARGs and VAGs. Bioinformatic analyses also revealed that the isolates exhibited substantial genetic diversity in phylogenetic groups, sequence types (ST) and serogroups and were harboring a variety of virulence-associated genes (VAGs). Thus, the studied isolates have a high potential for transferability of ARGs and VAGs.

Keywords: *Escherichia coli*; antimicrobial resistance genes; virulence-associated genes; mobile genetic elements; phylogenetic groups; Ethiopia

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Citation: Wolde, D.; Eguale, T.; Medhin, G.; Haile, A.F.; Alemayehu, H.; Mihret, A.; Pirs, M.; Smrdel, K.S.; Avberšek, J.; Kušar, D.; et al. Genomic Characterization of Extended-Spectrum β-Lactamase-Producing and Third-Generation Cephalosporin-Resistant *Escherichia coli* Isolated from Stools of Primary Healthcare Patients in Ethiopia. *Antibiotics* **2024**, *13*, 851. https://doi.org/10.3390/antibiotics13090851

Academic Editors: Linda Bester and Daniel Gyamfi Amoako

Received: 15 July 2024
Revised: 30 August 2024
Accepted: 3 September 2024
Published: 5 September 2024

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1. Introduction

Antimicrobial resistance (AMR) is a major global health problem that poses a significant threat to public health [1]. It is rapidly increasing and is expected to result in high healthcare costs and poor patient outcomes, potentially making AMR the leading cause of global mortality [2]. Africa and Asia are the two regions highly affected by AMR, with the potential for over 4.1 million annual deaths from AMR by 2050 [3]. *Escherichia coli* is one of the most important human pathogens and represents a major public health challenge. It is also one of the widely abundant commensal organisms in the gastrointestinal tracts of humans and animals, known for its ability to easily develop resistance to antimicrobial agents and to serve as a vector for the spread of AMR, which is a major problem for humans and animals [4]. In Ethiopia, there is an emergence and spread of resistance to common antimicrobials among *E. coli* isolates, leading to increased morbidity, mortality, and healthcare costs [5].

Infections with *E. coli* resistant to critically important antimicrobials, such as third-generation cephalosporins, have increased worldwide [6,7] and pose a challenge to clinical antibiotic therapy. *E. coli* strains resistant to third-generation cephalosporin antibiotics are frequently associated with the expression of extended-spectrum β-lactamases (ESBLs) [8]. ESBL production is the most problematic mechanism of antibiotic resistance, as many β-lactamase-encoding genes are typically carried on mobile genetic elements, such as plasmids, which can be easily transferred between different bacterial species [9]. Resistance to third-generation cephalosporins in *E. coli* can complicate the treatment of infections and lead to the use of a last-resort antimicrobial class such as carbapenems [10], which are not readily available in developing countries.

Bacterial populations acquire resistance to antibiotics either through chromosomal mutation or through horizontal gene transfer (HGT) from other bacteria that are either distant or closely related. This transfer is facilitated by mobile genetic elements (MGEs) [11]. MGEs are DNA elements that can move within or between bacterial cells, either from a chromosome to a plasmid or between plasmids through non-conjugative transposons, gene cassettes, and insertion sequence elements, or between DNA molecules through MGE elements capable of self-replication and conjugative transfer, such as plasmids and integrative conjugative elements [12].

MGEs carry genes that can confer resistance to antibiotics and play a critical role in facilitating the acquisition and spread of antimicrobial resistance genes (ARGs) through HGT [13]. The main mechanisms of HGT are conjugation, transduction, and transformation [14]. Conjugation drives the rapid evolution and adaptation of bacterial strains by facilitating the spread of diverse metabolic capabilities, including virulence, biofilm formation, and heavy metal and antibiotic resistance [15]. The conjugative transfer regions of the self-transmissible MGEs typically involve an origin of transfer (*oriT*) region, a conjugative type IV secretion system (T4SS), type IV coupling protein (ATPase), and a relaxase to deliver single-stranded DNA (ssDNA) into the recipient cell [16]. Understanding the profile of virulence-associated genes (VAGs), ARGs, and MGEs among *E. coli* strains circulating in a certain region is important for preventing the dissemination of antibiotic resistance, virulence factors, and other clinically relevant traits through *E. coli* bacterial populations. As there have been only a few studies conducted on this topic in Ethiopia, the aim of this study was to assess the distribution and diversity of ARGs, VAGs, MGEs, sequence types, serotypes and conjugative elements in 29 phenotypically confirmed ESBL-producing and/or third-generation cephalosporin-resistant *E. coli* isolates, obtained from patients attending primary healthcare units in Addis Ababa (n = 11) and Hossana (n = 18) using whole-genome sequencing.

2. Results

2.1. Phylogenetic Groups of E. coli Isolates

The *E. coli* isolates analyzed in this study belonged to seven phylogenetic groups. The predominant phylogenetic group was phylogroup B1 (n = 13; 44.8%), followed by

phylogroup A (*n* = 9; 31.03%). The other phylogenetic groups identified in this study were phylogroup C and E (*n* = 2 each), phylogroup B2 (*n* = 1), phylogroup D (*n* = 1), and phylogroup G (*n* = 1) (Figure 1). Phylogroups B2, C, D, and G were obtained from both non-diarrheic and diarrheic patients from Hossana. These phylogroups were found in a slightly higher proportion (60%) among females, and except for one of the phylogroup C isolate, all of them were detected in the age groups of 5–9 and 20–45 years (Table 1).

Table 1. Distribution of different *E. coli* phylogenetic groups among patients from study sites and their relations with other patients' characteristics.

Characteristics	Phylogroup (N = 29)						
	A N (%)	**B1** N (%)	**B2** N (%)	**C** N (%)	**D** N (%)	**E** N (%)	**G** N (%)
Study site							
Addis Ababa (*n* = 11)	7 (63.6)	3 (27.3)	0	0	0	1 (9.1)	0
Hossana (*n* = 18)	2 (11.1)	10 (55.6)	1 (5.6)	2 (11.1)	1 (5.6)	1 (5.6)	1 (5.6)
Sex							
Female (*n* = 13)	3 (23.1)	6 (46.2)	1 (7.7)	1 (7.7)	1 (7.7)	1 (7.7)	0
Male (*n* = 16)	6 (37.5)	7 (43.7)	0	1 (6.3)	0	1 (6.3)	1 (6.3)
Participants						1 (3.7)	
Diarrheic (*n* = 13)	3 (23.1)	7 (53.8)	0	1 (7.7)	1 (7.7)	1 (7.7)	0
Non-diarrheic (*n* = 16)	6 (35.7)	6 (35.7)	1 (6.3)	1 (6.3)	0	1 (6.3)	1 (6.3)
Age groups							
0–4 (*n* = 1)	0	0	0	1 (100)	0	0	0
5–9 (*n* = 6)	2 (33.3)	2 (33.3)	0	0	1 (16.7)	0	1 (16.7)
10–14 (*n* = 3)	1 (66.7)	2 (66.7)	0	0	0	0	0
15–19 (*n* = 2)	1 (50)	1 (50)	0	0	0	0	0
20–45 (*n* = 14)	5 (35.7)	5 (35.7)	1 (7.1)	1 (7.1)	0	2 (14.3)	0
46–65 (*n* = 3)	0	3 (100)	0	0	0	0	0

2.2. *Sequence Types of* E. coli *Isolates*

Twenty-two different STs were identified, of which one was a novel sequence type designated as ST15980. The most frequently identified ST was ST10 (*n* = 4; 13.8%), followed by ST48, ST224, ST345, and ST410 (*n* = 2; 6.9% each). ST131 was also detected in this study in one *E. coli* isolate obtained from a non-diarrheic patient in Hossana. The novel ST was also among isolates from Hossana. Of the total STs identified, only ST10 and ST48 were detected in *E. coli* isolates from both Addis Ababa and Hossana (Figure 1 and Supplementary Table S1).

