Department of Microbiology, Immunology and Parasitology, College of Health Sciences, Addis Ababa University

Molecular Characterization and Antimicrobial Resistance Profile of diarrheagenic *Escherichia coli* in under-five children in Central Ethiopia

By Tizazu Zenebe (M.Sc)

A thesis submitted to Addis Ababa University, College of Health Sciences, Department of Microbiology, Immunology and Parasitology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Microbiology

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Addis Ababa, Ethiopia

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Abstract

Background: Many children fail to reach their development milestones due to several infection-related health problems in developing countries. One major infection-related health problem in under-five children (UFC) is diarrhea with the highest mortality rate in sub-Saharan Africa including Ethiopia. Diarrheagenic *Escherichia coli* (DEC) is the major causes of diarrhea in UFC. Emergence of antimicrobial resistance (AMR) among DEC and other diarrheagenic bacterial strains including extended-spectrum ß-lactamase (ESBL) and carbapenemase producing *E. coli* strains are among the major threats to health globally. DEC pathotypes have emerged due to genomic plasticity of the *E. coli* and are associated with different life-threatening clinical diseases. The epidemiology of DEC and their AMR profile and the genetic background of DEC are not well characterized in Ethiopia. The present study aimed to determine molecular epidemiology and AMR profile of DEC and to provide some genetic insight of DEC pathotypes isolated from UFC from Central Ethiopia.

Materials and methods: A health facility-based cross-sectional study was conducted in Addis Ababa and Debre Berhan, Ethiopia between December 2020 and August 2021. A total of 476 stool specimens from UFC (391 diarrheic and 85 non-diarrheic) were collected. Bacterial isolation and identification were done by standard microbiology methods. Antimicrobial susceptibility testing was done using disk diffusion. Polymerase chain reaction (PCR) was used to further characterize isolates. In addition, a total of 28 selected DEC pathotypes were subjected to whole genome sequence (WGS) analysis. Data analysis was made using SPSS software program version 20. Different web based and available bioinformatic tools including bioinformatics resources were used in the genomic and phylogenetic analysis.

Results: Of the total 476 children with or without diarrhea, 89.9% (428/476) were positive for *E. coli;* of which 183 (42.8%, 183/428) were positive for one or more genes coding DEC pathotypes. The overall occurrence of DEC pathotypes was 38.4% (183/476). Of the total 391 of diarrheic UFC, the predominant bacterial isolates were *E. coli* (87.7%, 343/391), followed by *Shigella (*10%, 39/391), *Campylobacter* (3.8%, 15/391), and *Salmonella* (2%, 8/391). Of the 85 non-diarrheic children, only *E. coli* isolates were detected. All the six DEC pathotypes were detected. The predominant DEC pathotype was enteroaggregative *E. coli* (EAEC) (41.5%, 76/183), followed by enterotoxigenic *E. coli* (21.3%, 39/183), enteropathogenic *E. coli* (15.3%, 28/183), enteroinvasive *E. coli* (12.6%, 23/183), hybrid strains (7.1%, 13/183), Shiga toxin-producing *E. coli* (1.6%, 3/183), and diffusely-adherent *E. coli* (0.6%, 1/183). DEC was detected from 40.7% (159/391) of diarrheic and 28.2% (24/85) of non-diarrheic children (p= 0.020). DEC acquisition was significantly associated ($p<0.05$) with rainy seasons, low family income, poor childcare, early starting supplement food, availability of domestic animal in the compound where the child live, and lack of continuous water supply. The majority of the DEC pathotypes were resistant to ampicillin (95.1%, 174/183) and tetracycline (91.3%, 167/183). High rate of resistance to trimethoprim-sulfamethoxazole (58%, 44/76), ciprofloxacin (22%, 17/76), ceftazidime and cefotaxime (20%, 15/76) was seen among EAEC pathotypes. Multidrug resistance (MDR) was detected in 43.2% (79/183) of the DEC pathotypes, whereas ESBL and carbapenemase producers were 16.4% (30/183) and 2.2% (4/183), respectively. Among the 30 ESBL-producing isolates, 80% (24/30) bla_{TEM}, 73% (22/30) $bla_{CTX\text{-}M}$, and 60% (18/30) bla_{SHV} were detected. Carbapenemase-encoding genes, $bla_{NDM,}$ and $bla_{\text{OXA-}48}$ were found in 13% (4/30) of ESBL-producing DEC isolates. The WGS analysis of randomly selected 28 DEC pathotypes showed that the pathotypes were distributed in different phylogenetic groups, with varied sequence type, serotype, virulence gene (VG), and AMR genes (ARG) patterns. In the phylogenetic analysis, the hybrid strains

were grouped in to A (25%), B1 (25%), C (25%), and unknown (25%) phylogroup. The EAEC strains were grouped in A (33%), B1 (13%), B2 (27%), and D (27%) phylogenetic groups. The MLST of the hybrid strains identified were ST 10, ST517, ST 23, ST 155, ST 568, ST58, and ST10512. The MLST of the EAEC strains were ST 2555, ST 58, ST 1861, ST 38, ST 10, ST 154, ST 8746, ST 449, ST 4442, ST 10512, ST 3749, ST 10825, and ST 5614. The present study showed a close phylogenetic relationship with different DEC pathotypes originated from different sources and geographical areas. These strains carried a large number of VGs that encode vital virulence factors. Co-occurrence of ARGs that encode for resistance to commonly used antimicrobials, ESBL, and carbapenemase were identified. Mobile genetic elements (MGEs) including plasmids and transposons that were associated with ARGs and VGs were detected in 86% of the WGS analysed DEC pathotypes. The predominant plasmid replicon types in association with harbouring determinant genes (VGs and ARGs) were the IncQ1, Col156 and IncFll (pHN7A8). Tn2, IS6100, and ISSpu2 were the predominant transposon types associated with carrying VGs or/and ARGs in the present study.

Conclusion: All six common DEC pathotypes that have potential to cause severe diarrheal outbreaks and persistent infections were detected in UFC in the study area. The study showed that DEC has the potential to be a big concern in UFC in Ethiopia. The WGS analysis showed genetically diverse strains of DEC pathotypes that carry VGs and ARGs that have potential to spread through MGEs. The study also showed occurrence of resistant and possibly hyper-virulent DEC pathotypes in the area. Therefore, despite the treatment of diarrheic patients empirically it will go to conduct periodic surveillance of the DEC pathotypes in the different health care settings in Ethiopia.

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List of abbreviations

A/E----------Attaching and effacing

AAF----------Aggregative adhesion fimbria

AAU---------- Addis Ababa University

AIEC----------Adherent invasive *E. coli*

AMR ----------Antimicrobial Resistance

AOR---------- Adjusted odds ratio

ARG---------- Antimicrobial Resistance Genes

ATCC---------- American Type Culture Collection

BFP ----------Bundle forming pili

CFTR----------Cystic fibrosis transmembrane receptor

CGE ----------Center for Genomic Epidemiology

CLSI ----------Clinical and Laboratory Standards Institute guidelines

COR---------- Crude odds ratio

DA----------Diffuse adherence

DAEC----------Diffusely adherent *E. coli*

DC----------Developing Countries

DEC---------- Diarrheagenic *Escherichia coli*

DNA----------Deoxyribonucleic acid

EAEC----------Enteroaggregative *E. coli*

EAF----------EPEC adherence factor

EAST1----------Enteroaggregative *E. coli* heat stable toxin 1

EDTA----------Ethylene diamine tetra acetic acid

EHEC----------Enterohemorrhagic *E. coli*

EIEC----------Enteroinvasive *E. coli*

EPEC---------- Enteropathogenic *E. coli*

ESBL----------Extended-spectrum ß-lactamase

ETEC---------- Enterotoxigenic *Escherchia coli*

GEMS----------Global Enteric Multi-Center Study

GLASS---------- Global Antimicrobial Resistance and Use Surveillance System

HGT---------- Horizontal Gene Transfer

HUS---------- Hemolytic Uremic Syndrome

IEC----------Intestinal Epithelial Cells

IPC---------- Infection Prevention and Control

LDM---------- Laboratory Diagnosis Methods

LEE----------Locus of enterocyte effacement

LT----------Heat-labile enterotoxin

MALDI-TOF--------Matrix-assisted laser desorption/ionization-time of flight

MDR ----------Multidrug resistance

MGE ----------Mobile genetic elements

MLST---------- Multilocus sequence typing

OPD----------Out-Patient Department

PAI----------Pathogenicity Island

PCR ----------Polymerase Chain Reaction

PD----------Pneumonia and Diarrhea

SNP ----------Single nucleotide polymorphism

SP----------Surveillance Program

SSA----------sub-Saharan Africa

STEC----------Shiga toxin-producing *Escherichia coli*

ST----------Heat-stable enterotoxin

Stx----------Shiga toxin

T3SS----------Type Three Secretion System

UFC----------Under-Five Children

UPEC----------Uropathogenic *E. coli*

UTI----------Urinary tract infections

VAG----------Virulence genes

VTEC----------Verocytotoxin producing *E. coli*

WGS----------Whole Genome Sequence

WHO ----------World Health Organization

List of Annexes

Operational terms

Antimicrobial resistance genes (ARGs): genes that provide antimicrobial resistance to the bacteria if it is expressed, and can be transferred among the bacterial species through horizontal gene transfer.

Antimicrobial resistance (AMR): the characteristic that allow the bacteria to grow in the presence of antimicrobials to which they were naturally susceptible. .

Carbapenemases: ß-lactamase enzymes with capacities to hydrolyze the ß-lactam drugs including the carbapenem.

Diarrheagenic *E. coli* (DEC) pathotypes: Intestinal pathogenic *E. coli* strains characterized by the presence of specific sets of virulence related genes and the characteristics of the disease they cause.

Diarrhea: the passage of three or more loose or liquid stools per day (or more frequent passage than is normal for the individual).

Extended spectrum ß-lactamase (ESBL): Enzymes that confer resistance to most ß-lactam antimicrobials, including penicillins, cephalosporins, and monobactam.

Genome analysis: The identification, measurement or comparison of genomic features. In the present study, it includes identification of virulence genes, ARGs, mobile genetic elements, and phylogenetic analysis of the DEC pathotypes.

Hemolytic Uremic Syndrome (HUS): Characterized by haemolytic anemia, thrombocytopenia, and acute renal failure, and occurs due to Shiga-toxin producing *E. coli* (STEC) which lead to damages of the small blood vessels in the kidney.

Hemorrhagic colitis: Illness caused by STEC and characterized by abdominal cramp and pain, initially watery diarrhea and then bloody diarrhea.

Mobile genetic elements (MGE): Segments of DNA that contains genes that can move within a genome or from one bacterial species to other.

Multidrug resistance (MDR): Resistant to at least to one antimicrobial in three or more classes of antimicrobials.

Phylogenetic analysis: The study of evolutionary relationships of bacterial strains using phylogeny.

Sequence type: A bacterial strain assigned by a molecular typing technique called multilocus sequence typing based on allelic variation of housekeeping genes.

Serotype: A bacterial strain assigned by serotyping based on O-specific antigens and H-antigen.

ß-lactamase: Enzymes produced by bacteria and vary in their abilities to hydrolyze β-lactam antimicrobials.

Virulence genes: Genes that encode proteins that are involved in the pathogenesis of the specific bacteria.

Whole genome sequence (WGS): Procedure that determine the order of bases in the entire genome.

Chapter one

1. Introduction

1.1. Background

'All diseases begin in the gut' said by Hippocrates (1) to state that gut health has the ultimate importance in human life and reflecting a healthy gut for optimal health. Diarrhea due to enteric pathogens is among the major gastrointestinal diseases that affect the gut (2) and it has profound effects on the gut health (3). Some of the profound effects include dysbiosis, damages the gut and causes loss of body fluids which affects the intestinal absorption and nutrition (3) which leads to malnutrition and stunt growth in children (2, 4). During growth and development, it requires a healthy intestinal tract that allow absorption of vital nutrients for optimal growth and development of body organs including the brain, especially it is very critical in the first two years of life (3). In the first few years (the first 1000 days) of life, vital development (physical, emotional, social, and cognitive development) occurs and continued to occur significantly to the age of five (5). Consequently, the effect of diarrhea is more severe in under-five children (UFC) (5-7). Frequent diarrhea in UFC could result not only in impaired growth like stunting but also morbidity and mortality (6, 7). As a result of the huge burden of diarrhea and other infections, many UFC fail to reach their developmental milestones (5).

Globally, diarrhea is continued as major health problem in UFC (8-10). The contribution of diarrhea to deaths among UFC is significantly high in developing countries (DC) (11, 12)). It is among the leading causes of total year of life loss (8) and the problem is particularly high in sub-Saharan Africa (SSA) and South Asia (10). There were 5 million deaths of UFC with a global under-five mortality rate of 38/1000 (12). The under-five mortality rate is high in SSA (74/1000) in 2021(12). More children died in SSA and South Asia with 80% of under-five

deaths (12), in particular in 15 pneumonia and diarrhea (PD) high burden countries (13). Ethiopia is the fifth among these high PD burden countries in death of children in the world (13). Based on the global health estimates, diarrhea is the second leading causes of death in Ethiopia, next to lower respiratory infection (14). Regardless of regional difference (39-125/1000), the total mortality rate for UFC was 67 deaths per 1,000 live births in Ethiopia (15). A recent report showed 59/1000 mortality rate of UFC in Ethiopia (16).

Many international (17, 18) and local efforts (19) have been made to tackle the problem of diarrhea in children. However, still diarrhea continued as major health problems and health focuses or agenda nationally (19) and internationally (11, 17). There are challenges in prevention and treatment of diarrhea due to pervasive infrastructural, political, and socioeconomic barriers present in DC (9). The complex environmental, food, water, and sanitation related problems sustained diarrhea as severe health problems in children (20). The problem is more associated with lack of access to water supplies, poor hygiene and sanitation in DC (6). Climate variability also affects the availability of clean water and effective sanitation, and this in turn mediates the incidence of diarrheal diseases (21).

Bacteria, viruses, and parasites are the common enteric pathogens that cause diarrhea (22). Diarrheagenic *Escherichia coli* (DEC), *Campylobacter, Shigella,* and *Salmonella* have been commonly associated with diarrhea in UFC (23, 24). Due to the current epidemiological and antimicrobial resistance (AMR) alarms of *E. coli* strain, the primary focus of this study is DEC. *E. coli* was considered as commensal and resident of gastrointestinal tract in human and animal for longer period of time (25, 26). However, it has strains with pathogenic capacity and cause intestinal and extra-intestinal infections (26). The genomic plasticity of the bacteria resulted in occurrence of new pathotypes that are highly specialized pathogenic strains, cause outbreaks, and severe diseases, sometimes with fatal and long term consequences (25, 26). Nowadays DEC is considered as an emerging agent of gastroenteritis

(27). Regardless of a variety of infectious agents causing diarrhea, DEC are the major contributor (25). Different DEC pathotypes become responsible for childhood diarrhea due to well organized mechanisms of pathogenesis and their easy ways of transmission (25, 26). DECs have been determined as predominant isolates in UFC (28-31) and is responsible for more than 30% of acute diarrhea incidences in children in DC (32). Based on a report of large scale Global Enteric Multi-Center Study (GEMS), Enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* were found as leading causative agents of diarrhea in children (33). Same study showed high fatality rates associated with Enteropathogenic *E. coli* (EPEC) and ETEC. Many of the pathotypes are public health concerns and the issue of DEC pathotypes is becoming serious. Different surveillance programs (SP) are established for pathogenic *E. coli* to monitor and track outbreaks in developed countries include in United Kingdom (34) and United State of America (https://www.cdc.gov/ecoli/surveillance.html) but there is no such efforts in DC. Other diarrheagenic bacteria such as *Salmonella*, *Shigella*, and *Campylobacter* also have contribution to occurrence of diarrhea in children (35). Some studies showed that still *Shigella* and *Campylobacter* are strongly associated with acute diarrhea (30, 36).

The common laboratory diagnosis methods (LDM) used for *Salmonella*, *Shigella*, and *Campylobacter* detection are either not able or limited to detect DEC pathotypes, and in most case detection of DEC is possible only based on molecular assay (37). There is limited applications of currently available such LDM in routine laboratories in DC, including Ethiopia (32). However, in such resource limited countries, epidemiological data are needed to help effective patient management, and to identify and track causes of outbreaks of diarrheal diseases (38). This limited laboratory diagnostic capacity resulted in inaccurate diagnosis and ineffective treatment practices (39). In addition, absence of active SP in most DC that allow having an up-to-date data for monitoring the emergence of pathogenic and

resistance strains strengthen the problem. Thus, scarcity of epidemiological data for some bacterial strains with pathogenic capacity becomes a challenge in DC, including Ethiopia.

The other serious concern is the continuous increase of AMR among enteric pathogens (40). AMR become among the top ten threats to global health in recent years (41). Due to resistance to first-line and last-resort antimicrobials there is lack of effective antimicrobials globally (42). Misuse of antimicrobials (in human, animal and agriculture) is the main causes for development and spread of AMR (40). Other common causes for development of AMR include non-compliance with drug prescription, inadequate antimicrobial stewardship practice, poor prescription habits and poor regulation system (43). Bacteria use two major strategies to develop AMR, mutations in genes and acquisition of foreign deoxyribonucleic acid (DNA) through horizontal gene transfer (HGT) (44). Improving infection prevention and control (IPC) measures, and surveillance of AMR (generating data) could help in combating AMR (45).

The World Health Organization (WHO) listed extended-spectrum ß-lactamase (ESBL) and carbapenemase producing enterobacterales including *Escherichia coli* as priority-1 pathogens for research and development of new antimicrobials (46). *E. coli* is used as an indicator for monitoring occurrence of resistant bacterial strains and for this the bacteria is commonly used in SP (47). The surveillance assess the AMR level, estimate risk of transmission, and circulation of AMR among the target population (47). Global AMR and use Surveillance System (GLASS) (https://www. who.int/initiatives/glass/) and WHO one health approach (integrated approach) global surveillance focus on *E. coli* AMR SPs (48). The WHO global SP focuses on ESBL *E. coli* and aimed to determine the level of ESBL-producing *E. coli* in human (hospital and community), food (animals), and environment.

Globally, there is a serious and an urgent threat due to ESBL- and carbapenemase-producing *E. coli* strains (49). From MDR bacterial strains, the ESBL and carbapenemase-producing DEC pathotypes are among the emerging pathogens (50). Studies from East Africa have reported high levels of AMR to commonly used antimicrobials for Gram-negative bacteria (51). In Ethiopia, efforts against AMR began in 2009 and continued with developing different national strategic plans (52) including the recently revised AMR prevention and containment strategic plan using one health approach (2021-2025) in 2021 (53). Strengthening the knowledge and evidence on AMR and use through surveillance and research is among the main objectives stated in the national AMR surveillance plan. The present research project also aimed to provide evidences on the AMR profile of DEC and other diarrheagenic bacteria. This research project aimed to provide epidemiologic and AMR profile data for DEC and other diarrrheagenic bacteria. Thus, this research project focuses on generating data that could initiate good diagnosis and treatment practice, in particular in UFC which could contribute towards national efforts in the control of DEC and combat spread of AMR pathogens.