2.3. *Serotypes of* E. coli *Isolates*

A total of 23 different serotypes were recorded among the 29 *E. coli* isolates investigated in this study. Of the total number of tested isolates, 22 (75.8%) were typable for their O antigen and 28 (96.5%) for their H antigen, with 7 (24.1%) being 'rough' (lacking expression of the O antigen) and 1 (3.4%) lacking expression of the H antigen. The most frequently identified serotype was H30 (*n* = 3; 10.3%), followed by four serotypes, namely H34, O134:H53, O8:H21, and O9a:H30, each represented by two isolates (Figure 1). The serotype O153:H4 detected in this study was identified as ST131 (Supplementary Table S1).

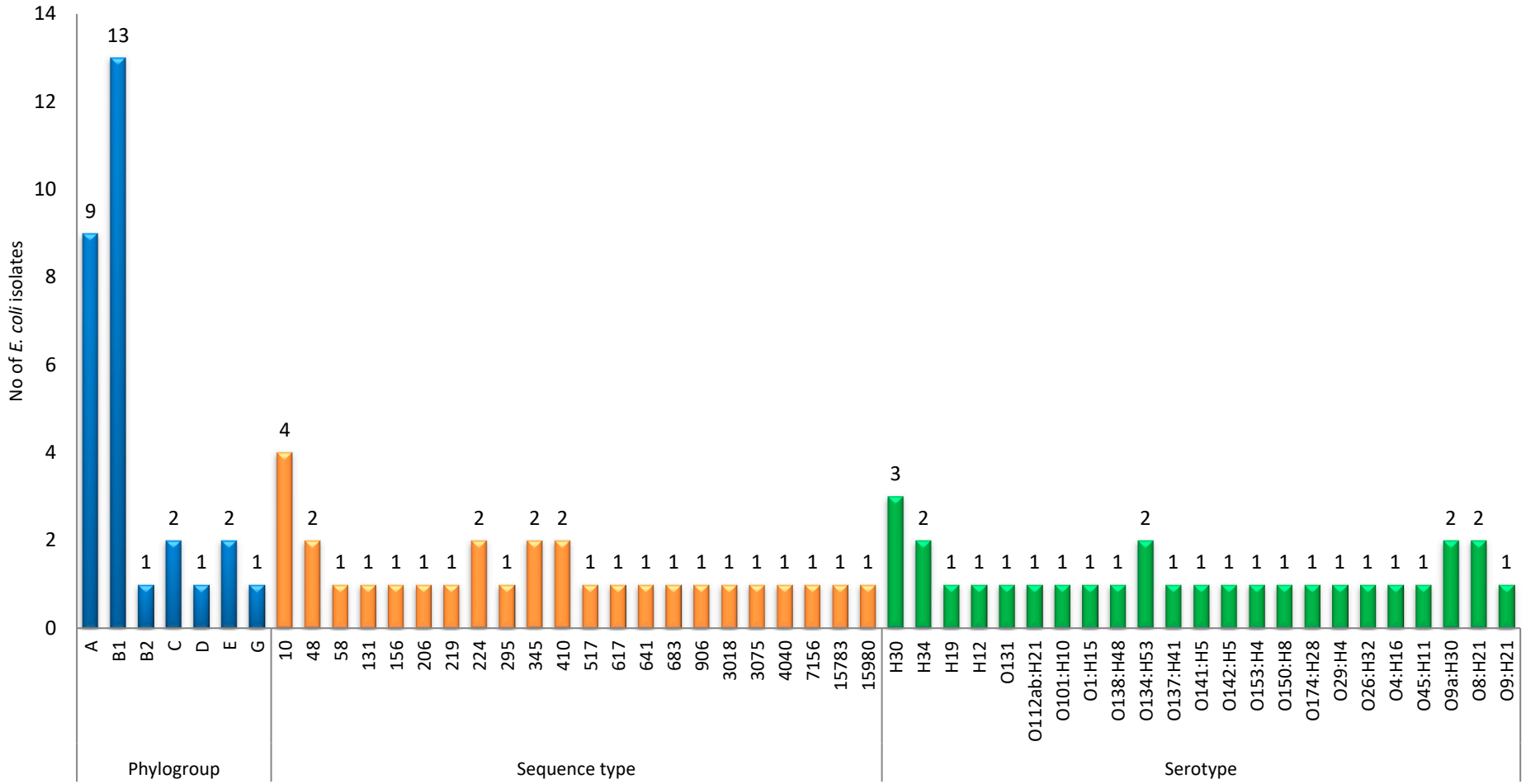

Figure 1. Phylogroups, sequence types, and serotypes of studied *E. coli* isolates.

2.4. Virulence-Associated Gene Profile of E. coli Isolates

A total of 72 different VAGs involved in adhesion (*n* = 23), protection against environmental stress and host immune response (*n* = 32), toxin production (*n* = 9), and in iron acquisition (*n* = 8) were identified in the *E. coli* isolates tested in this study. On average, all *E. coli* isolates carried 16 different VAGs, with a minimum of 7 and a maximum of 28 VAGs. The *terC* gene, which encodes for the tellurite resistance, and the *nlpI* gene, which encodes an outer membrane lipoprotein, were identified in all isolates tested. A total of 28 (96.6%) of the isolates contained *yehC* (chaperone, YhcD fimbrial cluster), 27 (93.1%) *csgA* (curlin major subunit CsgA), 26 (89.7%) *hlyE* (avian *E. coli* haemolysin), and 25 (86.2%) *yehB* (usher, YhcD fimbrial cluster). Forty-one of the total virulence-associated genes were detected in isolates from Hossana and Addis Ababa (Supplementary Table S1). Only seven genes, including the *cdt-IIIB* gene, which encodes the CdtB subunit of the cytolethal distending toxin complex, *cib* gene, which encodes a protein that protects *E. coli* cells from the cytotoxic effects of the cloacin DF13 bacteriocin, *colE5* (colicin E5 lysis protein Lys), *etsC* (putative type I secretory outer membrane protein) and *mcbA* (bacteriocin microcin B17) were detected exclusively in isolates from Addis Ababa. However, 27 different genes, including capsular polysaccharide-related genes (*kpsE*, *kpsMII*, and *kpsMII_K5*), the *tia* gene that encodes the Tia (toxigenic invasion locus) protein, which is involved in the adherence and invasion of host epithelial cells, and the *usp* (uropathogenic specific protein), were exclusively detected in isolates from Hossana (Supplementary Table S1). The most frequently detected VAGs (greater than 10%) in *E. coli* isolates from Addis Ababa and Hossana are presented in Figure 2. The study found that 28 VAGs were detected in 18.2% to 100% of the *E. coli* isolates from Addis Ababa, while 36 VAGs were detected in 11.1% to 100% of the isolates from Hossana.

The *afaB*, *afaC*, *afaE*, and *kpsMII_K5* genes were only found in the O153:H4 serotype. This serotype also possessed genes involved in the biosynthesis and uptake of yersiniabactin, aerobactin, and salmochelin siderophores. Seven VAGs (*fimH*, *nlpI*, *terC*, *yehA*, *yehB*, *yehC* and *yehD*) were detected in isolates from all phylogenetic groups. The *afaB*, *afaC*, *afaE*, and *kpsMII_K5* genes were only detected in phylogroup B2 isolates. Fourteen genes, including *usp*, *aslA*, *sigA*, *traT*, *hlyE*, *gad*, *chuA*, *cia*, *csgA*, *iha*, *ireA*, *iucC*, *iutA*, and *ompT*, were detected in phylogroup G. The *neuC* gene, which encodes a polysialic acid capsule biosynthesis protein, the *kpsMII* gene, which encodes a polysialic acid transport protein group 2 capsule and the *usp* gene, which encodes a uropathogenic specific protein, were only detected in phylogroups C, D, and G, respectively. Twenty-eight different VAGs were detected in a single ST131 *E. coli* isolate, among them the *sat* gene, which encodes the serine protease autotransporters of *Enterobacteriaceae* (SPATE), and the *senB* gene, which encodes the plasmid-encoded enterotoxin (Supplementary Table S1).

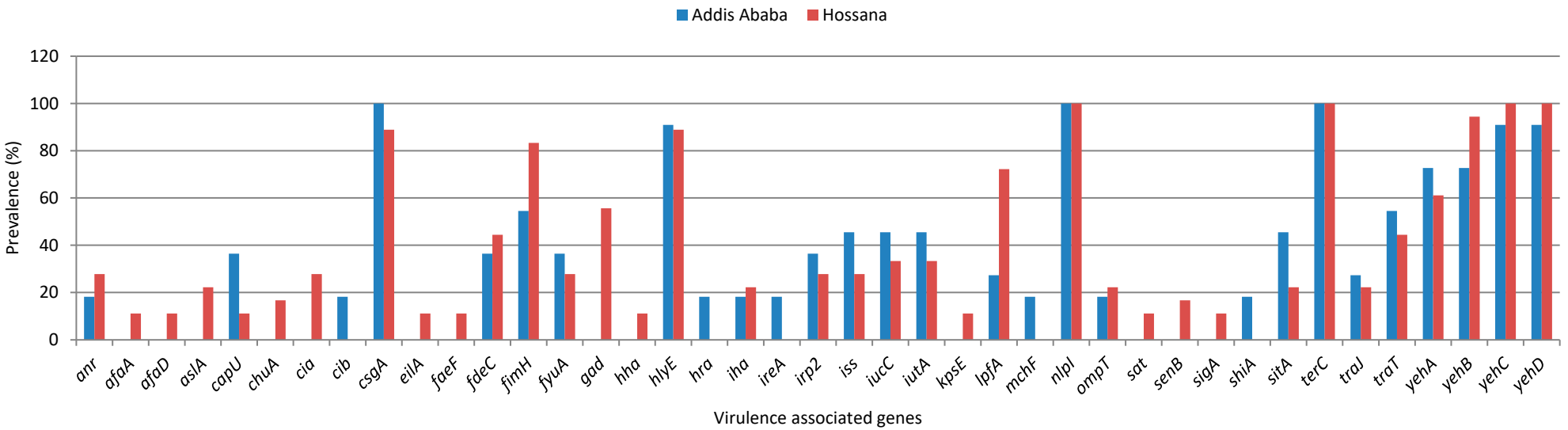

Figure 2. Virulence-associated genes detected with prevalence greater than 10% in studied *E. coli* isolates from Addis Ababa and Hossana.

2.5. Antimicrobial Resistance Genes

The distribution of ARGs among *E. coli* isolates is presented in Supplementary Table S2. ARGs were detected in 28 (96.6%) of *E. coli* isolates tested in this study. Thirty-one different horizontally transmitted acquired ARGs and ten chromosomal mutations in quinolone resistance-determining regions (QRDRs) were detected in *E. coli* isolates. Of the total *E. coli* isolates that carried ARGs, 19 (67.9%) had 5 or more different ARGs. The most prevalent ARGs detected were $bla_{\text{CTX-M-15}}$ (n = 22 isolates), followed by $bla_{\text{TEM-1B}}$ (n = 15 isolates), and *tet*(A) (n = 14 isolates). The $bla_{\text{CTX-M-15}}$ gene was identified in 78.9% of phenotypically confirmed ESBL-producing and 70% of third-generation cephalosporin-resistant *E. coli*. In total, 2 *E. coli* isolates from Hossana carried 14 different resistance genes encoding for resistance to antibiotics of different classes. Among third-generation cephalosporin-resistant *E. coli*, 90% carried β-lactamase and quinolone resistance genes. In this study, the plasmid-mediated quinolone resistance (PMQR) determinants, such as *qnrS1*, *aac(6′)-Ib-cr*, *qepA1*, *qepA2*, and *qepA4*, were detected. Among these, *qnrS1* was the most predominant, being detected in 13 (44.8%) of the isolates. Mutations in the QRDR and PMQR genes co-existed in 5 (20%) of the *E. coli* isolates carrying genes that determine resistance to quinolones. Of the *E. coli* isolates tested, 48.3% had chromosomal mutations in *gyrA*, *parC* and/or *parE*, which are associated with resistance to quinolones—ciprofloxacin and nalidixic acid. Double substitutions in *gyrA* (S83L + D87N) were observed in 8 (27.6%) of the *E. coli* isolates and these isolates also had an additional substitution in the *parC* (S80I) and *parE* (S458A) genes. Single amino acid substitutions, including S83L (n = 3), S83A (n = 1) and S83V (n = 1), were also observed in the *gyrA* gene. The substitutions at S80I (n = 6), E84K (n = 1), and A56T (n = 1) in *par*C, as well as S458A (n = 7), L416F (n = 1), and I529L (n = 1) in *parE*, were also observed (Table 2 and Supplementary Table S2).

This study found 7 *E. coli* serotypes (H30, O138:H48, O101:H10, O174:H28, O134:H53, O9a:H30, O8:H21) harboring 10 to 14 different ARGs. The predominant serotypes carrying the highest number (14) of ARGs were O9a:H30 and O8:H21. Furthermore, the *E. coli* isolates across all phylogenetic groups, with the exception of 1 from group B1, carried a range of ARGs, with groups A and B1 sharing 24 different ARGs. The analysis of sequence types revealed that ST224 and ST410 *E. coli* carried a high proportion of ARGs (Supplementary Table S2).

Table 2. Distribution of antimicrobial resistance genes and chromosomal mutations among studied *E. coli* isolates.