1.2. Statement of the problems

Diarrhea is the major health problem for UFC in Ethiopia (15, 54-57). Among the bacterial agents of diarrhea, *Shigella*, *Salmonella*, and *Campylobacter* have been studied in UFC in Ethiopia (58-63). There is, however, not that much investigation on the importance of DEC pathotypes in the diarrhea of UFC in Ethiopia (64). DEC is a bacterial pathogen with well-organized pathogenic capacity (25, 26), with genomic plasticity and emerging nature (25, 27), with high public concerns (34, 49), with huge contribution to childhood diarrhea (32, 33), with indicator capacity (47, 48), with high resistance threats (48, 65), and in frontline for research (65). Regardless of this, studies conducted in Ethiopia were focused on *Shigella* and *Salmonella* (59, 60). Except recent emerging few local reports (66-68), there is limited data

on DEC in the diarrhea of under-five children in Ethiopia. Limitation in diagnostic capacity of the routine laboratories and limitation in SP for DEC also causes limited data on the pathogen in the county. This scarcity of data makes health personnel, health professionals and other related stakeholders to give low attention or concerns to the pathogen. Providing epidemiological data on the pathogen could fulfil the data gaps and shift into better concerns on their potential health burden, particularly in UFC, where diarrhea is one of the major health problems.

On the other hand, Ethiopia is vulnerable to increasing temperatures and climate change (69) which exacerbates the effects of poor sanitation (15), interrupted water supply (70), and unimproved drinking water source (15). A systematic review and meta-analysis reported that the incidence of DEC was affected by an increasing mean monthly temperature (71). Intermittent piped water supplies (70), and contact with manure and animals (72) were found as potential risk factors for enteric pathogens including *E. coli* strains in the study areas. Isolation of high prevalence of presumptive *E. coli* isolates from diarrheic lambs (73) and from UFC with diarrhea (64, 74, 75) in the study areas were reported which can be considered as a potential evidence for the clinical importance of DEC in UFC in the country.

Moreover, the Ethiopia national IPC policy encourages conducting research to generate and utilize evidence for improving the IPC practices (76). Thus, determining molecular epidemiology of DEC pathotypes, providing molecular insight, and characterizing the contributing factors for DEC acquisition will have significant importance in the effective IPC measure, and patient management.

Antimicrobials most commonly prescribed for infections due to Enterobacterales include ß-lactam drugs, aminoglycosides and fluoroquinolones (77, 78). The Ethiopian guideline for treatment of diarrheal diseases also recommends antimicrobials from these categories (79).

However, AMR is a problem in Ethiopia (80, 81) due to the misuse of the antimicrobials (82). In Ethiopia, the available clinical laboratories are limited to allow diagnosis of DEC pathotypes and failure to avoid misdiagnosis and wrong treatment practices, and improve the patient outcome. In addition, treatment of DEC-related infections must be based on specific potential DEC pathotypes, at least empirically, to avoid severe risks for the patients, like the use of antimicrobials in STEC infections (83). Epidemiological data could at least initiate concerns for the right diagnosis and treatment practices.

In addition, *E. coli* strains are global targets for AMR research (48, 65), and used as indicator organisms in different SPs (47, 48), in particular ESBL and carbapenemase- producing *E. coli* strains. In Ethiopia, few studies characterized ESBL and carbapenemase- producing Enterobacterales (61, 84-86). There is limited data (66-68) on AMR profile of DEC pathotypes in the country. There is no report for ESBL and carbapenemase-producing DEC pathotypes as well as DEC harbouring any ß-lactamase encoding genes in the country. The revised AMR prevention and containment strategic plan aimed to strengthen the knowledge and evidence on AMR through surveillance and research (53). Thus, determining the AMR profile of DEC pathotypes including ESBL and carbapenemase-producing DEC pathotypes will have importance not only for infection prevention and patient management but also will be an input to the national strategic plan.

Genomic plasticity of bacteria contribute to emerging pathogens (87, 88) and to cause large epidemics and severe clinical diseases. Whole genome sequencing (WGS) pathogens can provide data for genome analysis (resistomes, virulomes, mobilomes, and clonality) and building a phylogeny (89), and can allow discriminating between different strains' profile, classifying novel pathogens, elucidating evolutionary history, showing zoonotic and geographical origin, and transmission chain. In Ethiopia, there is insufficient data for WGS analysis of DEC.

1.3. Objectives

a. General objective

- To determine molecular epidemiology of DEC and characteristics of extended spectrum β-lactamase and carbapenemase producing DEC in under-five children in Addis Ababa and Debre Berhan, Ethiopia.

b. Specific objectives

- To estimate occurrence of DEC in under-five children
- To determine distribution of DEC pathotypes
- To determine antimicrobial susceptibility profile of DEC isolated from under-five children
- To assess distribution of genes encoding for ESBLs, and carbapenemase production in DEC isolated from under-five children
- To determine genomic and phylogenetic profile of some selected DEC pathotypes
- To determine factors associated with acquisition of DEC pathotypes in under-five children

1.4. Hypothesis and Research questions

The presence of potential risk factors (70, 73) for enteric infections and isolation of presumptive *E. coli,* ranged from 24.1% to 62.7% (64) with resistance profile (84) and high diarrhea burden (54) in under-five children indicate the occurrence of DEC pathotypes with ESBL and carbapenemase-producing strains in under-five children in Ethiopia.

The research question is:

- What is the epidemiological characteristic of DEC (occurrence of DEC, the predominant pathotypes, the predominant extended spectrum β-lactamase and

carbapenemase producing DEC, genetic variation, their potential health threats in the area) in under-five children in Ethiopia?

1.5. Scope of the study

The primary aim of this research project was to determine the molecular epidemiology and AMR profile of DEC in UFC in Debre Berhan and Addis Ababa, Ethiopia. For this, culture, biochemical tests, antimicrobial susceptibility tests, Polymerase Chain Reaction (PCR) assay, and agarose gel electrophoresis were performed. The PCR assay was used for detection of specific virulence genes, ESBL and carbapenemase- encoding genes of DEC pathotypes. Some DEC pathotypes were selected for whole genome sequence (WGS) analysis. Multi-locus sequence type, serotype, phylotype, virulence genes, drug resistance genes and phylogenetic relationship analysis was conducted from the WGS data. Factors associated with acquisition of DEC bacteria were analysed.

1.6. Significance of the study

Epidemiological data related to etiologic agents of childhood diarrheal and their other characteristics in SSA is limited (6). Due to the scarcity of epidemiological data, there is low attention or concerns, and inapproparaite diagnosis and treatment practice on some bacterial strians with pathogenic capacity. Strong surveillance programs, and well organized IPC strategies are needed for understanding the occurrence of diseases with emerging strains and development of AMR (90). In resource limited countries like Ethiopia, interventional strategies planned to tackle occurrence of infection and AMR are not only insufficient but also absent in some cases. Ethiopia does not have a recent Demographic and Health Survey data to have up-to-date estimates of key demographic and health indicators. In 2021, Ethiopia developed the national IPC policy which include a comprehensive approach to prevent morbidity and mortality from transmissible infectious diseases (76). This policy encourages conducting research to generate data and utilization of the evidence to improve IPC practices.

Although Ethiopia established a National AMR Surveillance Plan, the implementation of the strategy is facing different challenges including problem in the integration of electronic data at the sentinel surveillance sites and poor microbiology supplies (52). Currently, LDM for DEC such as PCR assay are not routinely conducted in Ethiopia.

The present research provides evidences on epidemiology and resistance profiles of DEC pathotypes and an update on other diarrheagenic bacteria. The finding will call attention on the prevention and management of diarrheal diseases as central to improving child health in the country and may be enforced to plan primary preventive measures to reduce the burden of diarrhea as well. Laboratory identification or surveillance of aetiology is crucial to design specific prevention measures, vaccination strategies, to guide management of patients, and to control timely outbreaks through understanding the epidemiology of the pathogen. The finding of the present study will have significance in providing evidence for such strategies and interventions. It will also initiate expansion of sentinel surveillance sites. Providing data on genetic background of DEC will allow understanding the degree of genetic diversity (evolutionary relationship), tracking of infectious sources and transmission routes and indicating the clinical isolates that are circulating and potential to cause outbreaks. Thus, the present research has significance as part of the national efforts against occurrence of infections and AMR.

Chapter two

2. Literature review

2.1. Diarrhea

2.1.1. Overview of diarrhea

According to WHO diarrhea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than is normal for the individual (91). The three types of diarrhea based on clinical feature are acute watery diarrhea (lasts several hours or days), acute bloody diarrhea (dysentery), and persistent or chronic diarrhea (lasts 14 days or longer) (91). Diarrhea is also classified as secretory, osmotic, exudative, and inflammatory based on the underlying pathophysiological mechanism (92). Diarrhea could be explained based on severity (mild or severe), duration (lasts for less than 2 weeks or more than 4 weeks) or causes (disease related, medication related, diet related and infectious (93). Diarrhea can be classified in to infectious and non-infectious (93). Non-infectious diarrhea can be disease related, medication related, or diet related (93). Infectious diarrhea caused by enteric pathogens include bacteria, virus or parasites (22, 93). More frequent incidence of several infectious disease occurred in children with stunted growth (due to malnutrition) (94). Poor nutritional status leads to a greater risk of diarrhea (95) and other infections (4), and could contribute to child death.

2.1.2. The global situation of diarrhea in under five children

Regardless of significance reduction in global under-five mortality rate, the highest under-five mortality rate remains in the WHO African region (96). In 2021 and globally, 5 million UFC have died (12). Majority of under-five death (80%) was occurred in SSA and South Asia (12). The under-five mortality rate of SSA was much higher (74/1000) compared to the global (38/1000) in 2021 (12). Generally, it was higher for DC (67/1000) compared to high-income countries (5/1000) (12).

Among the causes of total year of life loss, diarrhea was the 5th leading causes in 2016 (8) and 2017 (10) globally. In 2019, there were 5.3 million deaths of UFC and diarrhea contributed 9.1% to the total deaths (11). The recent IVAC (international vaccine access center) report (2022) revealed that there were more than a million deaths per year, 3, 350 deaths per day, or 140 deaths per hour of children due to diarrhea or pneumonia (13). Higher number of under-five deaths (70%) was occurred in the 15 high PD burden countries (13). A study done in seven DC showed that acute watery, acute bloody and persistent diarrhea contributed between 12% and 56% to diarrheal death of children aged 1-4 years (97). A prevalence of 14.3% of diarrhea in UFC was reported in East Africa (98).

Many efforts have been made globally. The integrated global action plan for the prevention and control of PD (GAPPD) was published in 2009 and 2013 and set a global 75% reduction in the incidence of severe PD in UFC by 2025 (13, 99). The sustainable development goal also set a reduction of mortality of UFC to 25/1,000 live births by 2030 (18). There was a considerable reduction of death and mortality rate in UFC, from 2000 (9.92 million deaths and 75/1000 mortality rate) to 2019 (5.30 million and 38 /1000) due to many efforts (11). However, there was failure to achieve the global targets for preventing and ending the problem of diarrheal burden in many countries (11). All the 15 high-burden focus countries failed to meet the diarrhea GAPPD score of 82% and the mean diarrhea GAPPD score across 15 countries was 43% (13).

2.1.3. Diarrheal disease in under-five children in Ethiopia

Based on the 2016 Demographic and Health Survey data of Ethiopia, under-five mortality was 67/1000 live birth (15). The Ethiopian national new-born and child survival strategy was aimed to end all preventable child deaths by 2035 (19). In the strategy, it was planned for the reduction of under-five mortality from 2013 level (64/1,000) to 29/1000 by 2019/20 (19). However, the national strategy did not achieve the target; in 2019 it was 59/1000 (16).

Ethiopia is the fifth among high PD burden countries in the world (13). Global health estimates identified the top 10 causes of death in Ethiopia for both sexes aged 1 to 4 years (100). According to the estimate, diarrhea is the second (next to lower respiratory infection) leading causes of death (100). In Ethiopia, the national diarrhea prevalence in UFC (estimate of two weeks) is approximately 12%, with decreasing trends like 24% in 2000 to 18% in 2005, 13% in 2011 and 12% in 2016 (15). However, based on the different local studies and systematic review, the prevalence of diarrhea in UFC ranges from 11%-31% (54-56, 98). We did also a recent retrospective study to assess the burden of diarrhea in the present study areas and found diarrhea as the second (next to tonsillitis) causes for UFC to visit health facilities (Unpublished data). In Ethiopia, diarrhea contributed to 9% deaths of UFC (equal to the global burden) in 2019 (12).

2.1.4. Risk factors for diarrhea

The major transmission route for infectious diarrhea is faecal-oral route by direct contact with infected or carrier person or through contaminated food or water (101). Socio-economic, environmental and behavioural factors contributed to the occurrence of diarrhea (6). Among the common risk factors include early weaning, low maternal education, malnutrition, poor hand washing, poor sanitation, poor water storage practices, poor water supply, seasonal patterns, and younger age (6). Majority of diarrheal diseases (90%) are associated with inadequate personal hygiene, lack of access to clean water supply, and poor hygiene and sanitation conditions (6). Unclean water and unsafe sanitation were associated with 72% and 56% of death of UFC in 2016, respectively (10). Similarly in Ethiopia, different risk factors such as low access to improved toilet facilities (15), the presence of unimproved sanitation facilities (102), and the greater intermittent water supply and point of use water contamination were reported (70). The occurrence of intermittent water supplies increase

household water storage which leads to transmission of water borne pathogens, and put in danger the hygiene practices (103) for occurrence of diarrhea.

2.2. Diarrheagenic *E. coli* (DEC)

2.2.1. General microbiological overview of diarrheagenic E. coli

E. coli are rod-shaped bacterium with size of 0.5 μm diameter and 1.0 to 3.0 μm length (104). The bacteria can grow in 15-48 °C (optimal growth at 37 °C), facultative anaerobe (grow aerobic and anaerobic condition), non-motile or motile with peritrichous flagella, lactose fermenter or non-lactose fermenter, and with varied lysine decarboxylase and gas production (25, 87). It is Gram-negative, oxidase negative, urease negative, citrate negative, Voges-Proskauer negative, and hydrogen sulphide negative (25, 87, 104). However, the bacteria reduce nitrate to nitrite, positive for indole production, mannitol and methyl red test.

There are six common intestinal and two extra-intestinal pathotypes (25, 26). The six intestinal pathotypes are based on epidemiological evidence, phenotypic traits, clinical feature of the disease and specific virulence factors (Figure 1) (25, 87). These are EPEC, Shiga toxin-producing *E. coli* (STEC), ETEC, enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (25, 26, 105). STEC also named as verocytotoxin producing *E. coli* (VTEC) or Enterohemorrhagic *E. coli* (EHEC). The two extra-intestinal pathotypes are neonatal meningitis *E. coli* and uropathogenic *E. coli* (UPEC) (25, 26, 87). Other recently identified and poorly characterized pathotypes include Necrotoxigenic *E. coli* (NTEC), Cell-detaching *E. coli* (CDEC), and adherent invasive *E. coli* (AIEC) (105). *E. coli* are also serologically divided in serogroups and serotypes on the basis of specific antigens, mainly O antigens (somatic lipopolysacharide or cell surface) and H antigens (flagella), and occasionally K (capsular) antigens (106). All DEC pathotypes are transmitted through faecal-oral (Figure 2) (25, 87, 107).

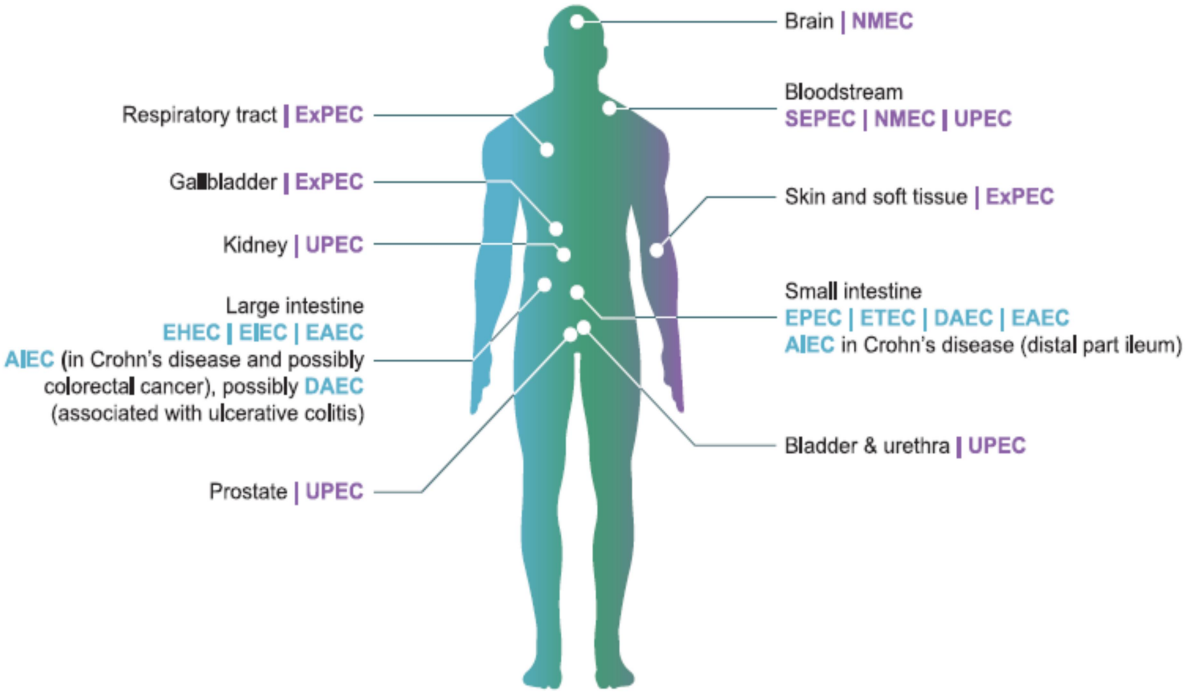


Figure 1. Different pathotypes with their clinical diseases associated in the human host (87). Abbreviations: EPEC, Enteropathogenic *E. coli*; ETEC, Enterotoxigenic *E. coli*; EIEC, Enteroinvasive *E. coli*; EAEC, Enteroaggregative *E. coli*; STEC, Shiga-toxin producing *E. coli*; DEAE, Diffusely adherent *E. coli*; ExPEC, Extraintestinal pathogenic *E. coli*; UPEC, uropathogenic *E. coli*; NMEC, Neonatal meningitis *E. coli*; SEPEC, Sepsis-associated *E. coli*

2.2.1.1. Enteropathogenic *E. coli* (EPEC)

In 1940s, a series of infantile diarrhea that was associated with a particular sero-groups (serotype test) and leads to recognize pathovars for the first time (108). EPEC is the first pathotypes identified from other DEC (25, 108). The term EPEC was used in 1955 (25). EPEC belongs to attaching and effacing (A/E) pathogens (25, 87). EPEC is able to form A/E lesions on the surface of intestinal epithelial cells (IEC) but unable to produce heat-labile (LT), heat-stable (ST) enterotoxins, and Shiga toxins (25, 87). EPEC are classified in to typical EPEC (tEPEC, *eae*+*bfpA*+*stx*–) and atypical EPEC (aEPEC, *eae*+*bfpA*–*stx*–) based on the presence or absence of EPEC adherence factor (EAF) plasmid which contains two operons, *bfp* (bundle-forming pili) and *per* (25). Typical EPEC possess EAF plasmid (25, 87). *bfp* encodes the type IV bundle forming pilus and *per* encodes a transcriptional activator called plasmid encoded regulator (Per) (25). EPEC is also LEE (locus of enterocyte

effacement) positive, a pathogenicity island (PAI) associated with A/E lesions (26). The LEE is a 35kb PAI and organized in five operons, encode type three secretion system (T3SS), ler (regulator), and effectors.