Antimicrobial Class	Antimicrobial Resistance Genes/Mutations Detected	No (%) of ESBL-Producing Isolates Possessing the Gene/Mutation (n = 19)	No (%) of 3rd-Generation-Cephalosporin-Resistant Isolates Possessing the Gene/Mutation (n = 10)	Total No (%) of Isolates Possessing the Gene/Mutation (n = 29)
β-lactams	$bla_{\text{CTX-M-15}}$	15 (78.9)	7 (70)	22 (75.9)
	$bla_{\text{CTX-M-3}}$	4 (21.1)	0	4 (13.8)
	$bla_{\text{TEM-1B}}$	10 (52.6)	5 (50)	15 (51.7)
	$bla_{\text{TEM-169}}$	2 (10.5)	0	2 (6.9)
	$bla_{\text{TEM-33}}$	2 (10.5)	0	2 (6.9)
	$bla_{\text{SHV-12}}$	0	1 (10)	1 (3.4)
	$bla_{\text{OXA-1}}$	1 (5.3)	1 (10)	2 (6.9)
Aminoglycosides	*aph(6)-Id*	8 (42.1)	3 (30)	11 (37.9)
	aph(3″)-Ib	8 (42.1)	3 (30)	11 (37.9)
	aac(6′)-Ib-cr	1 (5.3)	1 (10)	2 (6.9)
	aac(3)-IId	2 (10.5)	0	2 (6.9)
	aadA1	2 (10.5)	0	2 (6.9)
	aadA2	5 (26.3)	0	5 (17.2)
	aadA5	2 (10.5)	2 (20)	4 (13.8)

Table 2. *Cont.*

Antimicrobial Class	Antimicrobial Resistance Genes/Mutations Detected	No (%) of ESBL-Producing Isolates Possessing the Gene/Mutation (*n* = 19)	No (%) of 3rd-Generation-Cephalosporin-Resistant Isolates Possessing the Gene/Mutation (*n* = 10)	Total No (%) of Isolates Possessing the Gene/Mutation (*n* = 29)
Quinolones	*qnrS1*	8 (42.1)	5 (50)	13 (44.8)
	qepA1	0	1 (10)	1 (3.4)
	qepA2	0	1 (10)	1 (3.4)
	qepA4	1 (5.3)	1 (10)	2 (6.9)
	gyrA:p.S83L	7 (36.8)	4 (40)	11 (37.9)
	gyrA:p.D87N	5 (26.3)	3 (30)	8 (27.6)
	gyrA:p.S83A	1 (5.3)	0	1 (3.4)
	gyrA:p.S83V	1 (5.3)	0	1 (3.4)
	parC:p.S80I	5 (26.3)	2 (20)	7 (24.1)
	parC:p.E84K	0	1 (10)	1 (3.4)
	parC:p.A56T	1 (5.3)	0	1 (3.4)
	parE:p.S458A	5 (26.3)	2 (20)	7 (24.1)
	parE:p.L416F	0	1 (10)	1 (3.4)
	parE:p.I529L	0	1 (10)	1 (3.4)
Sulfonamides	*sul1*	7 (36.8)	3 (30)	10 (34.4)
	sul2	8 (42.1)	3 (30)	11 (37.9)
Trimethoprim	*dfrA1*	2 (10.5)	0	2 (6.9)
	dfrA12	5 (26.3)	0	5 (17.2)
	dfrA14	4 (21.1)	2 (20)	6 (20.7)
	dfrA17	2 (10.5)	2 (20)	4 (13.8)
	dfrB4	0	1 (10)	1 (3.4)
Tetracyclines	*tet*(A)	9 (47.4)	5 (50)	14 (48.3)
	tet(B)	4 (21.1)	1 (10)	5 (17.2)
Macrolides	*mph*(A)	5 (26.3)	2 (20)	7 (24.1)
	erm(B)	1 (5.3)	2 (20)	3 (10.3)
Amphenicols	*catA1*	2 (10.5)	1 (10)	3 (10.3)

2.6. Co-Occurrence of Antimicrobial Resistance Genes

Several ARGs were detected together in most of the isolates in the current study. All *E. coli* isolates carrying at least one aminoglycoside antibiotic resistance gene were shown to carry β-lactam, quinolone, sulfonamide, and trimethoprim resistance genes simultaneously. *E. coli* isolates that carried at least one β-lactam antibiotic resistance gene also carried quinolone resistance genes and sulfonamide resistance genes in 89.3% and 75% of the cases, respectively. The β-lactam antibiotic resistance genes were also detected in 39.3% of *E. coli* isolates that carried at least one trimethoprim resistance gene, 32.1% of *E. coli* isolates that carried at least one macrolide resistance gene and 10.7% of *E. coli* isolates that carried at least one amphenicol resistance gene (Supplementary Table S2).

2.7. Plasmids and Other Mobile Genetic Elements

In this study, *E. coli* isolates were found to harbor diverse MGEs, including plasmids, insertion sequences (IS), integrative conjugative elements (ICEs), and transposons. A total of 86 MGEs were detected in the *E. coli* isolates. Among these, 26 were identified as plasmids, 50 as IS, and 6 as composite transposons and 2 as unit transposons. The remaining two were ICEs and miniature inverted repeats. Except for one isolate, all tested *E. coli* isolates were confirmed to carry one to eleven plasmids. The *IncF* plasmid was the most abundant plasmid in *E. coli* isolates, with different replicon types and replicon variants, including *IncFII*, *IncFII(29)*, *IncFIB(AP001918)*, *IncFII(pRSB107)*, *IncFII(pAMA1167-NDM-5)*, *IncFII(pHN7A8)*, *IncFII(pCoo)*, *IncFIB(S)*, *IncFIC(FII)*, *IncFIA(HI1)*, *IncFII(pSE11)*, *IncFIA*, and *IncFIB(K)*. The isolates also carried other incompatibility group plasmids, including *IncI1* (*n* = 11), *IncI2* (*n* = 1), *IncY* (*n* = 9), *IncX1* (*n* = 3), *IncX3* (*n* = 1), and *IncX4* (*n* = 2). The *Col*

plasmids detected in the *E. coli* isolates in this study were *Col156* (*n* = 7), *Col(MG828)* (*n* = 5), *ColRNAI* (*n* = 2), and *Col(BS512)* (*n* = 4). A total of 26 plasmids' combination patterns were detected in *E. coli* isolates. Nine isolates carried two plasmids, while four isolates carried three plasmids. Nine isolates carried four or more plasmids, while a single isolate obtained from patient at Hossana carried eleven plasmids (Figure 3).

A total of 24 different plasmids were detected in *E. coli* isolates carrying genes resistant to quinolones, while 22 plasmids were detected in *E. coli* isolates carrying genes resistant to antibiotics of the aminoglycoside class. The *IncI2* plasmid was detected only in *E. coli* isolates harboring genes with resistance to the quinolones, whereas the *IncHI1A* and *IncHI1B(CIT)* plasmids were detected only in *E. coli* isolates harboring genes with resistance to the β-lactams. The dominant plasmid detected in all classes of antibiotic resistance gene-carrying *E. coli* was the *IncFIB(AP001918)* plasmid type.

Similar to plasmids, the *E. coli* isolates also carried several IS elements, miniature inverted repeats, integrative conjugative elements, and transposons. At least four MGEs, other than plasmids, were detected. The MITEEc1 was found in all tested *E. coli* isolates. A total of fifty different IS elements belonging to twenty-one IS families were identified. The most abundant IS family was IS3, followed by ISAs1 and IS630. The most prevalent IS elements were ISEc1 and IS609, detected in 72.4% and 58.6% of all *E. coli* isolates, respectively. Additionally, ISEc38, IS26, and ISEc9 were found in 37.9%, 34.5%, and 34.5% of the isolates, respectively. Isolates carrying twelve different MGEs were the most predominant (14.8%), followed by isolates carrying seven, eight, eleven, and thirteen different MGEs (11.1%) each. Notably, 1 *E. coli* isolate carried 17 different MGEs, while 15 isolates carried 10 to 17 MGEs (Figure 4 and Supplementary Table S3). There was a significant association between genes determining resistance to aminoglycosides and the presence of IS*3* (Fisher exact $p = 0.01$), IS*5075* (Fisher exact $p = 0.01$) and IS*629* ($X2 = 4.821$, $p = 0.028$). Similarly, a significant association was observed between genes determining β-lactam resistance and ISEc*31* (Fisher exact $p = 0.016$), ISEc*9* (Fisher exact $p = 0.048$), and ISSfl*8* (Fisher exact $p = 0.015$). In addition, resistance to quinolones was significantly associated with the presence of ISEc*9* (Fisher exact $p = 0.001$) and ISKpn*19* (Fisher exact $p = 0.003$).

2.8. Determination of Conjugative Transferable Elements

Of the 29 *E. coli* isolates tested, 22 (75.9%) had all the essential components necessary for conjugation, such as *oriT*s, relaxases, T4CPs, and T4SSs, indicating their potential for self-transferability. Fourteen (63.6%) of the *E. coli* isolates with conjugative transferable elements were obtained from eight diarrheic patients and six non-diarrheic patients in Hossana, while the remaining eight (three from diarrheic and five from non-diarrheic) were obtained from Addis Ababa. Four (13.8%) of the *E. coli* isolates were found not to carry *oriT*, whereas relaxases were not detected in three (10.3%) isolates. Only T4CP was detected in one *E. coli* isolate; *oriT*, relaxase, and T4SS were absent. Two (6.9%) isolates had both *oriT* and T4CP, but no relaxase and/or T4SS. Twenty-one (95.4%) *E. coli* with conjugative transferable elements had acquired ARGs. Virulence-associated genes were detected in all *E. coli* isolates with self-transferable and non-self-transferable MGEs.

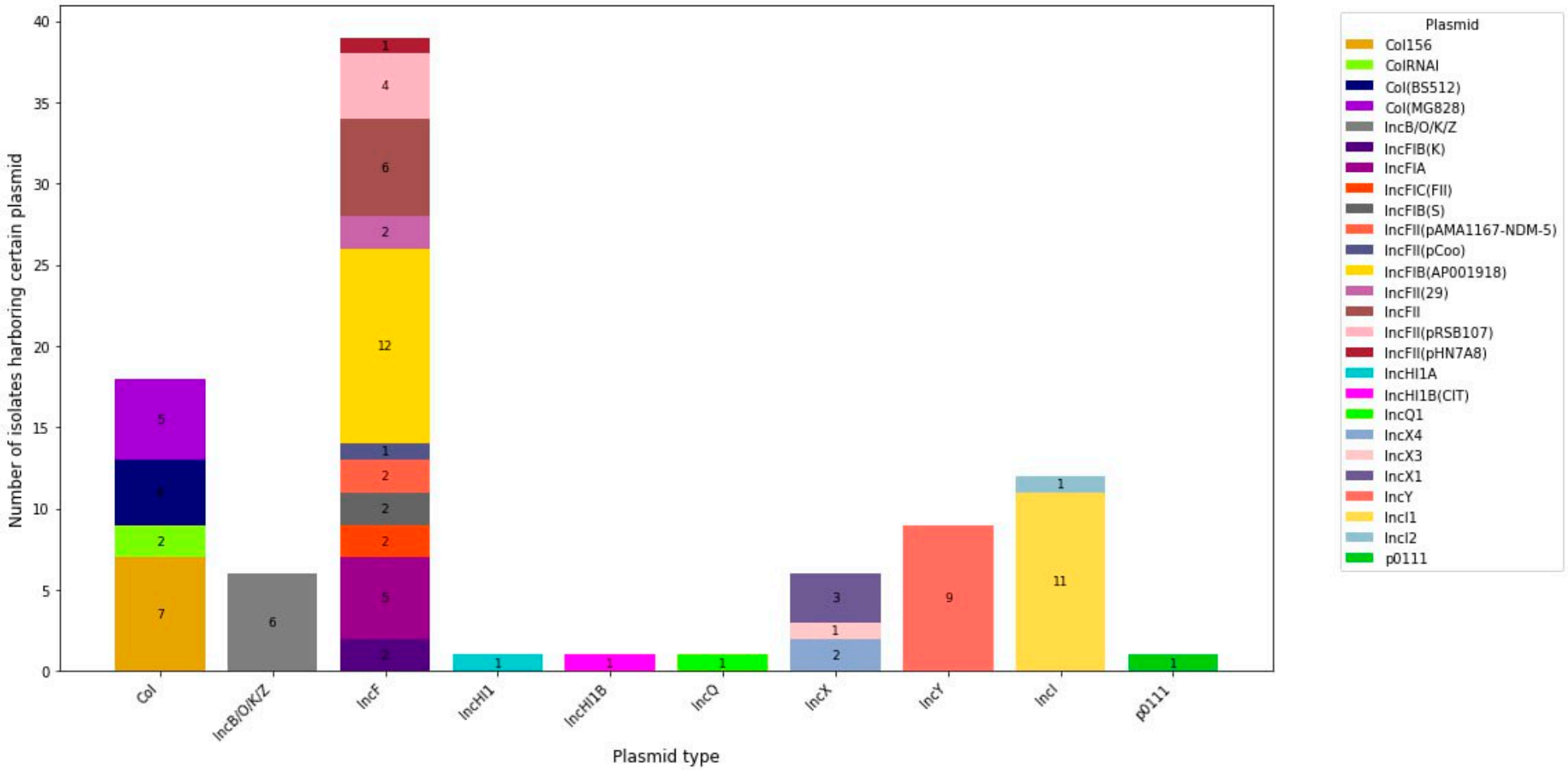

Figure 3. Number of studied *E. coli* isolates harboring certain plasmid types.