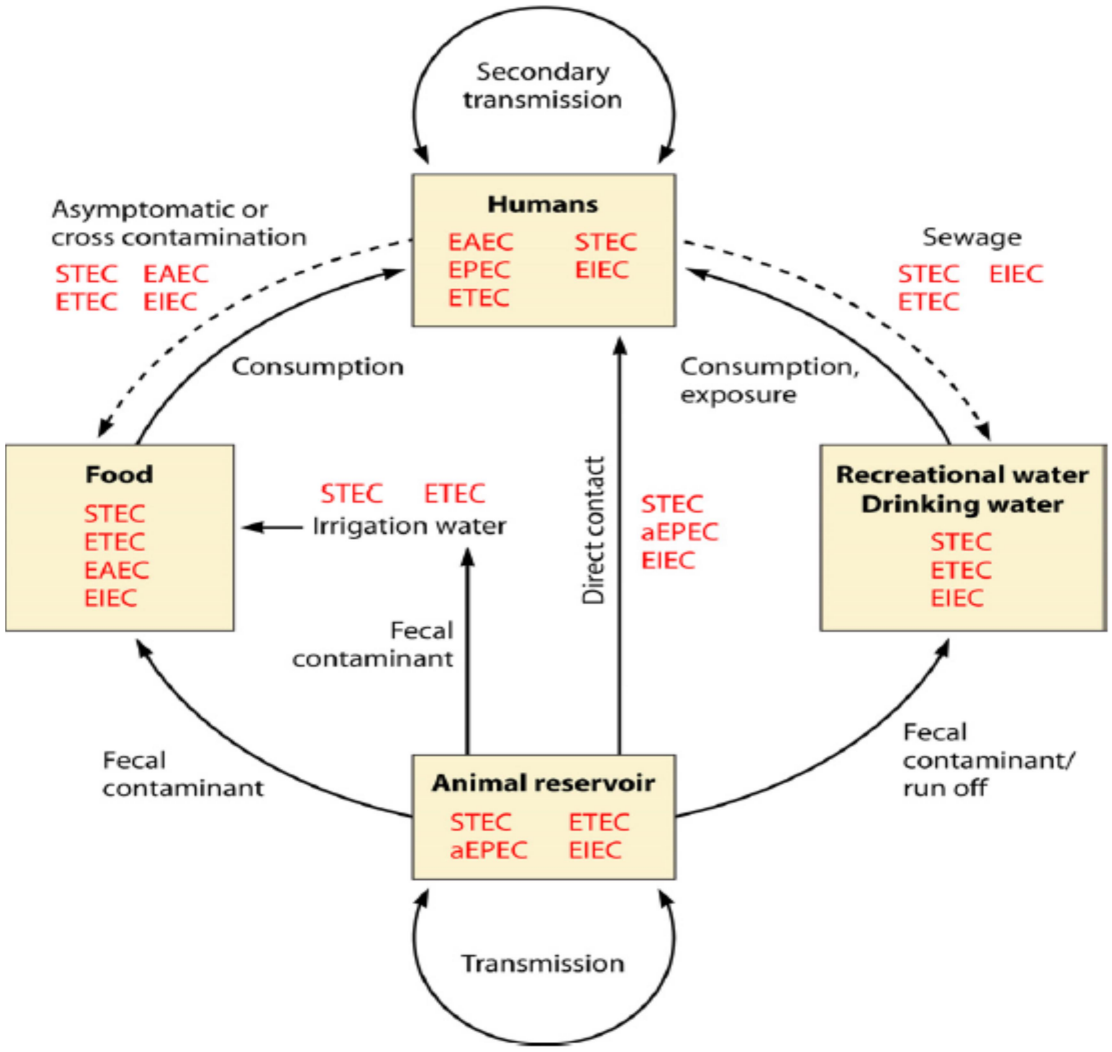


Figure 2. Potential reservoirs and modes of transmission for diarrheagenic *E. coli* (9). Abbreviations: EPEC, Enteropathogenic *E. coli*; aEPEC, atypical Enteropathogenic *E. coli*; ETEC, Enterotoxigenic *E. coli*; EIEC, Enteroinvasive *E. coli*; EAEC, Enteroaggregative *E. coli*; STEC, Shiga-toxin producing *E. coli*; DEAE, Diffusely adherent *E. coli*

The main transmission route for EPEC infection is faecal-oral route such as contaminated food and water, contaminated surfaces, human carriers, and weaning fluids (Figure 2) (25). Humans are the only reservoir for tEPEC but aEPEC may be isolated from human and animals such as dogs, rabbits, monkey, and sheep (25). A/E lesions formation on the surface of IEC is the hallmark of aEPEC and tEPEC pathogenesis (26). A/E lesions is an actin-rich

cup-like pedestal characterized by effacement of IEC microvilli with intimate adherence between the bacterium and the epithelial cell membrane (25). EPEC infection starts with local adherence (varied for tEPEC and aEPEC), and followed by signal transduction and intimate attachment, and then pedestal formation resulted (25, 26).

The initial attachment of tEPEC to enterocytes in the small bowel involves the bundle forming pilli and resulted in localized adherence pattern (Figure 3) (105). Other fimbriae or pilli involved in adherence include type I fimbriae, *E. coli* common pilli, EspA, and flagellin (25). Since typical EPEC possess bfp, it produces localized adherence whereas atypical EPEC (don't to have bfp) form localized like, diffuse, or aggregative adherence patterns (26). Bacterial translocated intimin receptor (Tir) which is translocated to the host cell cytoplasm through T3SS that expressed on the surface of the host cell as receptor will make the intimate attachment (105). This intimate attachment will cause recruitment of cytoskeletal proteins (actin dynamics) and accumulation of cytoskeletal proteins beneath the attached bacterial colonies and leads to actin cup or pedestal formation, which is called A/E lesion (25). Both LEE encoded effectors (Tir, map, EspF, Esp2, EspH, EspB) and non-LEE encoded effectors (NleB, NleC, NleD, NleH) involve in the pathogenesis of EPEC. In some atypical EPEC, EAEC heat stable toxin 1 (EAST1) could be produced.

The diarrhea resulted from EPEC infection is either secretory diarrhea or osmotic diarrhea, due to combination mechanisms. The secretary diarrhea resulted due to either alteration or disruption of epithelial electrolyte transport system through T3SS effectors and Nle effectors (25, 105). The osmotic diarrhea resulted due to the effacement of microvilli that could cause reduction of absorptive capacity and loss of fluid from lumen (105). The clinical outcome of EPEC is acute diarrhea (persistent diarrhea also occur) with clinical presentations such as dehydration, fever, and vomiting particularly in children under 2 years of age (26).

2.2.1.2. Shiga-toxin producing *E. coli* (STEC)

Cell culture based assay done for further differentiation of EPEC found that some EPEC strains were cytotoxic to vero cells (the toxin called Vero toxin or verocytotoxin) (108). Then, the toxin was characterized and found similar to Shigella toxin (structurally and functionally) and named as Shigella-like toxin, and this leads to recognition of STEC pathovars. STEC is also called VTEC due to the other name of the toxin, verocytotoxin. STEC contains shiga toxin 1 (stx 1) and shiga toxin 2 (stx 2) which are acquired by phage (25). EHEC is a subset of STEC. STEC is a highly infectious pathogen that colonizes the distal ileum and large bowel in human (105). STEC include O157:H7, non-O157 STEC (O26, O45, O111, O121, O145, and O103), and can be LEE positive (A/E pathogen) or negative (25). O157:H7 and non-O157 STEC (O103:H25, O104:H4, O11: NM, O157: NM, and O26:H11) caused outbreaks (25).

STEC transmitted through faecal-oral route (Figure 2) with low infectious dose, and found in animal, environment, food, and human (25). A major reservoir for STEC is cattle (meat and dairy). Exposure by direct contact with animal (cattle, swine, sheep, deer, rabbits, birds, dogs, rodents and insects) or with their faeces, environmental exposure (soil), contaminated food or water, and person to person (food handlers) can cause STEC infections. STEC outbreaks can occur through contaminated food and water, and environmental exposure (25). STEC require low infectious dose to cause infection. Viable but non culturable (VBNC) cell can produce stx but cannot grow in culture.

LEE positive forms A/E lesion through T3SS which is encoded by LEE but LEE negative use other unique set of virulence factors for causing Hemolytic Uremic Syndrome (HUS) and haemolytic colitis (25). The pathogenesis of STEC involves adherence, toxin production (shiga toxin, cytolethal distending toxins, and EHEC hemolysin), and A/E lesion formation (Figure 3) (25, 26, 105). Different strains contains different genomic islands and virulence

plasmids (105). The genome islands, LEE positive or negative, plasmid content, and stx variety determines the severity of the disease (25, 105). Stx is the vital virulence factor of STEC (25). There are two stx variants, stx 1 and stx 2 with subtypes (stx1: a, c, d; stx2: a to g) and both variants are encoded on prophages that are integrated into the chromosome. STEC can carry a single, or combination of variants or subtypes. The toxin variants could determine the severity of the disease (e.g. stx2 is associated with more severe diseases). Stx released from lysed bacterial cells during lytic cycle of the phage and this will occur during bacterial stress (e.g. use of antimicrobials).

Following the release of stx, it binds to host cell receptors (GB3), on renal cells and vascular endothelial cells, and cause inhibition of protein synthesis (109). Briefly, after internalization the toxin will be transported to host cell cytoplasm (retrograde). Subunit A will remove adenine residue from 28S rRNA (ribosomal RNA) which leads to inhibition of protein synthesis, resulted in cell death. Other virulence factors such as cytolethal distending toxin (damage DNA), EHEC hemolysin (pore forming toxin), and autotransporters (serine protease) involved in the pathogenesis of STEC infections. Mild watery diarrhea, bloody diarrhea, haemorrhagic colitis, and HUS could be caused by STEC. HUS characterized by thrombocytopenia, haemolytic anemia, and acute renal failure (25, 109).

2.2.1.3. Enteroinvasive *E. coli* (EIEC)

Strains that found to cause dysentery in children and adults and have ability to invade mammalian tissue culture cells and resulted in the recognition of EIEC in 1947, after 60 years of *Shigella* discovery (25, 108). EIEC are assumed as intermediate stage between *E. coli* and *Shigella* and are a potential pre-cursor of full-blown (fully mature) *Shigella* strains (26, 110). That means, *Shigella* is originated from *E. coli* through gene lose and gain mechanisms. And EIEC has some characteristics shared by *E. coli* and *Shigella*. Epidemiological data of EIEC is underestimated due to difficulty in distinguishing it from

Shigella (misclassification with *Shigella*) and the less severe clinical manifestations (25, 26). In spite of causing several outbreaks, EIEC have limited routes of transmission and distribution (26). EIEC comprises 21 serotypes based on O antigen pattern (exception that some has H antigen) and some O antigens are identical to *Shigella* O antigen. EIEC and *Shigella* use different strategies to block (loss or inactivation of antivirulence gene) lysine decarboxylase synthesis. *Shigella* delete or inactivated cadA gene but EIEC possess cadA but have an inactivated cadC (regulator).

EIEC is highly invasive and T3SS dependent pathogen and the virulence factors are responsible for bacterial invasion, escaping, cell spreading, inhibition of autophagy, regulation of immune response, and result in interacellular survival of the bacteria (26). The DEC pathotypes can be classified as non-T3SS dependent pathotypes (ETEC, EAEC, LEE-negative STEC, DAEC and AIEC) and T3SS dependent pathotypes (LEE-positive STEC strains, EPEC and EIEC) (110). EIEC and *Shigella* share the same pathogenesis mechanisms (25). EIEC contains key plasmid encoded virulence factors that involves in the disease pathogenesis (25). These include components of T3SS, chaperons (IpgA, IpgC, IpgE, Spals), transcriptional regulators (virF, virB, mxiE), translocators (IpaB, IpaC, IpaD) and T3SS independent virulence factors. The EIEC infections involves penetration of the epithelial barrier, induction of macrophage cell death, IEC invasion, suppression of the immune response, intra-and inter cellular movement, and modulation of epithelial integrity (25). EIEC can produce *Shigella* enterotoxin (ShE1 and ShE2), Pic and SepA (SPATE) (109). Unlike *Shigella species*, EIEC infections often causes self-limiting, and mild watery diarrhea (non-bloody) but rarely causes bloody (dysentery), shigellosis-like symptoms and complication (25).

2.2.1.4. *Enterotoxigenic E. coli* (*ETEC*)

A subset of EPEC strains were found to express enterotoxins based on animal pathogenicity studies and in vitro assay (25, 108). ETEC strains are characterized by production of colonization factors, ST and LT enterotoxins, as highly diverse pathotypes, with more than 100 somatic seroproups (O) and 34 flagella types (H) (25, 26). ETEC infection is transmitted faecal-oral route via asymptomatic carriers (e.g. food handlers), contaminated food, direct contact with animal, drinking water, and surface water (Figure 2) with higher infectious dose (10^6 to 10^8 cells) (25). ETEC has at least 25 distinct colonization factors which mediate adhesion to epithelial cells (110). Other outer membrane proteins Tia and TibA are involved in the intimate cell attachment and to induce ETEC invasion into epithelial cells (110).

ETEC colonize the small bowel epithelial lining using surface proteins mainly by colonization factors, and elaborate enterotoxins (the adherent ETEC) that causes ETEC-induced diarrhea (Figure 3) (25). The primary pathogenesis mechanisms for ETEC is secretion of heat stable (ST) and/or heat labile (LT) toxins (110). STa (with two variants, STh and STp) associated with human disease and STb associated with animals (25, 110). LT are two classes, LT-I (encoded by virulence plasmid) and LT-II (encoded in chromosomes acquired by phage) which has sub categories, LT-IIa, LT-IIb, and LT-IIc) with LT-I more being responsible to human illness (25, 110).

STa and LT cause elevations of intracellular messenger cyclic GMP and AMP concentration level, respectively (110). Briefly, STa bind to and the intestinal brush border guanylate-cyclase-C (GC-C) receptor and increase cGMP (110). This will resulted in phosphorylation of the cystic fibrosis transmembrane regulator (CFTR). Then, CFTR stimulate the ion channel and cause secretion that leads to diarrhea. LT toxins are AB5 toxins (one A subunit linked to a pentameric B subunit) and are transported across the bacterial outer membrane by

the type 2 secretion system (110). LT bind to ganglioside receptors on the mammalian cell via the LT-B subunit. The LT transported via the Golgi and endoplasmic reticulum (ER) to the cytosol and cause elevation of the intracellular cAMP concentration, and then phosphorylation of the CFTR. Adherence and colonization, toxin production (LT, ST) and phosphorylation of CFTR leads to diarrhea (25, 110). ETEC infections cause watery and traveler's diarrhea characterized by abdominal cramping, fever, headaches, nausea, vomiting and watery stools with quick incubation period (1 to 2 days) (25, 109). ETEC infections could result in malnourished and growth stunt in children (25).

2.2.1.5. Enteroaggregative *E. coli* (EAEC)

Assessment of attachment pattern to cultured cells resulted in identification of other pathotypes called EAEC (25, 108). EAEC is able to form biofilms on IEC through bacteria to bacteria adherence and bacteria adherence to the host cell surface, and form a characteristic adherence pattern called aggregative adherence pattern or stacked brick (Figure 3) (25). EAEC is transmitted by faecal-oral route through contaminated food or water (Figure 2) (25, 26). EAEC may be categorized into typical (which has aggR gene and more pathogenic), and atypical (which lacks aggR) (26).

EAEC is an emerging pathogen (26). The encoding genes of adhesisns, toxins, and other virulence factors are highly variable (25). Aggregative adhesion fimbria (AAF) and afimbrial adhesins are the major adherence factors (to IEC) used by EAEC (Figure 3) (25). Adherence to IEC, toxin production (enterotoxin and cytotoxin), and inflammation are the main pathogenesis mechanisms for EAEC (25, 105). Attachment of EAEC to IEC is mediated by both fimbrial (AAF) and afimbrial adhesins. AAF encoded by pAA virulence plasmids which also encode AggR (transcriptional factors that regulates AAF biogenesis). Adherences of EAEC to IEC stimulate inflammatory cytokine production (IL-8, CCL20) which cause recruitment of neutrophil and resulted in inflammatory diarrhea. EAEC produce different

toxins include pet (cytoskeletal altering toxin), EAST-1, ShE1 (*S. flexneri*) (induce cAMP and cGMP), and hemolysin.

Clinical presentations associated with EAEC include abdominal pain, low fever, prolonged diarrhea (depending on immunity, nutritional status and genetic susceptibility), vomiting and presence of mucus with or without blood. EAEC cause acute self-limiting watery diarrhea, traveller's diarrhea, and persistent diarrhea (25). EAEC (with AAF/I fimbriae) has emerged as agents of an outbreak of extra-intestinal disease, and community acquired urinary tract infections (UTI) (26).

2.2.1.6. Diffusely adherent *E. coli* (DAEC)

DAEC is another pathotypes that is able to attach to IEC different from classic patterns of adherence (local or aggregate adherence) (25). DAEC is characterized by its adherence over the entire surface of cultured epithelial HEo-2 cells in scattered pattern named diffuse adherence (DA) (25). DAEC infection transmission and reservoirs are not well characterized (25). DAEC epidemiology is not well known due to limitation in detection methods. DAEC is predominantly isolated from diarrheic children compared to healthy controls (25).

The pathogenesis of DAEC is associated with Afa-Dr adhesions which include fimbrial and afimbrial (Afa) adhesins (105). DAEC adhere via Afa/Dr adhesins to receptor on brush border epithelial cell include decay accelerating factor and crcinoembryonic antigens related cell adhesion molecules receptors. This leads to internalization, production of proinflammatory cytokines and brush border lesions (due to inflammation). DAEC causes watery diarrhea (persistent in children or asymptomatic carriers in adult), and chronic inflammatory intestinal disease such as Crohn's disease (25). Children with age between 18 months and 5 years are more prone to DAEC infections (105).

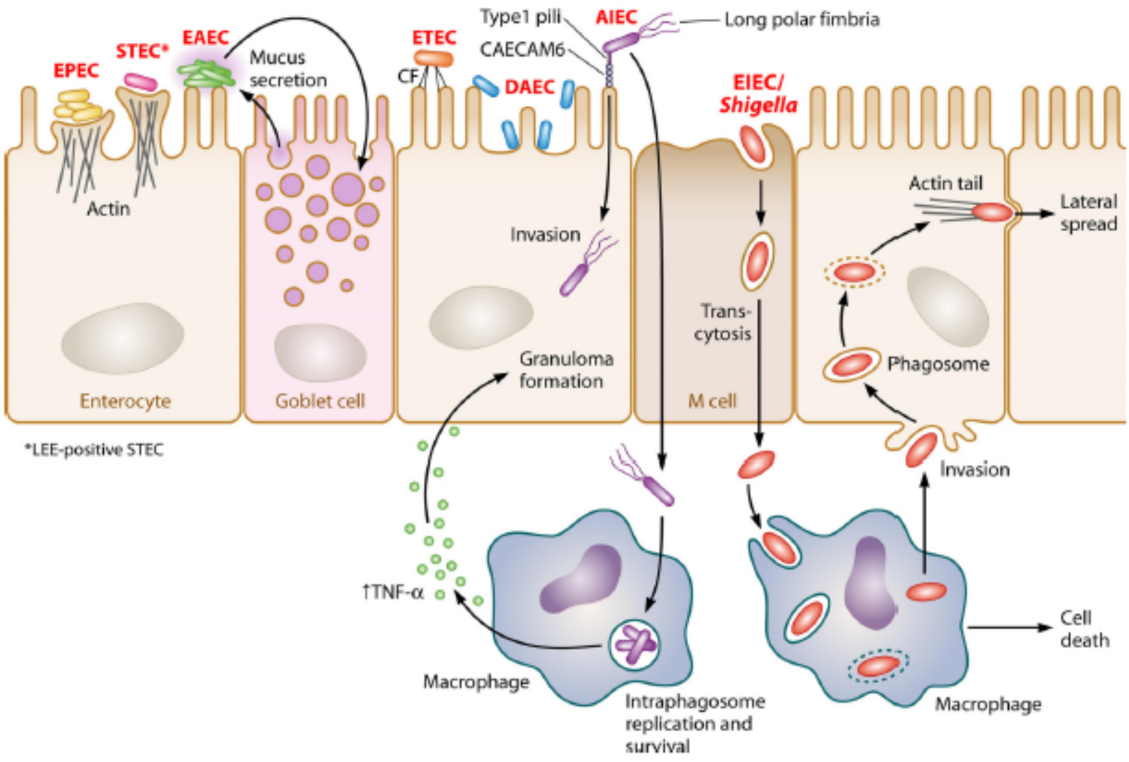


Figure 3. Adherence patterns of DEC pathotypes. Abbreviations: EPEC, Enteropathogenic *E.coli;* ETEC, Enterotoxigenic *E. coli*; EAEC, Enteroaggregative *E.coli*, DAEC, Diffusely adherent *E. coli*; AIEC, Adherent invasive *E.coli*, and EIEC, Enteroinvasive *E. coli* (25).

2.3. Other common diarrheagenic bacteria

Enteric pathogens that cause diarrhea include bacteria, virus, and parasites (22). DEC, *Campylobacter, Shigella, Vibrio cholerae,* and *Salmonella* from bacteria, *rotavirus, Human calcivirus (noroviruses* and *sapoviruses),* and *Adenovirus* from virus, and *Cryptosporidium parvum, Giardia intestinalis, Entamoeba histolytica,* and *Cyclospora cayetanensis* from parasites are common pathogens for diarrhea (22, 111). *DEC, Shigella, Salmonella*, and *Campylobacter* have been commonly associated as bacterial pathogens causing diarrheal disease among children in DC (23). There are many other potential diarrheagenic bacteria (38) and below some common bacteria associated with diarrhea have been mentioned.

2.3.1. Shigella

Shigella is a member of the order enterobacteriales and contain four species (112). These are *S. dysenteriae* (subgroup A with 16 serotypes), *S. flexneri (*subgroup B with 17

serotypes and sub-serotypes), *S. boydii* (subgroup C with 20 serotypes) and *S. sonnei* (subgroup D with 1 serotypes) (112). *S. flexneri* and *S. sonnei* are associated with endemic diarrhea in low- income and high- income countries, respectively whereas *S. dysentriae* serotype 1 is a pandemic and *S. boydii* is uncommon (23). But there are data that show a shift in the dominant species from *S. flexneri* to *S. sonni* in DC that have experienced improvement in socioeconomic conditions (113). *Shigella* was the third leading (next to rotavirus and Cryptosporidium spp.) cause of diarrheal death in UFC in 2015 (9).

2.3.2. Salmonella

Salmonella is belongs to the order Enterobacterales (114). The bacteria are a rod-shaped and Gram-negative facultative anaerobe. *Salmonella enterica* and *Salmonella bongori* are the two species (115, 116). *S. enteric* subdivided in to six subspecies. *Salmonella* strains has more than 50 serogroups and 2500 serotypes (117). Majority of *Salmonella* infections (90%) in human and animals are due to *Salmonella enterica subspecies enterica* (115).

Salmonella strains are grouped into typhoid *Salmonella* and non-typhoid *Salmonella.* The clinical presentation associated with salmonellosis include enteric fever, gastroenteritis, and bacteraemia (115). Non-typhoid *Salmonella* included *S. Choleraesuis, S. Enteritidis, S. Hadar,* and *S. Typhimurium*. Consumption of contaminated food products (beef, eggs, juices, pork, poultry meat, and vegetables) resulted in salmonellosis in human (115-117).

2.3.3. Campylobacter

Campylobacter, *Arcobacter* and *Helicobacter* are the three distinct genera of the family Campylobacteraceae (118). There are 22 species in the genus *Campylobacter* include *C. jejuni, and C. coli* (118). Most infections are caused by *C. jejuni* and *C. coli* in human. Campylobacter species are able to grow at 37-42 ˚C, with low oxygen tension (5% O2, 10% CO2, and 85% N2), optimally at pH 6.5–7.5, and sensitive to sodium chloride (NaCl) (118).

Biochemically, they are positive for oxidase, catalase, nitrate reductase and Voges-Proskauer reaction, but negative for methyl red, urease, indole and dihydrogen sulfide production (119).

Campylobacter species account for most diarrheal cases in human globally (36). It is among predominantely isolated bacteria from infants with diarrhea in DC (120). In East Africa, *Campylobacter* infections have been recorded in both rural and urban areas, particularly among children and the prevalence varies between countries (121). Prevalence of 2% in Sudan, 21% in Malawi, and 21% in South Africa of *Campylobacter* infection in UFC has been reported (121).

2.4. Epidemiology of Diarrheagenic bacteria

Nowadays, bacterial infections linked to one in eight global deaths (https://www.tropmedres.ac/news/bacterial-infections-linked-to-one-in-eight-global-deaths-according-to-gram-study). *Shigella*, *Salmonella*, *Campylobacter*, and *E. coli* were among the 33 bacterial pathogens associated with global mortality (122). Centre for disease control and prevention (CDC) established an epidemiological SP for tracking pathogens and identifying sources for bacterial enteric (intestinal) infections transmitted by food and other routes to decrease the burden of bacterial diarrheal illness by the year 2030 (https://www.cdc.gov/ncezid/dfwed/edeb/index.html). The SP includes *Shigella*, *Salmonella*, *Campylobacter*, and *E. coli*. A systematic review and meta-analysis done to determine enteric pathogens associated with gastroenteritis among UFC in SSA found 31% associated with bacterial pathogens (123). In the study, the bacterial pathogens were *Shigella*, *Salmonella*, *Campylobacter*, and *E. coli*. Many studies have been done elsewhere in the world that showed the role of different enteropathogens in UFC including in Uruguay (124), China (125), Angola (126), and Kenya (127). In these studies DECs were predominant isolates. Different prevalence of DEC (21%-45%) was reported in different studies done elsewhere in the world (128-130).

A study was conducted using research data conducted in the last five decades to determine prevalence of *E. coli* in UFC with diarrhea in Ethiopia (64). In this systematic review and meta-analysis study, there was varied isolation of *E. coli* (20.8% to 62.7%) among the studies (due to variation in detection methods), with 29% pooled isolation. Some old studies characterized few virulence factors for some DEC pathotypes (64). However, currently few data for DEC is becoming available, particularly, since 2022 in Ethiopia (66-68). Another systematic review and meta-analysis reported a 6.6% pooled prevalence of *Shigella* species in Ethiopia (61). A systematic review and meta-analysis on *Campylobacter* in Ethiopia showed pooled prevalence of 10.2% (63). According to the systemic review and meta-analysis study the pooled prevalence estimates of *Salmonella* in stool samples of diarrheic children, diarrheic adults and carriers were 8.72%, 5.68%, and 1.08% respectively (62). Data is available for *Shigella* (61), *Salmonella* (62), and *Campylobacter* (63) unlike for DEC pathotypes in Ethiopia. As a low-income country, Ethiopia has limitation in clinical laboratory capacity and SP and resulted in scarcity of epidemiological data on important pathogens. Thus, it is very important not only to provide new data on epidemiological profile of DEC pathotypes but also updating the epidemiology of other diarrheagenic bacteria in Ethiopia.

2.5. Detection methods for DEC pathotypes

Conventional methods include specific culture methods, antigen detection and microscopic examination that are used to detect bacteria, viruses and parasites in stool samples of patients with diarrhea (131). However conventional methods have limitation such as time consumption, false-negative result, and need personnel expertise (6, 37). Molecular LDMs permit the rapid, simultaneous detection of multiple pathogens with high sensitivity and specificity (37).

The diagnosis of diarrhea associated with *E. coli* is not easy, needs advanced diagnostic tools able to differentiating it from stool flora *E. coli* strains (25). *E. coli* are isolated from clinical samples such as fresh stool specimen, rectal swab, and surgical resection. Among the phenotypic methods are culture, biochemical assays, immunological O/H typing, adherence pattern, fluorescent actin stain, cytotoxicity assays, enzyme immunoassay, Sereny test, bacteriophage typing, and MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight) (25). Multilocus enzyme electrophoresis (MLEE), PCR (multiplex and real-time), microarrays, restriction fragment length polymorphism, pulsed-field gel electrophoresis (PFGE), ribotyping, Multilocus variable-number tandem repeat analysis (MVLA), Multilocus sequence typing (MLST) and whole-genome sequencing (WGS) are genotypic methods used for detection and typing of *E. coli* pathotypes (25, 132).

Microbial typing is important to control outbreaks, tracing cross contamination, recognizing virulent strains, knowing the source and routes of infections, and evaluating control measures (132). Molecular typing methods are used to address local and global epidemiology of pathogenic strains (132). Commonly used molecular typing methods include DNA-based typing methods (Plasmid DNA analysis and analysis of chromosomal DNA), amplification based typing method (PCR-based methods) and sequencing-based typing methods (MLST, SNP analysis, microarrays and WGS) (25).

Serotyping is important for classifying *E. coli,* epidemiological studies, determining virulence, and tracing outbreaks of diseases and sources of infection(133). However, serotyping has limitations, as the antisera generated against each specific O-group may cross-react, and many strains are non-typeable (133, 134). Currently, there are more than 186 O-antigens and 53 H-flagellar antigens of *E. coli* (134). Serotyping for *E. coli* is traditionally based on O-antigen (polysaccharide), K-antigens (capsular), and H- antigens (flagellar) (134). The conventional serotyping method is based on agglutination reactions of the O-antigen with

antisera that are generated in rabbits against each of the O-groups (134). Molecular O-typing and H-typing methods are based on the sequences of genes that encode for O-antigens and FliC, respectively (134). Nowadays, in silico serotyping of *E. coli* from WGS data enabled molecular O-typing and H-typing of *E. coli* (135).

Clermont method, the triplex PCR method allows doing simple and grouping of bacterial pathogens (136). The method is based on the presence or absence of chuA and yjaA and DNA fragment (TspE4.C2) (137). The phylogenetic analysis of *E. coli* based on triplex method (*chuA*, *yjaA* and TspE4.C2) and quadruplex method (*chuA*, *yjaA,* TspE4.C2 and arpA) grouped in to four phylogenetic groups (A, B1, B2, and D) and eight phylogroups, seven (A, B1, B2, C, D, E, F) belongs to *E. coli* and one (clade I) cryptic clade I, respectively (137, 138). The phylogroup distribution of *E. coli* could indicate the variation in disease causing ability, niches and lifestyles (137).

Clonal distribution of pathogenic *E. coli* could be determined with MLST based on DNA sequence variations and nucleotide sequences (seven housekeeping genes) (132). MLST determine the changes occurred in the allelic profile (genotypes) of the seven alleles (the founder) due to point mutation or recombination over time. Genotypes which have allelic profiles that differ from that of the founder at only one of the seven MLST loci, are called single-locus variants (SLVs), at two of the seven loci called double-locus variants, at three of the loci called triple-locus variants, and so on (139).

WGS is a powerful and comprehensive genomic analysis technique that involves determining the complete DNA sequence of an individual's genome (140). The current primary WGS strategies include short-read WGS using the Illumina technology (yields paired-end ~150 bp reads), and long-read WGS using Pacific Biosciences (PacBio) or Oxford Nanopore Technologies (yield 10-100 kb reads) (88). The basic principle for short-read sequencing involves sequencing by synthesis based on enrichment through hybridization, amplification,

or fragmentation and for long-read sequencing works on sequence detection either by synthesis or by electrical voltage change/impedance (140). Employing the short-read WGS using the Illumina HiSeq or NovaSeq platform are cost reasonable. There is a detailed step-by-step protocol used to generate Illumina-based WGS data for clinical isolates of bacterial pathogen including *Escherichia coli (141)*. WGS data analysis using an array of bioinformatics tools allow to elucidate the resistomes, virulomes, mobilomes, clonality, and phylogenies (89).

Multiplex PCR allows the compounding of multiple amplification reactions in a single tube by combining primer pairs (142). The multiplex PCR detected significantly more positive samples with bacterial, viral and parasitic infections as compared to conventional analysis (143). Performing multiplex PCR reactions have several advantages (faster to set up, use less reagents) compare to single-plex, and allow higher output (142). However, optimal conditions (annealing temperature, MgCl2 concentration, dNTP concentration, pH, elongation time, primer concentration, and polymerase) for each primer pair with their specific target (compromised) is the challenge (142). Multiplex PCR could detect pathogens such as DEC that are undetectable by conventional methods (143). Stool PCR testing is more sensitive than standard culture, has a short turnaround time, and can simultaneously detect multiple organisms, and likely to replace the conventional methods (144).

2.6. Management of childhood diarrhea

Diarrhea can be diagnosed based on clinical evaluation (assessment of the severity of illness), and laboratory evaluation (stool analysis and culture) (93, 111). Oral rehydration salts, nutritional support, and oral zinc therapy are used to treat acute diarrhea of children (6). In most case antimicrobials is not the primary management for acute diarrhea in children due to self-recovery nature of the disease, misdiagnosis as viral caused, adverse effect and risk of AMR (145). However there is a need to use antimicrobials in cases such as severe illness,

severe traveller's diarrhea, and prolonged diarrhea (145). Azithromycin, ciprofloxacin, levofloxacin, and rifaximin are antimicrobials mostly prescribed for traveler's diarrhea due to bacterial enteropathogens (146). Ciprofloxacin, cefixime, ceftriaxone, azithromycin and sulfamethoxazole/trimethoprim could be used for children with acute invasive bacterial or dysentery (severely ill) or immunocompromised patients (145). Diarrheal diseases can be prevented through clean water supply, sanitation and hygiene interventions as well as vaccination (6). Vaccine for rotavirus such as Rotarix and Rotavac are produced (6). Vaccines against bacterial agents are on progress such as ETVAX against ETEC and TSWC against *Shigella* (6).

2.7. Antimicrobial resistance (AMR)

Different options have been suggested for treating the infections caused by diverse pathogenic *E. coli* (147). Infections due to Enterobacterales could be treated by ß-lactam drugs, aminoglycosides and fluoroquinolones (77, 148). However, bacterial pathogens that are resistant to ß-lactam antimicrobials (penicillin, cephalosporin, carbapenem and monobactam) become a treat to global health and resulted in health crises (149). AMR caused loss of many people, high expenditure, and hinders the control of infectious diseases (150). It creates a huge clinical (high morbidity and mortality) and financial (increase healthcare costs) burden globally (151).

AMR mechanisms are classified based on biochemical route involved in resistance and genetics (152). The biochemical aspect includes modifications of the antimicrobial molecule, decrease permeability and efflux, changes and/or bypass of target sites whereas the genetic base includes resistance via mutation (intrinsic or innate) and HGT (acquired) (152). Bacterial resistance that occur via acquisition of exogenous genes is by mobile genetic elements (MGE) such as plasmid, integrons, transposons, and bacteriophages and a combinations of these mechanisms (40). All Pathogenic, environmental and commensal

bacterial species can be a reservoir of AMR genes (153). Intestinal microbiota acquire AMR genes from exogenous resistant bacteria or become resistant through selection of antimicrobial-resistant mutants (154). Misuse of antimicrobials, lack of clean water and sanitation, inadequate IPC contributed to AMR (150).

ß-lactamases conferring resistance to third generation cephalosporins (mainly due to ESBL), cephamycins (AmpC) and carbapenems (carbapenemases) have been documented in several studies (147). Resistance to ß-lactams among Enterobacterales occur through different mechanisms including inactivation of the antimicrobials by ß-lactamases (148). ß-lactamases are the predominant ß-lactam drug resistance mechanism in Enterobacterales (148). Nowadays ß-lactamase producing Enterobacterales are the most serious and critical threats to the world (148). HGT mediate the transfer of ß-lactamases in Enterobacterales (155). ß-lactamases (include penicillinases, extended spectrum cephalosporinase, carbapenemase, and oxacillinase) are enzymes that hydrolyze β-lactam antimicrobials (155). There are many different ß-lactamases that are classified in to different groups based on amino acid sequence, substrate and inhibitor profile and variation in their active sites (use serine or require divalent zinc ions) for hydrolysis (155). Based on amino acid sequence (Ambler classification), β-lactamases are grouped in to A, B, C, and D whereas the Bush-Jacoby classification divides (biochemical function) them in to four groups (with subgroups) (Figure 4) (156). Ambler class A (penicillinase) enzymes hydrolyze ampicillin, and 1st, 2nd, and 3rd generation of cephalosporins, and monobactams, and inhibited by ß-lactamase inhibitors (148, 157). Class A β-lactamses include penicillinase (TEM-1 and SHV-1), ESBLs (e.g. CTX-M) and carbapenemases (e.g. KPC). Ambler class B enzymes could hydrolyze carbapenems (157). Ambler class C ß-lactamase (AmpC enzymes) hydrolyze ß-lactamase inhibitors, all penicillins, cephamycins, 1st, 2nd, and 3rd generations, but are sensitive to cefepime and carbapenems (148, 157). Ambler class D ß- lactamase contains varied enzymes

that can to be grouped to other classes (157) and hydrolyze oxacillin; poorly inhibited by clavulonic acid (152).

ESBL are enzymes that hydrolyse penicillins, 1st, 2nd and 3rd generation of cephalosporins and aztreonam but inhibited by ß-lactamase inhibitors (clavulanic acid, tazobactam or sulbactam) (157). Most common ESBLs are grouped in class A (155). There are many types of ESBLs include SHV (except SHV-1), TEM (except TEM-1 and TEM-2), CTX-M, OXA (158). TEM-types, SHV-types (sulfhydryl variable), and CTX-M-types (hydrolytic activity against cefotaxime) are clinically important types of ESBLs (157, 158). Currently there are more than 183 TEM-types with most common TEM-type found in *E. coli* (148) and more than 178 SHV varieties, mostly found in Enterobacterales (148, 157). The term CTX reflects the potent hydrolytic activity of CTX-M ESBL against cefotaxime (158). Based on their amino acid sequence there are more than 128 CTX-M- types with five classes; CTX-M-1, CTX-M-2, CTX-8, CTX-M-9, and CTX-M-25, and found in different Enterobacterales (157, 158).