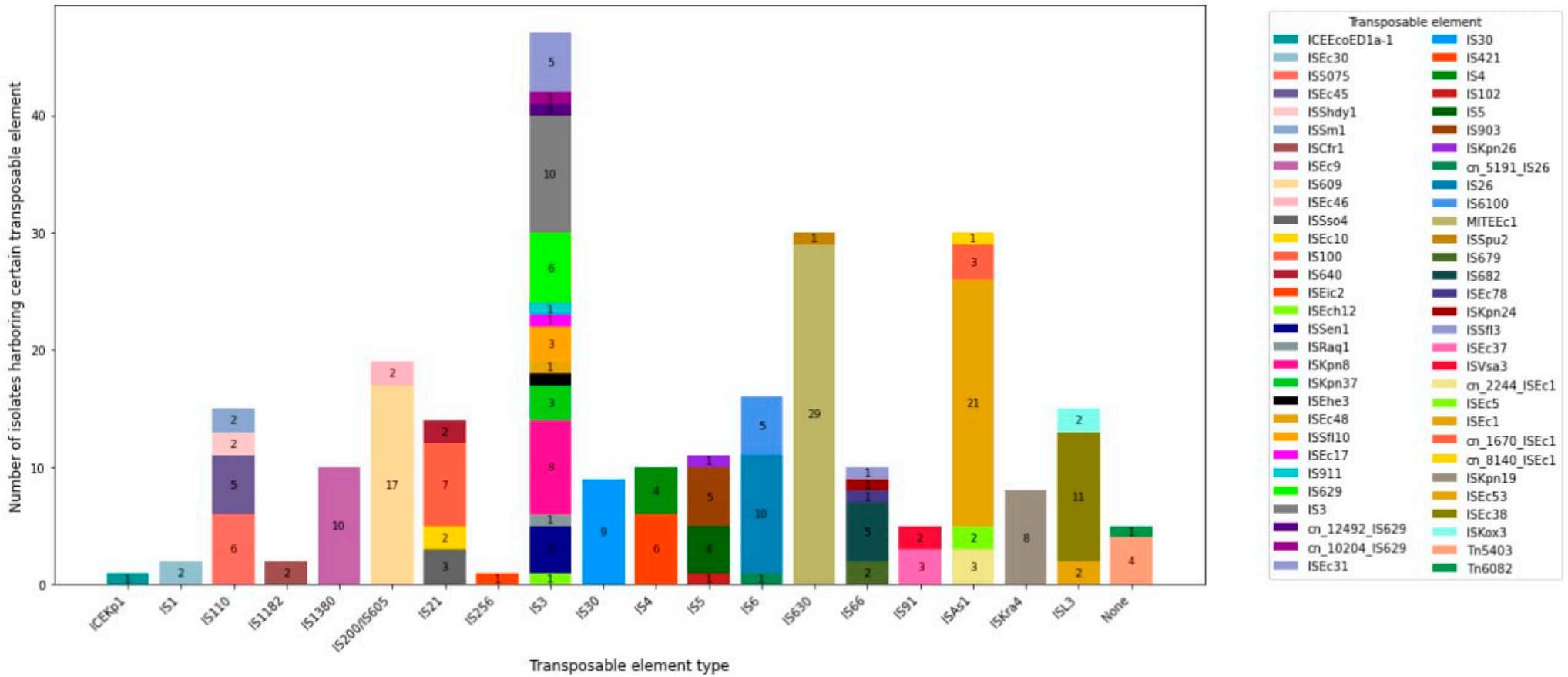

Figure 4. Number of studied *E. coli* isolates harboring certain types of transposable elements.

3. Discussion

E. coli is an extremely diverse bacterial species in which only about 6% of the genes are shared by all strains. The remaining genes, accounting for more than 90%, are variable "accessory genes" that are differentially present in the various *E. coli* strains [17]. The results of the study revealed a high level of genetic diversity among the *E. coli* isolates, which were classified into seven distinct phylogenetic groups. The findings showed that 82.8% of the *E. coli* isolates belonged to phylogroups A, B1, and C, which are generally regarded as phylogenetic groups in which most intestinal pathogenic *E. coli* strains are found in humans [18]. Of all the phylogenetic groups, B1 was the predominant one. This is consistent with previous findings from southwestern Nigeria [19]. In contrast, a study conducted in South Africa among diarrheic children confirmed that a predominant strain characterized by increased virulence and the ability to cause a wide range of infections belonged to phylogroup B2 [20]. The differences can be explained by health, diet and environmental, social, and geographical conditions [21]. The phylogroup B2 *E. coli* strain identified in our study belonged to the ST131. In addition, phylogroup G, which is known to contain highly virulent and AMR strains, was also detected in the current study [22], which implies a higher likelihood of severe and invasive *E. coli* infections.

In the current study, the *E. coli* isolates showed 22 different STs. One of them was a novel ST designated as ST15980, which was isolated from a non-diarrheic patient in Hossana. This isolate showed a broader spectrum of resistance to antimicrobials. The isolate carried resistance genes (*aph(6)-Id*, *aph(3″)-Ib*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, *qnrS1*, *sul2*, *dfrA14*) conferring resistance to aminoglycosides, β-lactams, quinolones, sulfonamides, and trimethoprim, which complicates treatment with a wide range of common antibiotics and poses a serious health problem. This isolate also typically carried plasmids from incompatibility groups (*IncI1*, *IncY*) and other MGEs such as (*IS26*, *IS30*, *ISEc38*), which often harbor genes conferring resistance to various antibiotics and have the ability to be efficiently transferred between bacterial cells [23]. Many VAGs have also been detected in ST15980, enabling it to adhere to host tissues and cells, evade the host immune system, and cause tissue damage. These include *hlyF*, the gene encoding hemolysin, which may be associated with increased production of outer membrane vesicles and contributes to the release of cytolethal distending toxin and other chemicals [24]; and the *terC* gene, which encodes a subunit of the tellurite resistance protein complex, which may contribute to its fitness and allow it to evade the host's primary immune response [25], making the strain more virulent and resistant to immune defense.

The prominent ST131, which is a highly virulent and extensively antimicrobial-resistant strain that has spread explosively throughout the world [26], was also found in our study. It is known to cause extraintestinal infections, including urinary tract and bloodstream infections [17]. The ST131 isolated in this study carried 28 VAGs, including *afa* (Dr binding adhesins), *iutA* (aerobactin receptor), and *kpsMT II* (group 2 capsule synthesis), which are characteristic of extraintestinal pathogenic *E. coli* (ExPEC) [18], demonstrating its ability to colonize and persist in the intestine, as confirmed by other studies [19]. Additionally, this isolate contained several genes, including *sat* (secreted autotransporter toxin), *fimH* (type 1 fimbriae), *fyuA* (yersiniabactin receptor), *iha* (adhesin siderophore receptor), *ompT* (outer membrane receptor) *iucC* (aerobactin), *iutA* (aerobactin receptor), and *tratT* (serum resistance associated), that are frequently found in *E. coli* ST131 isolates. Studies have indicated the growing prevalence and spread of the ST131 clonal group in various African countries. In Malawi, a genomic epidemiology study at Queen Elizabeth Central Hospital, a tertiary care center and referral hospital in Lilongwe, found ST131 in 14.9% and 32.8% of the *E. coli* isolates sequenced, respectively [27,28]. Additionally, a study conducted in South Africa found that 18% of *E. coli* isolated from urinary tract infections in inpatients and outpatients belonged to ST131 [29]. Furthermore, a study found that ESBL-producing *E. coli* isolates from Tanzania and Uganda belonged to the ST131 strain, accounting for 8.4% and 2.9% of isolates, respectively [30]. Considering the increased prevalence and pandemic

nature of the ST131 strain, which poses a significant threat to public health, due attention should be given to prevent and control its dissemination.

A high degree of diversity was observed in the *E. coli* population with 23 different serotypes. The predominant *E. coli* serotype was H30, which showed increased resistance to different classes of antibiotics. They harbored a plasmid encoding the $bla_{\text{CTX-M-15}}$ gene, one of the most widespread and predominant ESBL-encoding genes [31]. They also carried genes that determine resistance to aminoglycosides, quinolones, sulfonamides, trimethoprim, tetracyclines, macrolides, and amphenicols. The result also showed that this serotype harbors different groups of plasmids and MGEs containing genes for various VAGs encoding protectins, iron acquisition, cytotoxins, or adhesion factors [32]. The high diversity of ARGs and VAGs and their distribution among various serotypes and STs suggest multiple sources of resistant and virulent bacteria and the flow of these genes between different bacterial populations [33].

Antimicrobial resistance is one of the greatest global threats to public health. The fundamental reason why some bacteria are able to resist the effects of antibiotics is that they have specific genes that code for resistance mechanisms [34]. The resistance genes were detected in 96.6% of the *E. coli* isolates tested in this study. This rate is comparable to a previous report in which resistance genes were found in 84.9% of *E. coli* isolates from humans in the Central Zambia region [35]. However, a lower rate (38%) of ARGs was detected in *E. coli* isolates obtained from human samples in a systematic review and meta-analysis conducted in South Africa [36]. Bioinformatic analysis of the whole-genome sequence of the isolate (MBL protocol number 181) that was found to be phenotypically resistant to ampicillin, cefuroxime, cefotaxime, ceftriaxone, and trimethoprim-sulfamethoxazole did not reveal any ARGs, indicating that this isolate might possess a not yet identified ARG. Another possible explanation could be that the MGE carrying the ARG conferring this specific resistance phenotype was lost in sub-culturing the *E. coli* isolates.

E. coli has developed various mechanisms to resist the effects of antibiotics. In many cases, a single strain of *E. coli* can carry ARGs that confer resistance to different classes of antibiotics [37]. This ability significantly complicates the treatment of *E. coli* infections, as clinicians have fewer effective antibiotic options to choose from [38]. The current study found a high co-detection rate of multiple ARGs within the *E. coli* isolates tested. For example, all the isolates carrying aminoglycoside resistance genes were also confirmed to harbor resistance genes for β-lactams, quinolones, sulfonamides, and trimethoprim. Many of the detected resistance genes, including those encoding aminoglycoside-modifying enzymes (AMEs), β-lactamases, and determinants of quinolone resistance, sulfonamides, trimethoprim, and tetracyclines resistance, are often located on MGEs [39–41]. Furthermore, plasmids belonging to different incompatibility groups, and other MGEs, which have an important role in the transmission of ARGs, were simultaneously detected in the isolates in the current study. This observation suggests that the diverse MGEs may have the capacity to disseminate different antibiotic classes, further exacerbating the problem of multi-drug-resistant *E. coli* infections and the need to target plasmids to limit acquisition and transmission of antimicrobial resistance, as already reviewed by Vrancianu et al. [42].

Approximately 75.9% of plasmids were found to be self-transferable, possessing key genetic elements such as *oriT*s, relaxases, T4CPs, and T4SSs. This high proportion of self-transferable plasmids indicates their substantial potential for self-transfer and dissemination within bacterial populations. Additionally, it was observed that 95.4% of the conjugative plasmids examined were found to carry acquired ARGs. This suggests that these conjugative plasmids have the capacity to potentially spread and disseminate ARGs among different bacterial species, which poses a significant concern in the context of antimicrobial resistance.

The main limitation of this study was the inability to conclusively determine whether ARGs were plasmid-encoded or chromosomally located. This technical limitation restricts the understanding of the genetic mechanisms underlying the observed multi-drug resistance. Further investigations employing advanced sequencing techniques would be needed

to address these limitations and provide a more comprehensive understanding of the antimicrobial resistance landscape in the region.

4. Materials and Methods

4.1. E. coli *Isolates*

The current study is a continuation of a previous cross-sectional study conducted in Addis Ababa and Hossana from October 2021 to September 2022, in which 260 *E. coli* strains were isolated from patients of all ages and sexes recruited according to patient flow from 13 randomly selected health centers from 4 sub-cities (40% of the total) in Addis Ababa and all 4 health centers in Hossana [43]. For the current study, all *E. coli* isolates that showed phenotypic resistance to third-generation cephalosporin antibiotics or were identified as ESBL-producing were selected from the previous collection, because *E. coli* isolates exhibiting these characteristics are critically important for human medicine. The sampling method used in the current study is shown in Figure 5.

4.2. DNA Extraction, Whole-Genome Sequencing, Raw Data Pre-Processing, De Novo Assembly, and Quality Control

Twelve selected isolates exhibiting phenotypic resistance to third-generation cephalosporins were subjected to whole-genome sequencing (WGS). Genomic DNA extraction, WGS, raw data pre-processing, de novo assembly and quality control was performed as previously described for the 19 ESBL-producing isolates possessing ESBL genes [43]. The whole-genome sequence data of the two sequenced third-generation cephalosporin-resistant *E. coli* isolates did not pass the preliminary quality assessment criteria and were excluded in further bioinformatic analysis.

4.3. Bioinformatics Analysis

The ClermonTyping tool available at http://clermontyping.iame-research.center/ (accessed on 7 June 2024) was used to determine the phylogenetic groups of *E. coli* isolates [44]. Sequence typing was performed based on the seven gene Achtman scheme using the website https://pubmlst.org/ (accessed on 7 June 2024) [45] and EnteroBase https://enterobase.warwick.ac.uk/ (accessed on 27 June 2024) [46]. The detection of *E. coli* virulence-associated genes and serotypes was performed using VirulenceFinder 2.0 and SerotypeFinder available at www.genomicepidemiology.org (accessed on 5 June 2024) with the default parameters [47,48]. In addition, the acquired antimicrobial resistance genes as well as chromosomal point mutations causing resistance were determined using ResFinder 4.0, available at http://genepi.food.dtu.dk/resfinder (accessed on 5 June 2024), with default parameters [49]. Plasmid replicon was identified to infer plasmid presence using PlasmidFinder 2.1, available at https://cge.food.dtu.dk/services/PlasmidFinder/ (accessed on 15 June 2024), with minimum identity of 95% and minimum coverage of 60% [50]. Integrated mobile genetic elements were predicted in the assembled genomes using MobileElementFinder version 1.0 with MGEdb v1.0.2, available at https://cge.food.dtu.dk/services/MobileElementFinder/ (accessed on 15 June 2024) [51].

4.4. Statistical Analysis

The data were captured in Microsoft Excel and analyzed using SPSS software (Version 25. IBM SPSS Inc., New York, NY, USA). Descriptive analyses using frequency and percentages were used to summarize the variables. The chi-square or Fisher exact test was used to assess the association between ARGs and MGEs in *E. coli* isolates. A p-value of <0.05 was considered statistically significant.