Carbapenems were used to treat infections caused by ESBLs producing bacteria or cephalosporin resistant infections (155). Among the global health concerns, the emergence and spread of carbapenem resitant *E. coli* strains is in the frontline (159). Carbapenem-resistant- Enterobacterales are able to hydrolyse any carbapenem antimicrobial (160). Carbapenemases are ß-lactamases that hydrolyze penicillins, cephalosporins, monobactam and carbapenems (159). Based on Ambler classes, carbapenemase class A and D enzymes need a serine-based hydrolytic mechanism whereas class B enzymes need zinc ions for catalytic activity (161). There is a rare detection of CMY-10 (hydrolyze imipenem) grouped as carbapenemase class C (161). Class A contain both chromosomally encoded (IMI, SME and NMC), and plasmid encoded (KPC and GES) carbapenemase (161). Class B carbapenemases include NDM, VIM, IMP, and SIM (155, 161). Class D carbapenemases

(also called OXA β-lactamase) are plasmid-encoded β-lactamase, serine β-lactamase, hydrolyze oxacillin, and poorly inhibited by clavulanic acid (155, 161).

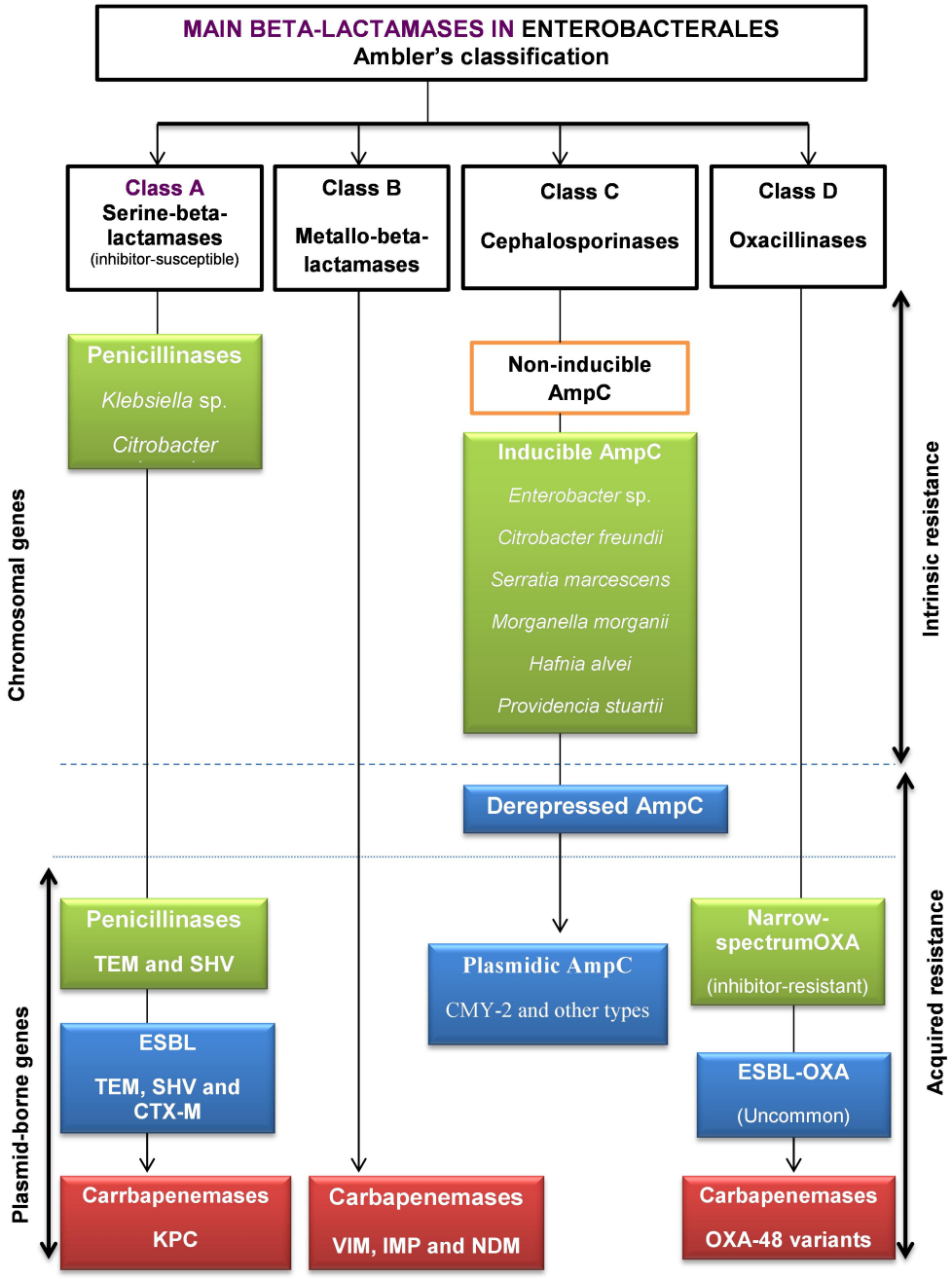


Figure 4. Common ß-lactamases in Enterobacterales (156). The color of the boxes indicates the enzyme-conferred resistance (hydrolysis-spectrum). Green represents the enzymes that hydrolysis the penicillin and first generation cephalosporin, blue for the second, third and fourth generation cephalosporin, and red for the carbapenem and other beta-lactam.

The epidemiology of ESBLs is determined in different regions of the world. Different ESBLs are reported globally, including in Europe (CTX-M-3, CTX-M-15, and SHV-5),

South and Central America (SHV-2 and SHV-5), North America (TEM-10, TEM-12, and TEM-26), Africa (CTX-15 and TEM-63), Australia (SHV), and Asia (CTX-M-15 and SHV) (157). Imipenemase metallo-β-lactamase (IMP) and Verona integron-encoded metallo-β-lactamase (VIM) are also reported as an emergence of metallo-β-lactamases in different countries (160). ESBL-related genes in Bangladesh (162) and an increasing number of cases with enteric bacteria per year in Norway, with predominant NDM and OXA-48-like carbapenemase variants (163) were reported. In another study also carbapenem-resistant DEC pathotypes positive for carbapenemase genes such as blaNDM-1, blaKPC-2, blaCTXM-65, and blaTEM-1 were identified (164). Globally, there is limited data on molecular epidemiology of β-lactamase genes in DEC pathotypes from under-five children. The present study aimed to determine molecular epidemiology of ESBL and carabapenemase genes in DEC pathotypes isolated from UFC in Addis Ababa and Debre Berhan, Ethiopia.

Chapter three

3. Methodology

3.1. Study design

A health facility based cross sectional study design was employed. In addition, some non-diarrheic controls were used for comparing the role of DEC to diarrhea in UFC. Some virulent genes of DEC pathotypes are carried by pathotypes that didn't cause diarrhea (25). And the detection of DEC pathotypes in this study was based on virulence genes (VG); for this we included children without diarrhea as controls in the study.

3.2. Study area and period

The study was conducted in two different areas of central Ethiopia, Addis Ababa and Debre Berhan (Figure 5) from December 2020 to August 2021. Addis Ababa is a capital city of Ethiopia; headquarter of African Union and focal point for many international organizations. Administratively, Addis Ababa is divided into 11 sub-cities. It has 11 government hospitals and 92 health centers (6-13 in each sub cities). The recent population estimate for Addis Ababa is 5,227,794 (https://worldpopulationreview.com/world-cities/addis-ababa-population). The study sites from Addis Ababa were Tekelehaymanot health center (Lideta Sub-city), Kazanchis health center (Kirkos Sub-city) and Arada health center (Arada Sub-city). The Sub-cities and health centers from each sub-city were randomly selected using lottery method to be included in the study. Debre Berhan is located in the Semen Shewa Zone of the Amhara Region, about 130 kilometers North East of Addis Ababa. It is the administrative center of the Semen Shewa Zone of the Amhara Region. The total population estimate is 95, 000 (https://edaethiopia.org/index.php/program-offices/8-debrebirhan). There are 2 public and 1 private hospitals, 3 health centers, 16 private clinics, and 5 health posts in the Town. The health facilities involved from Debre Berhan were Ayer Tena health Center and kebele 04 Health Center. In these study areas, previous studies attempted to show the

presence of presumptive *E. coli* isolates among diarrheic children. However there was limitation in characterizing the epidemiology of DEC pathotypes in the study areas as well as in other parts of the country.

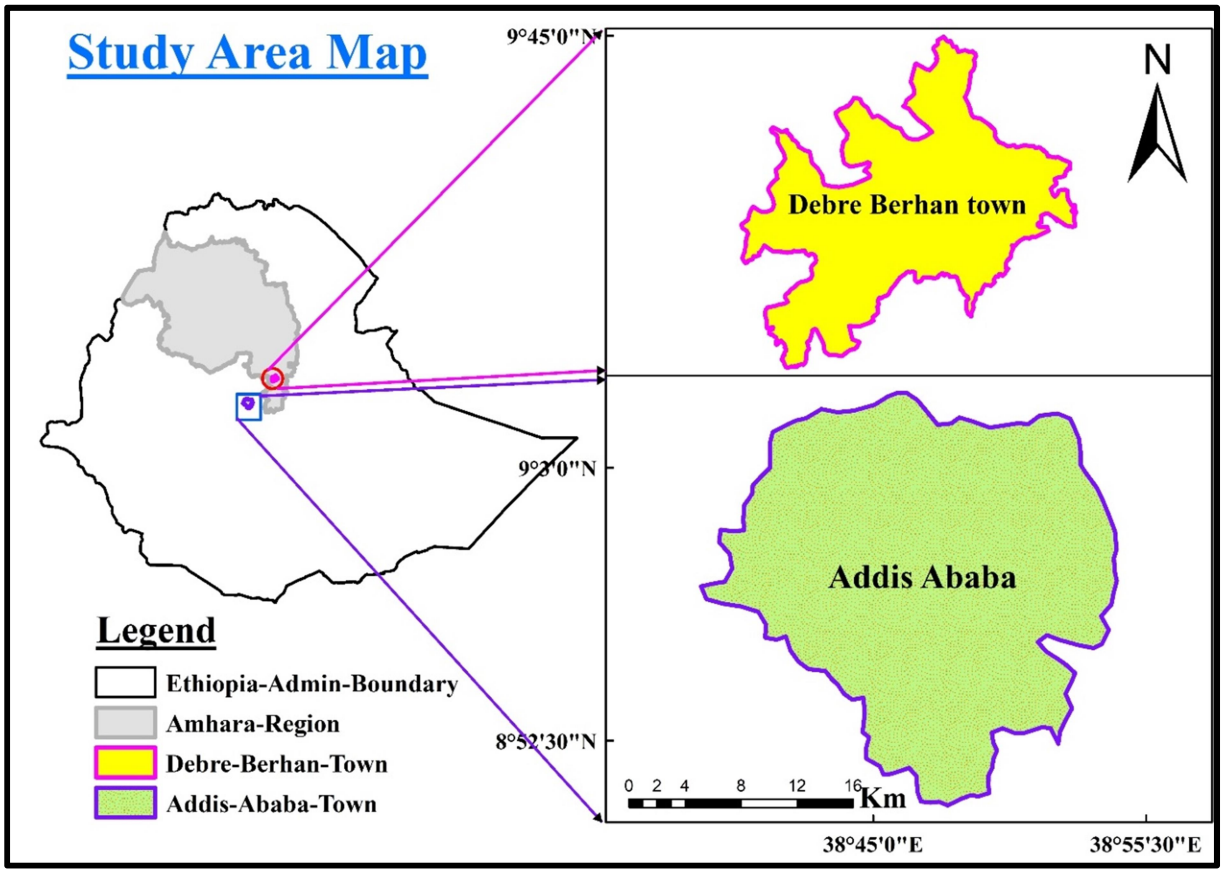


Figure 5. Map of the study areas, Addis Ababa and Debre Berhan, Ethiopia.

3.3. Study population

The study population were all children with age less than 5 (UFC) who visited the under-five out-patient department (OPD) of the selected health facilities.

3.3.1. Inclusion criteria

Children with age of five and below, who come to under-five OPD of the health facility due to diarrhea, and who have not received antimicrobials were included in the study. Some non-diarrheic children who came to health facility due to cases other than diarrhea were also included as controls.

1.1.1. Exclusion criteria

Children who have received any antimicrobials in the last two weeks before the beginning of the diarrhea and prior to data collection, and whose parents/ guardians that were not willing to consent were excluded from the study.

3.4. Variables

3.4.1. Dependent variables

DEC pathotypes and AMR

3.4.2. Independent variables

Socio-demographic variables, clinical features, feeding practice, source of drinking water and resistance genes.

3.5. Sample size determination

The sample size for the study was determined based on prevalence of bacterial enteropathogens of previous studies done in in Addis Ababa (P= 37.2%) (165) and Debre Berhan (P=41.6%) (75), and using statistical formula (166), precision =5%, and 95% confidence level. Average prevalence and 5% contingency was taken. A total of 476 UFC, 391 diarrheic and 85 non-diarrheic UFC were enrolled.

$n = \frac{Z^2 P(1-P)}{d^2}$, n=sample size, Z= level of confidence, P=proportion, d=degree of precision

The sample size was allocated by proportion for each study area based on the estimated target population of Debre Berhan town and selected sub-cities of Addis Ababa. For rough estimation, target population of the selected sub-cities of Addis Ababa and Debre Berhan have been used from the Ethiopian 2007 census data (http://www.statsethiopia.gov.et/census-2007-2/). Finally, 277 for Addis Ababa (226 diarrheic and 51 non-diarrheic) and 199 for Debre Berhan (165 diarrheic and 34 non-diarrheic) were allocated. Sample size for each

health center in the study areas was taken based on the patient' flow presented during the data collection.

3.6. Sampling procedure and data collections

Three sub-cities (Lideta, Kirkos and Arada) and one health center from each selected sub-city were randomly selected using lottery method from Addis Ababa. A total of three health centers namely Tekelehaymanot health center from Lideta Sub-city, Kazanchis health center from Kirkos Sub-city, and Arada health center from Arada Sub-city were selected. Two health centers (kebele 04 and Ayer Tena health center) from Debre Berhan were included in the study.

Convenience sampling method was applied to enrol the study participants in paediatrics department of each selected health facilities. UFC with diarrhea attending the under-five OPD during the data collection period was enrolled for the study by trained nurses. In addition to diarrheic children, non-diarrheic children that came to the same department due to different medical cases other than diarrhea were enrolled as control for the study. The trained nurse informed the parent/guardians about the objective of the study, potential risks and benefits of participation and then asked to obtain a written consent. Diarrhea was defined as the passage of three or more liquid stools within 24 hours, or any number (more than three times) of loose stools accompanied with mucous within 24 hours as per WHO definition (91). The diarrhea was categorized as acute watery diarrhea (lasts several hours or days, and includes cholera), acute bloody diarrhea (also called dysentery), and persistent or chronic diarrhea (lasts 14 days or longer) (91).

The socio-demographic, clinical features and other factors obtained from the parents/guardians of every child were collected using standardized structured questionnaires following medical examination. The socio-demographic data including age, sex, and family

income were collected. Clinical features including time when illness started, duration of diarrhea, stool frequency, stool consistency, dehydration status, fever, vomiting, nausea, thirst, abdominal distension, and previous treatment were also collected. In addition, feeding practice, presence of animal in the house and source of drinking water were included in the questionnaire. Study participants were given their own unique code to ensure confidentiality for the study participants. Each questionnaire was labelled with the participant's code and not personal identifiers.

3.7. Specimen collection, handling and transportation

Laboratory technicians were provided refresher training on how to collect the stool samples from study participants (Annex 1). Before collection, important instructions and information was given to the parents for having supports during the actual stool collection. For the children with age older than 1 year (or child who can sit on potty), stool specimen was collected by placing plastic wrap over the rim of a potty. For infants (with age less than 1 year) with diarrhea or for children who were not able to use potty, stool samples were collected using disposable diaper by putting the plastic side next to the skin. Then, using two small wooden sticks at least one small teaspoon (5 g or 5 ml) of faeces (containing blood, mucus or pus) was transferred to Cairy-Blair transport media from the plastic wrap or diaper and stored in cold box with ice-bag until it is transferred to the laboratory to be processed. Care was taken during stool collection to avoid urine, soil, or water mixing with the stool. In case where a child was unable to produce stool, a rectal swabs was taken using sterile cotton swabs. The cotton swabs was inserted in the rectum sphincter about 1-2 cm and rotated and removed and then after observing physically it was placed in the Cary-Blair's transport medium (167, 168). When cholera was suspected about 1 ml of specimen was transferred into 10 ml of sterile alkaline peptone water.

The collected stool samples were labelled properly and transported with cold-chain to Microbiology Laboratory of Tikur Anbessa Specialized Hospital (TASH), Addis Ababa University (AAU) (for the Addis Ababa site) and Microbiology Laboratory of Debre Berhan Referral Hospital (for the Debre Berhan site) for phenotypic analysis (including bacterial isolation and identification, and antimicrobial susceptibility test). The phenotypic analysis (bacterial isolation and identification, and antimicrobial susceptibility test) was conducted in Microbiology laboratory of Tikur Anbessa Specialized Hospital and Debre Berhan Referral Hospital. PCR assay was done at Armauer Hansen Research Institute. The WGS analysis was carried out in Eurofins Genomics laboratory (Germany) in collaboration with Orebro University, Sweden.

3.8. Laboratory investigations

3.8.1. Bacterial isolation and identification

Selenite F enrichment broth (1-2 gm of stool sample or 10% by volume) was used for *Salmonella* (Annex 2). Faecal suspension was prepared by taking the wooded sticks (cotton swabs) from the transport media containing the stool sample and rinsed thoroughly in 1 ml of saline. For the liquid stool sample, saline was not used. Then, stool sample was inoculated onto MacConkey and Xylose-Lysine-deoxycholate (XLD) media, and incubated at 37 $^{\circ}$C for 18 -24 hours for the isolation of *E. coli, Shigella* and *Salmonella*. When cholera was suspected several loopfuls of the peptone water culture (taken from the surface) was subcultured on thiosulphate citrate bile-salt sucrose (TCBS) agar and incubated aerobically at 35–37 °C overnight (168, 169). If large yellow and green colonies were grown, oxidase test was conducted. For the oxidase positive, the isolates were tested for salt tolerance with different concentration of NaCl supplemented in nutrient broth (0%, and 8%). If the colony was yellow on TCBS and there was growth in 0% and no growth at 8% NaCl-nutrient broth, then it was taken as presumptive *Vibrio cholerae*. If the colony was green on TCBS and there

was growth in NaCl concentrations of 8%, and no growth in 0%, this was considered presumptive for *Vibrio parahaemolyticus*. On TCBS *V. cholera* produced yellow shiny colonies with yellow in the medium but after prolonged incubation (≥ 48 hrs) turns to green (168, 169). On XLD *Shigellae* form pink-red colonies (did not ferment xylose, lactose, sucrose except *S. sonnei*), *Salmonallae* form similarly pink-red colonies (but they ferment xylose with gas production), hydrogen sulphide (H2S) producing *Salmonella* forms red colonies with black centers, and *E. coli* produces yellow colonies (due to carbohydrate fermentation, some strains may be non-lactose fermenters). On MacConkey agar *Shigellae*, *Salmonellae* and other non-lactose fermenting organisms, produced colourless colonies. *E. coli* and other lactose fermenting organisms produced pink colonies on MacConkey agar. From the pure culture, representative colonies (3-5 colonies) were taken and series of conventional biochemical test were done.