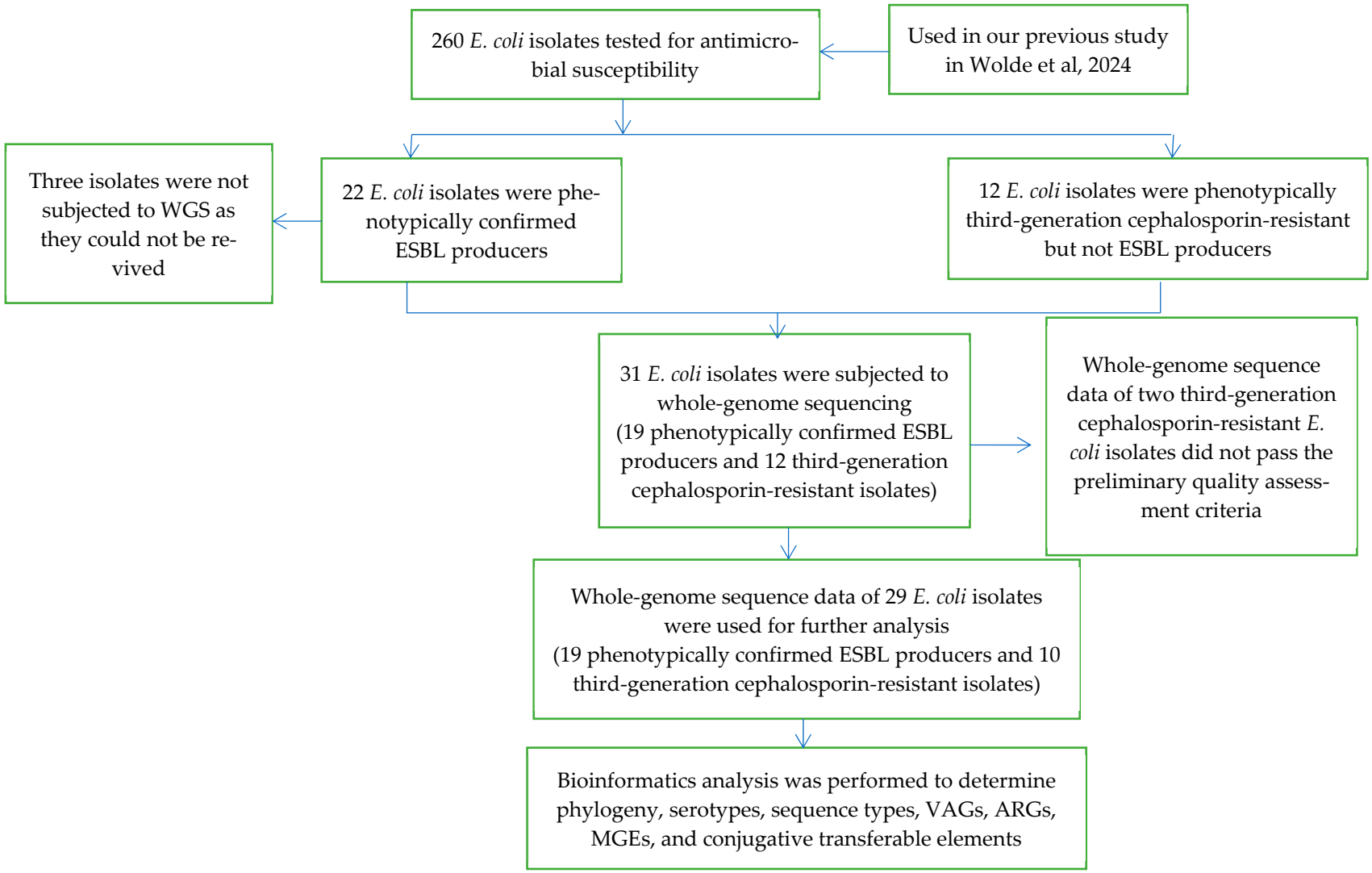

Figure 5. Flow diagram showing the selection of *E. coli* isolates for the study.

5. Conclusions

The overall finding of the current study showed the high genetic diversity of ESBL-producing and third-generation cephalosporin-resistant *E. coli* in patients from the two study areas. The observed diversity in the ARGs and MGEs among different STs and serotypes suggests the possible exchange of genes amongst different isolates, posing a significant challenge to infection control and to effectively treating infections. Additionally, a high proportion of self-transferable conjugative elements suggests a significant potential for horizontal gene transfer, with implications for the spread of clinically important traits, such as virulence and antimicrobial resistance. Therefore, the findings from this study highlight the pressing need for comprehensive strategies to address the challenge of AMR in the study area. In the future, based on the findings of this study, enhanced surveillance covering a large geographic area with a representative number of samples in collaboration with various stakeholders and sectors will be proposed to address this growing public health challenge. Implementation of strategies to reduce the spread of ARGs and VAGs is necessary to address this public health threat.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13090851/s1, Supplementary Table S1: VAGs detected in *E. coli* isolates; Supplementary Table S2: ARGs detected in *E. coli* isolates; Supplementary Table S3: MGEs detected in *E. coli* isolates.

Author Contributions: Conceptualization, D.W., M.P., J.A. and M.S.E.; methodology, D.W., K.S.S., J.A., M.P., T.C.K. and D.K.; software, D.W., G.M., T.C.K., T.J., A.S. and M.S.E.; validation, T.E. and M.S.E.; formal analysis, D.W., K.S.S., T.C.K. and M.S.E.; investigation, D.W., M.P., K.S.S., J.A., D.K., T.C.K., T.J. and A.S.; resources, M.P., J.A., A.S. and M.S.E.; data curation, D.W., A.F.H., H.A., A.M., K.S.S., J.A., D.K., T.C.K., T.J., A.S. and M.S.E.; writing—original draft preparation, D.W.; writing—review and editing, D.W., T.E., G.M., A.F.H., H.A., A.M., M.P., K.S.S., J.A., D.K., T.C.K., T.J., A.S. and M.S.E.; visualization, D.W., T.C.K. and M.S.E.; supervision, T.E., A.F.H., G.M, A.M. and M.S.E.; project administration, M.S.E.; funding acquisition, M.S.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially supported by Addis Ababa University through the thematic research project and by the Slovenian Research and Innovation Agency (research core funding no. P4-0092 'Animal health, environment and food safety'; no. P1-0198 'Molecular biology of microorganisms; no. P3-0083 'Host-parasite relationship'; and the APC was funded by P1-0198). D.W. was a grant recipient from the Swedish International Development Cooperation Agency.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of ALIPB, AAU (protocol code ALIPB IRB/66/2013/21 and date of approval 19 August 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The generated sequencing raw data and assembled genomes were submitted to SRA—Sequence Read Archive (accession number: PRJNA1105046, URL https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1105046, accessed on 10 May 2024).

Acknowledgments: The authors would like to thank Marko Kolenc from the Institute of Microbiology and Immunology, University of Ljubljana and Tom Koritnik from the National Laboratory of Health, Environment, and Food, Ljubljana, Slovenia for their assistance in DNA extraction and sequencing isolates, respectively. The authors are also thankful to Nigel Dyer, research fellow, Bioinformatics Research Technology Platform, University of Warwick (EnteroBase), for his support in defining a new sequence type detected in our study.

Conflicts of Interest: The authors declare no conflicts of interest.

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