The conventional biochemical tests including oxidase test, triple sugar iron agar, urea, lysine, motility, indol , manitol, and Simon's citrate were used to identify bacterial isolates (Annex 3) (167, 168). The results were interpreted based on their biochemical result and positive control strains were used for confirmation of the biochemical test results. Gram staining and microscopy examination was also done to confirm the Gram negative bacteria. Randomly selected 170 *E. coli* isolates (40%, 170/428) were sent to Sweden for MALDI-TOF mass spectrometry analysis and were confirmed *E. coli* isolates. Confirmed *E. coli* isolates from each cultured plate were stored at -80 °C in brain heart infusion broth containing 16% (v/v) glycerol. Other diarrheagenic bacterial isolates were also preserved in the same manner for further analysis. For the *Campylobacter*, stool samples were preserved at -80 °C to detect it using PCR assay.

3.8.2. DNA (Deoxyribonucleic acid) extraction for PCR-based detection

3.8.2.1. For detection of *Campylobacter*

Stool samples (after thawing) were pre-treated according to previously described procedure (170). Briefly, 20 mg frozen sample was taken in to a sterile tube containing 5 ml of ice-cold phosphate-buffered saline (PBS). After vortexing, it was centrifuged at 500 rpm for 4 min. The supernatant was transferred to new 5 ml sterile tube. The pellets were resuspeneded in 5 ml PBS buffer, mixed thoroughly and centrifuged at 500 rpm for 4 min and repeated twice. All the supernatants were centrifuged at 9000 rpm for 5 min. Finally, the cell pellet was resuspended in molecular grade water for extraction (Annex 4).

A previously used DNA extraction protocol (171) called TE boil extraction method (T method) with slight modification was used. The pellet was suspended in 200 µL TE buffer (10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA), and the mixture was briefly mixed on a vortex mixer. The suspension was boiled at 94 °C for 10 min in a dry block incubator (Thermo Fisher scientific, California) and placed in a freezer at −20 °C for 10 minutes, then placed at room temperature for one minute and centrifuged at 14,000 g for 5 min. Then, 100 µL of the supernatant was transferred into nuclease free Eppendorf tube and stored at −20 °C until use for PCR assay.

3.8.2.2. For detection of DEC pathotypes

Genomic DNA extraction was performed using boiling method as describe by Rúgeles *et al* (172). Briefly an overnight liquid culture suspension of DEC isolates was boiled at 94 °C for 10 min in a dry block incubator (Thermo-fisher scientific, California) and placed in a freezer at −20 °C for 10 minutes, then placed at room temperature for one minute and centrifuged at 14,000 g for 5 min. Then, 100 µL of the supernatant was transferred into nuclease free Eppendorf tube and stored at −20 °C until use for PCR assay (Annex 4). The purity of the extracted DNA was assessed by Nanodrop.

3.8.3. PCR assay for detection of DEC virulence genes

DEC identification was performed using multiplex PCR assay following a previously described procedure (172). Briefly, detection of DEC VGs were performed in three separate PCR reactions (multiplex) using the primer sequences shown in Table 1. The three PCR reactions were set based on the base pair sizes of the target genes. PCR reaction 1 contained primer mix 1 (M-1) for detection of EPEC and STEC targeting VGs *bfp, eae* and *stx.* PCR reaction 2 contained primer mix 2 (M-2) for ETEC and EIEC targeting VGs *lt*, *st*, *virF* and *ipaH*. PCR reaction *3* contained primer mix 3 (M-3) for detection of EAEC and DAEC targeting VGs *aggR, astA* and *daaF*. Multiplex PCR reaction 1 and 3 were carried out with 20 µl reaction mixture containing 10 µl Platinum™ II Hot-Start PCR Master Mix (2X) (Thermo Fisher Scientific), 1.2 µl of forward primer mix (0.4 µl each primer), 1.2 µl of reverse primer mix (0.4 µl each primer), 1 µl of template DNA, and 6.6 µl of molecular grade water (Annex 5). Whereas multiplex PCR reaction 2 was also carried out with 20 µl reaction mixture containing 10 µl Platinum™ II Hot-Start PCR Master Mix (2X) (Thermo Fisher Scientific), 1.6 µl of forward primer mix (0.4 µl each primer), 1.6 µl of reverse primer mix (0.4 µl each primer), 1 µl of template DNA, and 5.6 µl of molecular grade water. The PCR thermal conditions were set with an initial denaturation of 94 °C for 2 min, followed by 35 cycles of 92 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec and a final extension at 72 °C for 5 min in a PCR machine (Biometra TRIO Thermal Cycler, Analytik Jena). PCR products were separated using 1.7% (w/v) agarose gel in Tris Borate EDTA buffer (pH 8.2) stained with ethidium bromide (10 µg/ml) and visualized with UV transilluminator system (Bio-Rad) (Annex 6). Extracted DNA samples positive for the target genes of the tested DEC pathotypes (some ATCC strains and some previously whole genome sequenced strains) as positive control and CCUG 24T *E. coli* as negative control were used during the PCR assay.

3.8.4. PCR assay for detection of Campylobacter

The PCR assay for detection of *Campylobacter* species was done using the target genes, aspartokinase (*asp*), specific of *C. coli*, the hipuricase gene (*hipO*), specific of *C. jejuni* (173), and a universal 16S rRNA for the genus *Campylobacter* (174). The primer sequences are presented in Table 1. A previously used procedure for PCR assay with slight change was used (174). Single-plex PCR assay for detection of the 16S rRNA target gene was done. For 16S rRNA positive samples, duplex PCR assay for detection *asp* and *hipO* genes was performed. PCR reaction was carried out with 25 µl reaction mixture containing 12.5 µl Platinum™ II Hot-Start PCR Master Mix (2X) (ThermoFisher Scientific), 0.8 µl of forward primer mix (0.4 µl each primer), 0.8 µl of reverse primer mix (0.4 µl each primer), 1 µl of template DNA, and 9.9 µl of molecular grade water (Annex 5). The PCR thermal conditions were set at 94 °C for 3 min to the initial denaturation followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 s, and extension at 72 °C for 30 sec, with a final extension at 72 °C for 5 min. Extracted DNA samples of positive control (*C. jejuni* ATCC 3329) and previously identified known *Campylobacter* species (*C. jejuni* and *C. coli*) were used as positive control during the optimization of the PCR assay. The PCR products were analyzed by gel electrophoresis on 1.7% (w/v) agarose gel in Tris Borate EDTA buffer (pH 8.2) stained with ethidium bromide (10 µg/ml) and visualized with UV transilluminator system (Bio-Rad). Gels were run for 1 hour at 120 V and visualized with UV transilluminator system (Bio-Rad). The size of the products was confirmed by comparison with the molecular marker GeneRuler 100-bp DNA Ladder.

3.8.5. Antimicrobial susceptibility tests

Antimicrobial susceptibility test (Annex 7) for all DEC pathotypes was carried out using the Kirby–Bauer disc diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI) (175). The antimicrobials tested were ampicillin (AM 10 µg),

ceftazidime (CAZ 30 µg), cefotaxime (CTX 30 µg), ertapenem (ETP 10 µg), meropenem (MEM 30 µg), amoxacillin-clavulunate (AMC 20/10 µg), gentamicin (GM 10 µg), teteracycline (T 30 µg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75 µg), ciprofloxacin (CIP 5 µg), chloramphenicol (C 30 µg), and cefepime (FEP 30 µg). All antimicrobial discs were from Oxoid Ltd, Basingstoke, United Kingdom. The results were interpreted using CLSI guidelines (175). *E. coli* ATCC 25922 was used as reference strain. Multidrug resistance (MDR) was defined as non-susceptible to at least one agent in three or more antimicrobial categories (176).

3.8.6. Phenotypic detection of ESBLs and Carbapenemases production

Isolates resistant to cefotaxime (30 µg) and ceftazidime (30 µg) were tested for extended spectrum ß-lactamase production using the combination disk method (175). *Klebsiella pneumoniae* ATCC 700603 (ESBLs positive) and *E. coli* ATCC 25922 (ESBLs negative) were used for quality control. Briefly, the combination disk method was done on Mueller Hinton agar by using ceftazidime (CAZ) and cefotaxime (CTX) alone and with ceftazidime + clavulanic acid (CAZ/CLA) and cefotaxime + clavulanic acid (CTX/CLA) as recommended by CLSI 2020 (175). The production of carbapenemase was tested using a modified carbapenem inactivation method (175). *K. pneumonia* ATCC BAA-1705 (carbapenemase positive) and *K. pneumonia* ATCC BAA-1706 (carbapenemase negative) were used for quality control. Briefly; 1 µL loopful colony of test isolate from overnight blood agar plate was suspended in 2 mL of nutrient broth (Oxoid UK) and 10 µg of meropenem disk was fully immersed to the nutrient broth. The tubes were incubated at 37 °C in ambient air without agitation for 4 hours ± 15 min. Subsequently, the meropenem disks were removed using a 10 µL inoculation loop and applied to Mueller-Hinton agar plates (Oxoid, UK) freshly inoculated with a 0.5 McFarland suspension of *E. coli* ATCC 25922.

The results from the ESBL confirmation and carbapenemase production were interpreted according to CLSI guidelines (175).

3.8.7. Detections of ESBL and Carbapenemase Genes

Conventional PCR assay for detection of different ESBLs (*bla*$_{\text{CTX-M}}$, *bla*$_{TEM}$, and *bla*$_{\text{SHV}}$) and carbapenemase (*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, and *bla*$_{\text{OXA-48}}$) genes was performed in two separate PCR reactions. Reaction one contained the three ESBL genes (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$) and reaction two contained the three carbapenemase genes (*bla*$_{\text{KPC}}$, bla$_{\text{NDM}}$, and *bla*$_{OXA-48}$) in separate tube. The sets of specific primers used for the detection of ESBL genes (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, and *bla*$_{\text{SHV}}$) and carbapenemase genes (*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, and *bla*$_{OXA-48}$) are shown in Table 1.

The PCR assay was performed in a final volume of 20 µL containing 10µLPlatinum™ II Hot-Start PCR Master Mix (2X) (ThermoFisher Scientific), 1.2 µL of each primer (0.2 µM), 1.5 µL of template DNA, and 6.1µL of nuclease-free water. For both reactions, the PCR thermal conditions were set with initial denaturation at 94 °C for 15 minutes followed by 35 cycles each of denaturation at 92 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec, and final extension at 72 °C for 5 minutes. PCR products were separated on 1.7% (w/v) agarose gel in Tris Borate EDTA (Ethylene diamine tetra acetic acid) buffer (pH 8.2) stained with ethidium bromide (10 µg/ml) and visualized with UV transilluminator system, GelDoc (Bio-Rad). *K. pneumoniae* ATCC 700603 (ESBLs positive) and *K. pneumonia* ATCC BAA-1705 (carbapenemase positive) were used during the optimization of the PCR assay.

Table 1. Target genes for PCR amplification, primers and their characteristics.

PCR reaction mix	Strains	Target genes	Primer sequence	Amplicon size (bp)	Ref.
M-1	EPEC/ STEC	*eae*	F: 5'-CTGAACGGCGATTACGCGAA-3' R: 5'-CGAGACGATACGATCCAG-3'	917	(172)
	EPEC	*bfp*	F: 5'-AATGGTGCTTGCGCTTGCTGC-3' R: 5'-GCCGCTTTATCCAACCTGGTA-3'	326	
	STEC/EHEC	*stx*	F:5′-GAGCGAAATAATTTATATGTG-3′ R: 5′-TGATGATGGCAATTCAGTAT-3′	518	
M-2	ETEC	*lt*	F: 5'-GCACACGGAGCTCCTCAGTC-3' R: 5'-TCCTTCATCCTTTCAATGGCTTT-3'	218	
		st	F: 5'-GCTAAACCAGTAGAG(C)TCTTCAAAA-3' R: 5'-CCCGGTACAG(A)GCAGGATTACAACA-3'	147	
	EIEC	*virF*	F: 5'-AGCTCAGGCAATGAAACTTTGAC-3' R: 5'-TGGGCTTGATATTCCGATAAGTC-3'	618	
		ipaH	F: 5'-CTCGGCACGTTTTAATAGTCTGG-3' R: 5'-GTGGAGAGCTGAAGTTTCTCTGC-3'	993	
M-3	EAEC	*aggR*	F: 5'-GTATACACAAAAGAAGGAAGC-3' R: 5'-ACAGAATCGTCAGCATCAGC-3'	254	
		astA	F:5'-CCATCAACACAGTATATCCGA-3' R: 5'-GGTCGCGAGTGACGGCTTTGT-3'	111	(177)
	DAEC	*daaF*	F: 5'-GAACGTTGGTTAATGTGGGGTAA-3' R: 5'-TATTCACCGGTCGGTTATCAGT-3'	542	(172)
Campylobacter	Campy	16S rRNA	F:5'- AGTTGGAAACGACTGCTAATACTC-3' R:5'- TTAATGGTTAAGCCATTAGATTTCAC-3'	450	(174)
	C. jejuni	*hipO*	F:5'-GACTTCGTGCAGATATGGATGCTT-3' R:5'-GCTATAACTATCCGAAGAAGCCATCA-3'	344	(173)
	C. coli	*asp*	F: 5'-GGTATGATTTCTACAAAGCGAG-3' R: 5'-ATAAAAGACTATCGTCGCGTG-3'	500	
AMR genes	ESBL genes	bla_{CTX-M}	F: 5'-CGCTGTTGTTAGGAAGTGTG-3' R: 5'GGCTGGGTGAAGTAAGTGAC-3'	754	(178)
		bla_{TEM}	F: 5'-TTTCGTGTCGCCCTTATTCC-3' R: 5'-ATCGTTGTCAGAAGTAAGTTGG-3'	403	(179)
		bla_{SHV}	F: 5'-CGCCTGTGTATTATCTCCCT-3' R: 5'-CGAGTAGTCCACCAGATCCT-3'	293	
	Carba genes	bla_{NDM}	F: 5'-GGTTTGGCGATCTGGTTTTC-3' R: 5'-CGGAATGGCTCATCACGATC-3'	621	(180)
		bla_{KPC}	F: 5'-CGTCTAGTTCTGCTGTCTTG-3' R: 5'-CTTGTCATCCTTGTTAGGCG-3'	798	
		bla_{OXA-48}	F: 5'-TGTTTTTGGTGGCATCGAT-3' R: 5'-GTAAMRATGCTTGGTTCGC-3'	177	(181)

Abbreviations: bp, base pair; PCR, Polymerase Chain Reaction; EPEC, Enteropathogenic *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli*; EAEC, Enteroaggregative *Escherichia coli*; STEC, Shiga-toxin producing *Escherichia coli*; DEAE, Diffusely adherent *Escherichia coli; ESBL,* Extended spectrum beta lactamase; Carba, Carbapenemase.

3.8.8. Whole Genome Sequence (WGS)

3.8.8.1. DNA extraction for WGS analysis

Quick-DNA™ Miniprep Plus Kit (Zymo-Spin™ Technology) was used to extract DNA for WGS (Annex 8). Briefly, bacterial samples (*DEC* isolates) of randomly selected 28 DEC pathotypes were shipped to Sweden. Resuspension of samples (1-5 x 10^6 cells in 200 µl) was done using DNA Elution Buffer. Then following the protocol, DNA was eluted with nuclease free water, assessed for integrity and quality, and stored at -20 °C for use. The DNA extracts quality were assessed using Nanodrop (OD 260/280 ≥ 1.8-2.0 and an OD 260/230 ≥ 1. 8-2.2). The integrity of the gDNA was then assessed on 1% (w/v) agarose gel in Tris Borate EDTA buffer (pH 8.2) stained with ethidium bromide (10 µg/ml) and visualized with UV transilluminator system (Bio-Rad). Finally, at least 1 µg of DNA per sample with concentration greater than 10 ng/µl, pure (OD 260/280≥1.8; OD 260/230≥1.9), high molecular RNA-free DNA, was dissolved in RNase-, DNase-and protease-free Tris-HCl buffer (PH 8.0-8.5) prepared for WGS analysis. The extracted DNA samples were shipped to Eurofins Genomics Europe Sequencing GmbH, Germany to conduct the WGS. The WGS was done by Genome Sequencer Illumina NovaSeq 6000 which supports to generate up to 250 bp paired end reads.

3.8.8.2. Genome analysis

After WGS, quality assessment and trimming of the raw reads was done using Galaxy database (https://usegalaxy.org) and using PATRIC, the Fastq utility (Annex 9). Genomic assembly and annotation was done using PATRIC bioinformatics platform (https://www.patricbrc.org). De novo assemblies were performed with Spades (version 3.5.0) (182). *In Silico* Clermont Phylotyper used to identify phylogroups (183) which is a web based application (http://clermontyping.iame-research.center/). MLST sequence types (STs) were analysed using MLST 2.0 database hosted by the Center for Genomic Epidemiology (CGE)

(https://cge.food.dtu.dk/services/MLST/). SerotypeFinder 2.0 was used to identify serotype of the DEC pathotypes (https://cge.food.dtu.dk/services/SerotypeFinder/). VG and AMR genes (ARG) were identified using VirulenceFinder 2.0 (https://cge.food.dtu.dk/services/VirulenceFinder/) and ResFinder 4.1 (https://cge.food.dtu.dk/services/ResFinder/). In addition, the antibiotic-resistance database (CARD, https://card.mcmaster.ca/) was used to identify ARGs. The coding sequence of the strains were analysed using BLASTP against the virulence factors genes of *E. coli* listed in Virulence Factor Data Base, VFDB (184). MGE were identified on CGE website (https://cge.food.dtu.dk/services/MobileElementFinder/). To compare VG and ARG between DEC hybrid strains and other DEC pathotypes short-reads of publicly available DEC pathotypes were downloaded from enterobase (http://enterobase.warwick.ac.uk) and GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

3.8.8.3. Phylogenomic Analyses

Single nucleotide polymorphism (SNP) based phylogenetic tree was done using CSI Phylogeny 1.4 pipeline (185). The CSI Phylogeny 1.4 pipeline is an online bioinformatics platform used for construction of phylogenetic tree (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). For this WGSs of all the selected DEC pathotypes were uploaded and analysed on the platform. Selection of SNPs was based on default parameters on the CSI Phylogeny which include a minimum distance of 10 bp (10x) between each SNP, a minimum of 10% of the average depth, mapping quality was above 25, SNP quality above 30, minimum Z-score of 1.96, and all insertions and deletions were excluded. The *Morganella morganii subsp. morganii* KT genome (Accession number: CP004345.1) (186) served as the out group to root the tree enabling the easy configuration of the phylogenetic distance between the isolates on the branches.

Public databases (GenBank and enterobase) were used to obtain WGSs of comparative strains. To compare the phylogeny of the DEC pathotypes of the present study, other DEC pathotypes short-reads of publicly available DEC trains were downloaded from enterobase (187). The present hybrid strains were compared phylogenetically first with publicly available 10 hybrid strains (188-190) from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and then together with 51 other DEC pathotypes from enterobase database (http://enterobase.warwick.ac.uk) (downloaded on 2022/07/20). Similarly, the EAEC strains were compared phylogenetically, first among themselves and then with 51 different DEC pathotypes from enterobase database (http://enterobase.warwick.ac.uk) (downloaded on 2022/07/20). SNP-based phylogenetic analysis was performed using CSI Phylogeny 1.4 pipeline for all strains. Then with fastTree phylogeny output results were taken in Newick format and the phylogeny tree was visualized with annotations for isolate information using the Interactive Tree of Life (iTOL v4). Hierarchical clustering of the strains according to VGs and ARGs was constructed using dendrogram and associated heatmap based on the presence or absence of strains VGs or ARGs with GraphPad Prism 9.5.

3.9. Data analysis

Data analysis was made by transforming the data from Excel into SPSS Statistics version 20. Descriptive statistics such as frequency and percent distribution were used and presented in tables and graphs. The difference between variables was computed by using a Chi-Square statistic and a P-value less than 0.05 was considered significant. Bivariate and multivariate analyses were done using a binary logistic regression model to identify determinants of factors. Independent variables for the final model (multivariate logistic regression) were identified using a bivariate logistic regression model with $p<0.25$. Model fitness by the Hosmer and Lemeshow goodness-of-fit (P-value=0.348) and multi-co-linearity

of the independent variables using the Variance Inflation Factor and the Tolerance tests were checked. Crude odds ratio (COR) and adjusted odds ratio (AOR) were used to present the results. Different Public databases and online bioinformatics platforms include CGE were used for analysis of WGS data. CSI Phylogeny 1.4 pipeline for construction of phylogenetic tree was used. Annotation of the phylogenetic tree was done using FigTree v1.4.2 and the Interactive Tree of Life (iTOL v4). Dendrogram and associated heatmap were made by GraphPad Prism 9.5.

3.10. Ethical consideration

The study was approved by AAU, College Of Health Science Institutional Review Board (IRB) (protocol number: 025/20/DMIP) and the Ethiopian National Research Ethics Review Committee (Ref.No. RED/1.14/9428/21). Informed verbal assent and written consents were obtained from parents/guardian at the time of data collection. All patients were informed about the purpose of the study, benefit and possible discomfort, and their right to withdraw. Confidentiality of any information related to the study participants was preserved. We also obtained permission letter from each selected health facilities in Addis Ababa and Debre Berhan, Ethiopia.

Chapter four

4. Results

4.1. Epidemiology of diarrheagenic *E. coli*

4.1.1. Socio-demographic characteristics of the study population

A total of 476 samples were included for analysis in this study, where 277 (58.2%) were from Addis Ababa and 199 (41.8%) were from Debre Berhan. Of these, 274 (57.6%) were male and 202 (42.4%) were female (Table 2). Majority of the UFC were 24-59 months (60.5%). From the 476 participants, 391 (82%) were diarrheic and 85 (18%) were non-diarrheic. Of the total under-five diarrheic children, 226 were from Addis Ababa and 165 were from Debre Berhan. From the non-diarrheic UFC, 51 were from Addis Ababa and 34 were from Debre Berhan. Data and sample was collected from 69% of children during dry season and from 31% of children during rainy season. The family income was categorized using the previous study done by Fekadu and Lemma (191) and the Ethiopian birr was converted to US dollars with exchange rate during time of data collection (Table 2). Number of children in the family was one in 40.3%, two in 38.9%, and three or more than 3 in 20.8%.

Table 2. Socio-demographic data of under-five children participated in the study.

Characteristics		**Addis Ababa (n=277)**	**Debre Berhan (n=199)**	**Total (N=476)**
Sex	Male	153 (55.2%)	121 (60.8%)	274 (57.6%)
	Female	124 (44.8%)	78 (39.2%)	202 (42.4%)
Age	0-12 months	45 (16.2%)	34 (17.1%)	79 (16.6%)
	13-24 months	71 (25.6%)	38 (19.1%)	109 (22.9%)
	24-59 months	161 (58.1%)	127 (63.8%)	288 (60.5%)
Number of the child in the family	One	126 (45.5%)	93 (46.7%)	219 (46.0%)
	Two	117 (42.2%)	77 (38.7%)	194 (40.8%)
	≥Three	34 (12.3%)	29 (14.6%)	63(13.2%)
Order of the child birth in the family	First	125 (45.1%)	101 (50.8%)	226 (47.5%)
	Second	98 (35.4%)	68 (34.2%)	166(34.9%)
	Third	41 (14.8%)	22 (34.9%)	63 (13.2%)
	≥Four	13 (4.7%)	8 (4%)	21 (4.4%)
Educational level of the mother or guardian	Illiterate	23 (8.3%)	29 (14.6%)	52 (10.9%)
	Primary	89 (32.1%)	85 (42.7%)	174 (36.6%)
	Junior and Secondary School	101 (36.5%)	42 (21.11%)	143 (30.0%)
	Preparatory school	15 (5.4%)	3 (1.5%)	18 (3.8%)
	College or University	49 (17.7%)	40 (20.1%)	89 (18.7%)
Marital status of the parents or guardian	Married	234 (84.5%)	180 (90.5%)	414 (87.0%)
	Unmarried	25 (9%)	13 (6.5%)	38 (8%)
	Divorced	10 (3.6%)	6 (3%)	16 (3.4%)
	Widowed	8 (2.9%)	0 (0%)	8 (1.7%)
Occupational level of the mother or guardian	Employed	96 (34.7%)	48 (24.1%)	144 (30.3%)
	Self-employed	21 (7.6%)	60 (30.2%)	81(17%)
	Other *	160 (57.8%)	91 (45.7%)	251 (52.7%)
Monthly family income	<$36 (low)	112 (40.4%)	70 (35.2%)	182 (38.2%)
	$37-115(middle)	126 (45.5%)	81 (40.7%)	207 (43.5%)
	> $115(high)	39 (14.1%)	48 (24.1%)	87 (18.3%)

∗Other includes farming, doing paid work, and non-regular businesses

4.1.2. Occurrence of *E. coli* and other diarrheagenic bacteria

Among the 391 diarrheic UFC, the predominant bacterial isolates were *E. coli* (87.7%), followed by *Shigella (*10%), *Campylobacter jejuni* (3.8%), and *Salmonella* (2%) (Table 3). At genus level, *Campylobacter* was positive for 28 samples (16S rRNA positive); however, only 15 samples were positive for *Campylobacter jejuni* (15/391, 3.8%). Varied distribution

of bacterial isolation was observed across the study areas. The isolation of *E. coli* in Addis Ababa (81%) and Debre Berhan (97%), and *Salmonella* in Addis Ababa (0.4%) and Debre Berhan (4.2%) was statistically significant ($p<0.001$). There was no difference in isolation rate for *Shigella* and *Campylobacter jejuni* between Addis Ababa and Debre Berhan ($p>0.05$).

Table 3. Occurence of bacterial isolates from under-five children with diarrhea.

Bacterial isolates	**Addis Ababa (n=226)**	**Debre Berhan (n=165)**	**Total (n=391)**
E. coli (n=343)	81% (183/226)	97% (160/165)	87.7% (343/391)
Shigella (n=39)	11.1% (25/226)	8.5% (14/165)	10% (39/391)
Salmonella (n=8)	0.4% (1/226)	4.2% (7/165)	2% (8/391)
Campylobacter jejuni (n=15)	2.7% (6/226)	5.5% (9/165)	3.8% (15/391)
Shigella/Campylobacter jejuni	0.9% (2/226)	1.2% (2/165)	1% (4/391)
Salmonella/Campylobacter jejuni	0%	0.6% (1/165)	0.26% (1/391)

4.1.3. Molecular epidemiology of DEC

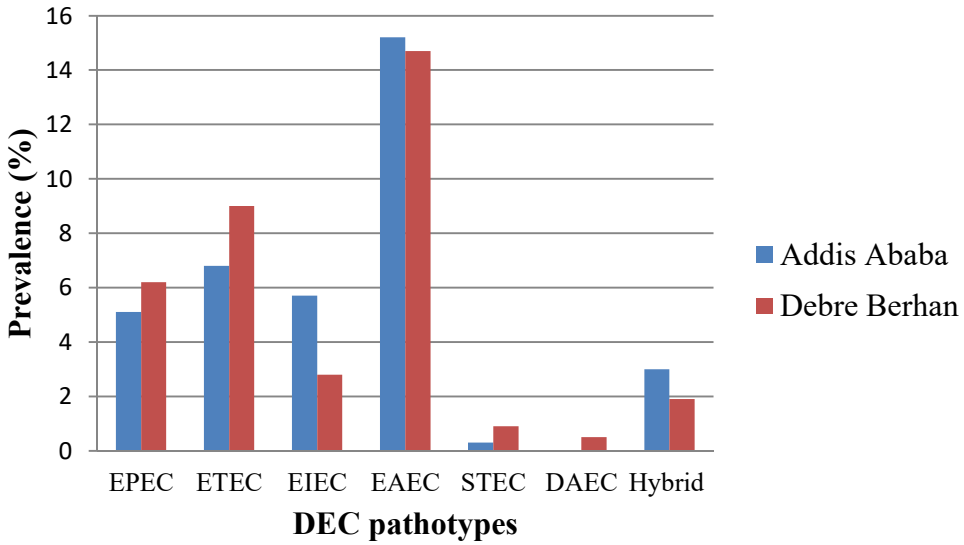
Of the 476 specimens analysed by culture, 89.9% (428/476) were positive for *E. coli*. All 428 *E. coli* isolates were tested by PCR, of which 183 (42.8%, 183/428) were positive for DEC pathotype (Table 4, Fig 6 a and b). Over half of the DEC were isolated from male (56.8%, 104/183) and the remaining 43.2% (79/183) were from female UFC. Higher number of DEC (59.6%, 109/183) was isolated from children with age ranges of 24-59 months. The occurrence of DEC was not statistically different with sex and age groups. The overall occurrence of DEC in the present study was 38.4% (183/476). From the total DEC isolated, 58.5% (107/183) were from Addis Ababa and 41.5% (76/183) were from Debre Berhan. The

occurrence of DEC was 40.7% (159/391) and 28.2% (24/85) in diarrheic and non-diarrheic UFC, respectively (p=0.021) (Table 4). From diarrheic UFC, the occurrence of DEC was 42% (95/226) in Addis Ababa and 38.8% (64/165) in Debre Berhan (p>0.05). The occurrence of DEC during the dry season and rainy seasons was 38% (124/326) and 39% (59/150), respectively.

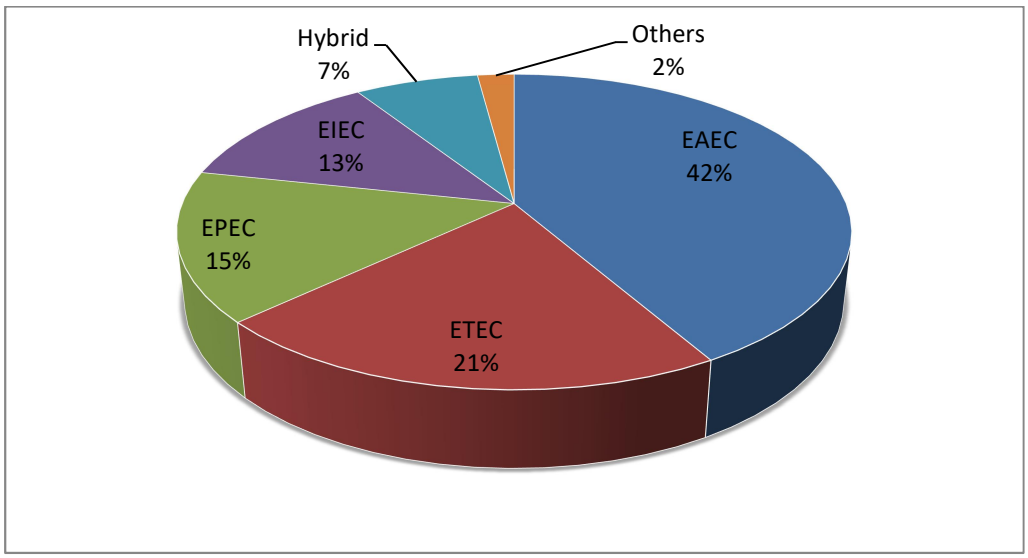
Table 4. Distributions of different DEC pathotypes identified from diarrheic and non-diarrheic under-five children in Addis Ababa and Debre Berhan, Ethiopia.

DEC Pathotypes	**Diarrheic**			**Non-diarrheic**			**Total diarrheic**	**Total non-diarrheic**
	AA (n=95) N (%)	DB (n=64) N (%)	Total (n=159) N (%)	AA (n=12) N (%)	DB (n=12) N (%)	Total (n=24) N (%)	(n=391) N (%)	(n=85) N (%)
EPEC (eae+)	14 (14.7)	12 (18.8)	26 (16.3)	1 (8.3)	1 (8.3)	2 (8.3)	26 (6.6)	2 (2.3)
ETEC (lt+)	12 (12.6)	15 (23.4)	27 (17)	3 (25)	4 (33.3)	7 (29.2)	27 (6.9)	7 (8.2)
ETEC (st+)	3 (3.2)	0(0)	3 (1.9)	0 (0)	0 (0)	0 (0)	3 (0.8)	0 (0)
ETEC (st+lt+)	2 (2.1)	0(0)	2 (1.3)	0(0)	0 (0)	0 (0)	2 (0.51)	0 (0)
EIEC (virF+ipaH+)	15 (15.8)	6 (9.4)	21 (13.2)	2 (16.7)	0 (0)	2 (8.3)	21 (5.4)	2(2.3)
EAEC (aggR+)	11 (11.6)	12 (18.8)	23(14.5)	1(8.3)	1 (8.3)	2 (8.3)	23 (5.9)	2 (2.3)
EAEC (astA+)	6(6.3)	6 (9.4)	12 (7.5)	4(33.3)	4(33.3)	8 (33.3)	12 (3.1)	8 (9.4)
EAEC (aggR+astA+)	23(24.2)	8 (12.5)	31 (8.2)	0 (0)	0 (0)	0(0)	31(7.9)	0 (0)
STEC (eae+stx+)	1 (1.1)	2 (3.1)	3 (1.9)	0(0)	0 (0)	0 (0)	3 (0.78)	0 (0)
DAEC (daaF+)	0 (0)	0(0)	0(0)	0 (0)	1 (8.3)	1 (4.2)	0 (0)	1 (1.2)
Hybrid	8 (8.4)	3 (4.7)	11 (6.9)	1(8.3)	1 (8.3)	2 (8.3)	11 (2.8)	2 (2.3)

Abbreviations: DEC, Diarrheagenic *Escherichia coli*; EPEC, Enteropathogenic *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli*; EAEC, Enteroaggregative *Escherichia coli*; STEC, Shiga-toxin producing *Escherichia coli*; DEAE, Diffusely adherent *Escherichia coli;* AA, Addis Ababa; DB, Debre Berhan.



a)



b)

Figure 6. The distributions of DEC pathotypes (a) shows occurrence of DEC pathotypes in Addis Ababa and Debre-berhan (b) Overall distribution of DEC pathotypes among under-five children in the two study sites.. Abbreviations: DEC, Diarrheagenic Escherichia coli; EPEC, Enteropathogenic Escherichia coli; ETEC, Enterotoxigenic Escherichia coli; EIEC, Enteroinvasive Escherichia coli; EAEC, Enteroaggregative Escherichia coli; STEC, Shiga-toxin producing Escherichia coli; DEAE, Diffusely adherent Escherichia coli. Other include STEC and DAEC.

The predominant DEC pathotype was EAEC (41.5%, 76/183), followed by ETEC (21.3%, 39/183%), EPEC (15.3%, 28/183), EIEC (12.6%, 23/183), hybrid strains (7.1%, 13/183), STEC (1.6%, 3/183), and DAEC (0.6%, 1/183). The hybrid pathotypes of DEC isolated in the present study were 5 ETEC/EAEC hybrids, 5 ETEC/EPEC hybrids, and 3 EPEC/EAEC hybrids. Sample gel image result for DEC pathotypes is shown in Figure 7. The occurrence of each DEC pathotypes were higher among diarrheic compared to non-diarrheic UFC (p=0.030). The distribution of DEC pathotypes among the study areas (in Addis Ababa and Debre Berhan) was not statistically significant (p>0.05). A clinical presentation of UFC positive for DEC is presented in Table 5. Among the diarrheic under five children, co-infection with other diarrheagenic bacteria (*Shigella, Salmonella,* and *Campylobacter jejuni*) was common. Seven DEC (5 EAEC and 2 EPEC) with *Shigella*, two DEC (EAEC and ETEC) with *Salmonella*, and one DEC (EAEC) with *Campylobacter jejuni* were detected.

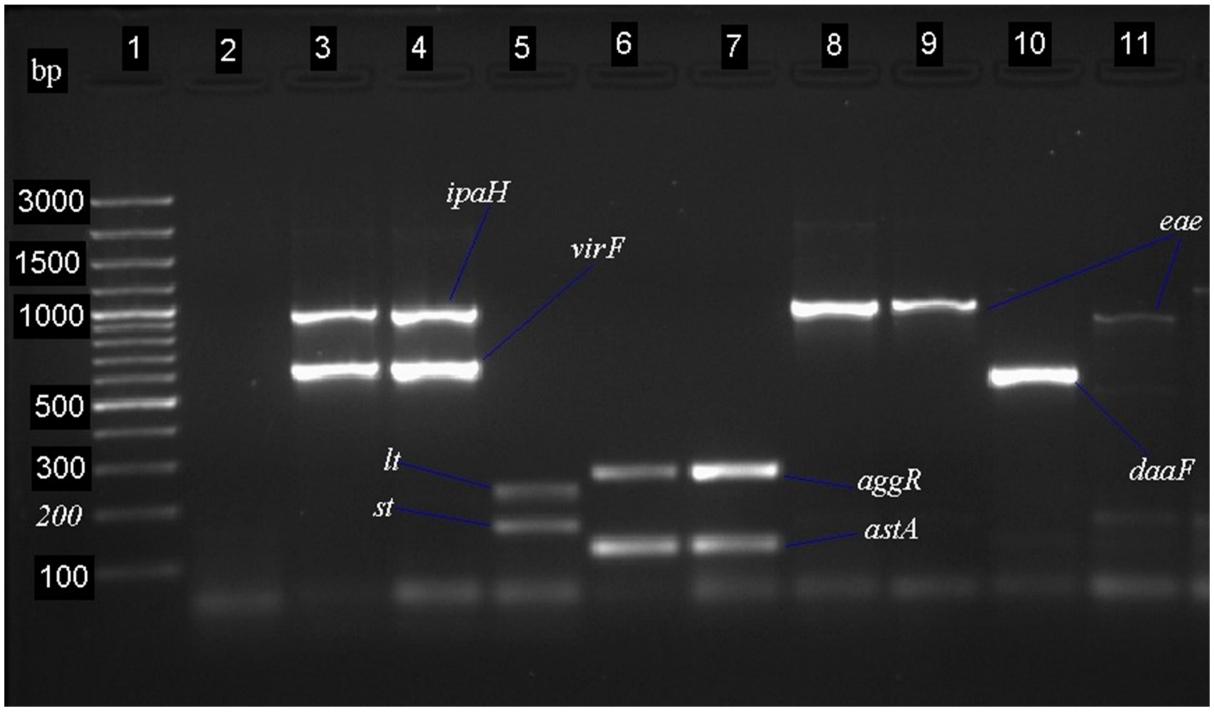


Figure 7. Gel images of PCR products of DEC pathotypes. Lanes: 1=molecular marker (GeneRuler 100 bp Plus DNA Ladder; Invitrogen Life Technologies); 2= (negative control, CCUD 24T Escherichia coli, *3 and 4=EIEC, 5*=ETEC, *6 and 7*=EAEC, *8, 9 and 11*=EPEC, *and 10* =DAEC. *Numbers on the left column denote fragment sizes (bp).*

Table 5. Clinical presentation of under-five children who were positive for DEC pathotype during their health facility visit

Characteristics (n, %)*	**EPEC**	**ETEC**	**EIEC**	**EAEC**	**Hybrid**
Total (n)	28	39	23	76	13
Fever	10 (35.7)	11 (28.2)	7 (30.4)	30 (39.5)	3 (23.1)
Vomiting	12 (42.9)	11 (28.2)	10 (43.5)	23 (30.2)	6 (46.2)
Nausea	6 (21.4)	1 (2.6)	1 (4.3)	14 (18.4)	0 (0%)
Increased thirsty	6 (21.4)	8 (20.5)	6 (26.1)	23 (30.3)	4 (30.8)
Abdominal pain	5 (17.9)	4 (10.3)	4 (17.4)	8 (10.5)	0 (0)
Time of illness					
1-2 days	15 (53.6)	22 (56.4)	16 (69.6)	40 (52.6)	7 (53.9)
3 days	7 (25)	14 (35.9)	4 (17.4)	25 (32.9)	6 (46.2)
≥4 days	6 (21.4)	3 (7.7)	3 (13)	11 (14.5)	0 (0)
Stool frequency per 24 hrs.					
1-2 times	12 (42.8)	7 ((18)	8 (34.8)	19 (25)	3 (23.1)
3 times	10 (35.7)	18 (46.2)	8 (34.8)	21 (27.6)	6 (46.2)
≥4 times	6 (21.4)	14 (35.9)	7 (30.4)	36 (47.4)	4 (30.8)
Dehydration					
Mild	6 (21.4)	7 (17.9)	5 (21.7)	25 (32.9)	2 (15.4)
Moderate	12 (42.9)	12 (30.8)	8 (34.8)	31 (40.8)	10 (76.9)
Severe	0 (0)	3 (7.7)	1 (4.3)	2 (2.6%)	1 (7.7)
None	10 (35.7)	17 (43.6)	9 (39.1)	18 (23.7)	0 (0)
Stool consistency					
Watery	13 (46.4)	11 (28.2)	2 (8.7)	29 (38.2)	6 (46.2)
Mucoid	10 (35.7)	17 (43.6)	7 (30.4)	28 (36.7)	2 (15.4)
Semisolid (loose)	1 (3.6)	2 (5.1)	3 (13)	5 (6.6)	1 (7.7)
Bloody	2 (7.1)	4 (10.3)	8 (34.8)	9 (11.8)	3 (23.1)
Formed	2 (7.1)	5 (12.8)	3 (13)	5 (6.6)	1 (7.7)

*STEC (n=3) and DAEC (n=1) are not included since % is not relevant due to low n value. Abbreviations: DEC, Diarrheagenic *Escherichia coli*; EPEC, Enteropathogenic *Escherichia coli;* ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli;* EAEC, Enteroaggregative *Escherichia co*li; STEC, Shiga-toxin producing *Escherichia coli*; DEAE, Diffusely adherent *Escherichia coli*

4.1.4. Factors associated with DEC acquisition

UFC were more likely to acquire DEC during the rainy season compared to dry season (AOR=0.529, CI= 0.335; 0.835) (Table 6). Children who lived in a family with a monthly income of below $36 were more likely to acquire DEC (AOR=2.364, CI=1.354; 4.126) compared to children who lived in a family with a monthly income greater than $115. Children who were cared for by their mother had a lower risk of DEC (AOR=0.423, CI=0.211; 0.846) compared to those cared for by others, including grandmother, close family member, and day-care. UFC who began their supplementary food before six months were

more likely to acquire DEC (AOR=3.660, CI=1.322; 10.128) than those older than 12 months. Children living in a compound with domestic animals had two times the likelihood (AOR=1.555, 1.016; 2.381) of being positive for DEC compared to those without contact to domestic animals. Poor access to a clean water supply was significantly associated with acquiring DEC. Children whose families obtained water in shift had more likely to get DEC (AOR=1.735, OR=1.046; 2.879) compared to those with a continuous daily supply of water.

Table 6. Demographic factors associated with DEC positive under-five children in Addis Ababa and Debre Berhan, Ethiopia.

Variables		**DEC**		**Univariate analysis**		**Multivariate analysis**	
		Yes (n=183) N (%)	No (n=293) N (%)	COR[a] (95%CI)	P-value	AOR[b] (95%CI)	P-value
Study area	Debre Berhan	76 (41.5%)	123 (42.0%)	1.019 (0.700, 1.481)	0.923	1.075 (0.640, 1.806)	0.784
	Addis Ababa	107 (58.5%)	170 (58.0%)	1.00		1.00	
Sex	Male	104 (56.8%)	170 (58.0%)	1.050 (0.723, 1.525)	0.798	1.184 (0.782, 1.770)	0.409
	Female	79 (43.2%)	123 (42.0%)	1.00		1.00	
Age	0-12 months	31(16.9%)	48 (16.4%)	0.943 (0.566, 1.571)	0.946	0.929 (0.527, 1.638)	0.967
	13-24 months	43 (23.5%)	66 (22.5%)	0.935 (0.595, 1.469)		1.000 (0.616, 1.622)	
	24-59 months	109 (59.6%)	179 (61.1%)	1.00		1.00	
Seasons	Winter	84 (45.9%)	146 (49.8%)	0.540 (0.351, 0.832)	0.004	0.529 (0.335, 0.835)	**0.009**
	Spring	40 (21.9%)	56 (19.1%)	1.084 (0.665, 1.767)		0.999(0.597, 1.671)	
	Summer	59 (32.2%)	91 (31.1%)	1.00		1.00	
Family income (monthly)	<$36 (low)	61 (33.3%)	121 (41.3%)	2.212 (1.447, 4.118)	0.002	2.364 (1.354, 4.126)	**0.008**
	$37-115(middle)	74 (40.4%)	133 (45.4%	2.212 (1.329, 3.681)		2.042 (1.191, 3.502)	
	> $115(high)	48 (26.2%)	39 (13.3%)	1.00		1.00	
Child care by	House worker	70 (38.3%)	142 (48.5%)	0.742 (0.385, 1.432)	0.002	0.731 (0.366, 1.458)	**0.009**
	Mother	98 (53.6%)	110 (37.5%)	0.411 (0.214, 0.787)		0.423 (0.211, 0.846)	
	Others[c]	15 (8.2%)	41 (14.0%)	1.00		1.00	
Begin supplementary food	<6months	22 (12.0%)	59 (20.1%)	2.458 (0.947, 6.379)	0.059	3.660 (1.322, 10.128)	**0.019**
	6-12months	150 (82.0%)	222 (75.8%)	1.357 (0.583, 3.155)		1.826 (0.740, 4.508)	
	>12months	11 (6.0%)	12 (4.1%)	1.00		1.00	
Domestic animals	Yes	69 (37.7%	156 (53.2%)	1.881 (1.291, 2.742)	0.001	1.555 (1.016, 2.381)	**0.042**
	No	114 (62.3%)	137 (46.8%)	1.00		1.00	
Obtaining water	In shift	107 (58.5%)	198 (67.6%)	1.480 (1.010, 2.170)	0.044	1.735 (1.046, 2.879)	**0.033**
	Daily	76 (41.5%)	95 (32.4%)	1.00			

[a] Crude Odd Ratio
[b] Adjusted Odd Ratio
[c] including grandmother, close family member, and day care

4.2. Antimicrobial resistance (AMR) profile

4.2.1. Antimicrobial susceptibility profile of DEC and others bacterial species

Table 7 showed the phenotypic antimicrobial susceptibility test results of DEC, *Shigella* and *Salmonella*. The DEC pathotypes were resistant to ampicillin (95.1%, 174/183), ceftazidime and cefotaxime (16.4%, 30/183), tetracycline (91.3%, 167/183), ciprofloxacin (14.2%, 26/183), and trimethoprim-sulfamethoxazole (42.6%, 78/183). ETEC, EIEC, EAEC, and hybrid strains showed resistance to trimethoprim-sulfamethoxazole from 31% (4/13) to 58% (44/76). Resistance to ceftazidime and cefotaxime was higher in DEC isolates obtained from diarrheic compared to non-diarrheic children (p=0.01). All the STEC and DAEC, 15% ETEC (6/39) and 22% EAEC (17/76) were resistant to ciprofloxacin. EPEC (11%, 3/28), ETEC (15%, 6/39), EAEC (20%, 15/76), and all STEC were resistant to ceftazidime and cefotaxime. ETEC (5%, 2/39) and EAEC (3%, 2/76) were resistant to meropenem and ertapenem. Majority of isolates were more susceptible to amoxicillin-clavulanate, ciprofloxacin, ceftazidime, cefotaxime, and cefepime compared to other antimicrobial compounds (Table 7).

Table 7. Antimicrobial susceptibility profile of DEC pathotypes , *Shigella* , and *Salmonella* isolated from diarrheic and non-diarrheic under -five children, Addis Ababa and Debre Berhan, Ethiopia.

Discs	**EPEC (n=28)**		**ETEC (n=39)**		**EIEC (n=23)**		**EAEC(n=76)**		**Hybrid *(n=13)***		**Others (n=4)**		***Shigella* (n=39)**		***Salmonella* (n=8)**	
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R%	S%	R%	S%
AMP	25(89)	3 (11)	36(92)	3 (8)	21(91)	2 (9)	75 (99)	1(1)	13(100)	0(0)	4 (100%)	0 (0%)	34 (87%)	5 (13%)	8 (100%)	0 (0%)
AMC	2 (7)	26(93)	4(10)	35(90)	1(4)	22 (96)	8(11)	68(90)	0(0)	13(100)	0 (0%)	4 (100%)	7 (18%)	32 (82%)	1 (13%)	7 (87%)
CIP	1(4)	27(96)	6(15)	33(85)	2(9)	21 (91)	17 (22)	59 (78)	1(8)	12(92)	0 (0%)	4 (100%)	4 (10%)	35 (90%)	2 (25%)	6 (75%)
GM	2(7)	26 (93)	10(26)	29(74)	3(13)	20(87)	31(41)	45(59)	2(15)	11(85)	4 (100%)	0 (0%)	3 (8%)	36 (92%)	1 (13%)	7 (87%)
SXT	4 (14)	24(86)	13(33)	26(67)	9(39)	14(41)	44(58)	32(42)	4(31)	9(69)	4 (100%)	0 (0%)	15 (38%)	24 (62%)	5 (63%)	3 (37%)
C	2(7)	26(93)	9(23)	30(77)	2(9)	21(91)	28(37)	48(63)	3(23)	10(77)	4 (100%)	0 (0%)	3 (8%)	36 (92%)	0 (0%)	8 (100%)
TE	25 (89)	3(11)	36(92)	3(8)	21(91)	2(9)	70(92)	6(8)	11(85)	2(15)	4 (100%)	0 (0%)	33 (85%)	6 (15%)	7 (88%)	1 (12%)
CAZ	3(11)	25(89)	6(15)	33(85)	2(9)	21(91)	15(20)	61(80)	1(8)	12(92)	3 (75%)	1 (25%)	3 (8%)	36 (92%)	0 (0%)	8 (100%)
CTX	3(11)	25(89)	6(15)	33(85)	2(9)	21(91)	15(20)	61(80)	1(8)	12(92)	3 (75%)	1 (25%)	3 (8%)	36 (92%)	0 (0%)	8 (100%)
FEP	1(4)	27(96)	1(3)	38(97)	0(0)	23(100)	5(7)	71(93)	1(8)	12(92)	0 (0%)	4 (100%)	2 (5%)	37 (95%)	0 (0%)	8 (100%)
MEM	0(0)	28(100)	2(5)	37(95)	0(0)	23(100)	2(3)	74(97)	0(0)	13(100)	0 (0%)	4 (100%)	1 (3%)	38 (97%)	0 (0%)	8 (100%)
ETP	0(0)	28(100)	2(5)	37(95)	0(0)	23(100)	2(3)	74(97)	0(0)	13(100)	0 (0%)	4 (100%)	1 (3%)	38 (97%)	0 (0%)	8 (100%)

Abbreviations: AMP, Ampicillin; AMC, Amoxacillin-Clavulanate; CIP, Ciprofloxacin; GM, Gentamicin; SXT, Trimethoprim-Sulfamethoxazole; C, Chloranphenicol; TE, Tetracycline; CAZ, Ceftazidime; CTX, Cefotaxime; FEP, Cefepime; MEM, Meropenem; ETP, Ertapenem

4.2.2. Multidrug resistance (MDR), ESBL, and Carbapenemase production among DEC pathotypes

Seventy-nine (43.2%, 79/183) of the DEC pathotypes were MDR (Table 8). Of the MDR strains, 42 % (33/79) were from Debre Berhan and 58 % (46/79) were from Addis Ababa. The occurrence of MDR strains was 43% (33/76) and 43% (46/107) in Debre Berhan and Addis Ababa (P- 0.242), respectively. The predominant MDR were EAEC (55.7%, 44/79), followed by ETEC (21.5%, 17/79), EIEC (8.9%, 7/79), EPEC (5.1%, 4/79), STEC (3.8%, 3/79), hybrid (3.8%, 3/79), and DAEC (1.3%, 1/79). The MDR was 14.3% (4/28) in EPEC, 43.6% (17/39) in ETEC, 30.4% (7/23) EIEC, 57.9% (44/76) in EAEC, 100% (3/3) in STEC, 100% (1/1) in DAEC, and 15.4% (2/13) in hybrid strains.

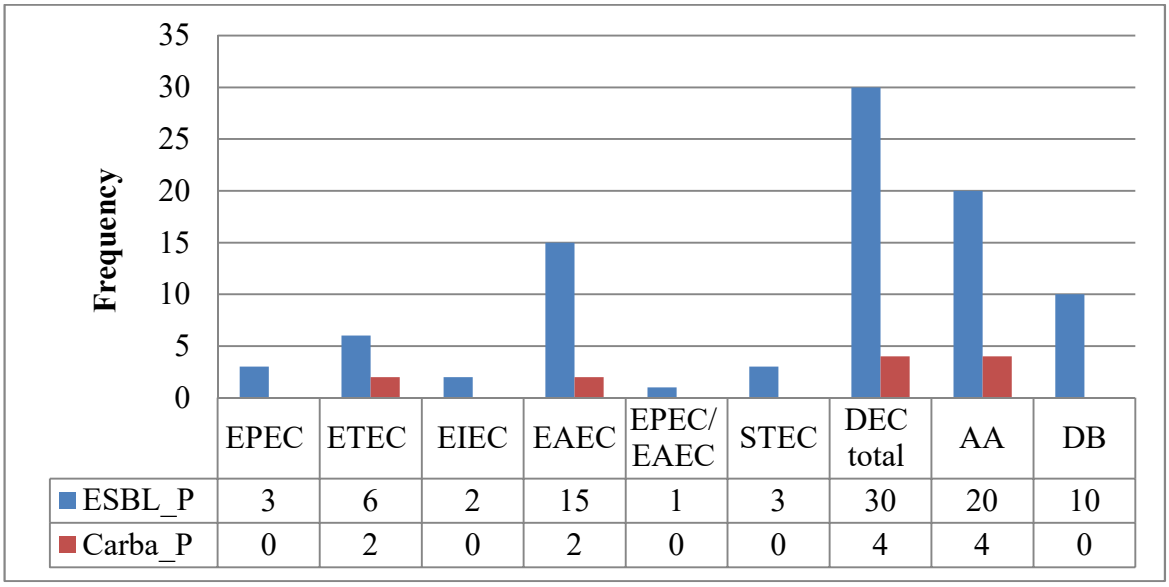
Table 8. Antibiogram profile of DEC pathotypes isolated from under- five children in Debre Berhan and Addis Ababa, Ethiopia.

DEC path types	Level of antimicrobial resistance, n (%)								
	R0	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**≥ R8**
EPEC (n=28)	2 (7%)	4(15%)	18 (64%)	2 (7%)	2 (7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ETEC (n=39)	2 (5%)	3(8%)	17 (44%)	8 (20%)	3(8%)	2 (5%)	2 (5%)	2 (5%)	0 (0%)
EIEC (n=23)	1 (4%)	3 (13%)	12 (52%)	2 (9%)	2 (9%)	2 (9%)	1 (4%)	0 (0%)	0 (0%)
EAEC (n=76)	1 (1%)	6 (8%)	27 (34%)	18 (23%)	17 (22%)	3 (4%)	3 (4%)	0 (0%)	3 (4%)
Hybrid (n=13)	0 (0%)	3 (23%)	7 (54%)	2 (15%)	1 (8%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Others* (n=4)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	1 (25%)	2 (50%)	0 (0%)	0 (0%)
Total (n=183)	6 (3.2%)	19 (25%)	81 (44.3%)	32 (17.5%)	26 (14.2%)	8 (4.4%)	8 (4.4%)	2 (1.1%)	3 (1.6%)

∗Other includes STEC (n=3) and DAEC (n=1). R0: Sensitive to all antimicrobials tested (or stands for resistance for zero antimicrobial); R1, R2, R3, R4, R5, R6, R8: Resistant to one, two, three, four, five, six, eight and greater than eight antimicrobials, respectively. Abbreviations: DEC, Diarrheagenic *Escherichia coli;* EPEC, Enteropathogenic *Escherichia coli;* ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli;* EAEC, Enteroaggregative *Escherichia coli;* STEC, Shiga-toxin producing Escherichia coli; DEAE, Diffusely adherent *Escherichia coli*

A total of 30 out of 183 (16.4%) of the DEC pathotypes or 38% (30/79) of the MDR isolates were ESBLs producers. More specifically all the three STEC isolates (100%), 19.7 % (15/76) of EAEC, 15.3% (6/39) of ETEC, 10.7% (3/28) of EPEC, 8.7% (2/23) of EIEC, and 7.7% (1/13) of hybrid strains were ESBL producers (Figure 8). Among the ESBL producers, the predominant pathotypes were EAEC (50%, 15/30), followed by ETEC (20%, 6/30), EPEC (10%, 3/30), STEC (10%, 3/30), EIEC (6.7%, 2/30), and the hybrid strains (3.3%, 1/30). A

total of 4 (2.2%, 4/183) of the DEC pathotypes were carbapenemase producers, and all were from Addis Ababa. Representative gel image of the PCR products for ESBL-producing strains is presented in Figure 9.



	EPEC	ETEC	EIEC	EAEC	EPEC/ EAEC	STEC	DEC total	AA	DB
ESBL_P	3	6	2	15	1	3	30	20	10
Carba_P	0	2	0	2	0	0	4	4	0

Figure 8. Distribution of ESBLs- and carbapenemase-producing DEC pathotypes isolated from under -five children, Addis Ababa and Debre Berhan, Ethiopia 2020/21. Abbreviations: ESBL_P, Extended spectrum ß-lactamases producing; Carba_P, Carbapenemase producing; AA, Addis Ababa; DB, Debre Berhan; DEC, Diarrheagenic Escherichia coli; *EPEC, Enteropathogenic Escherichia coli*; *ETEC, Enterotoxigenic Escherichia coli*; *EIEC, Enteroinvasive Escherichia coli*; *EAEC, Enteroaggregative Escherichia coli*; *STEC, Shiga-toxin producing Escherichia coli*; *DEAE, Diffusely adherent Escherichia coli*.

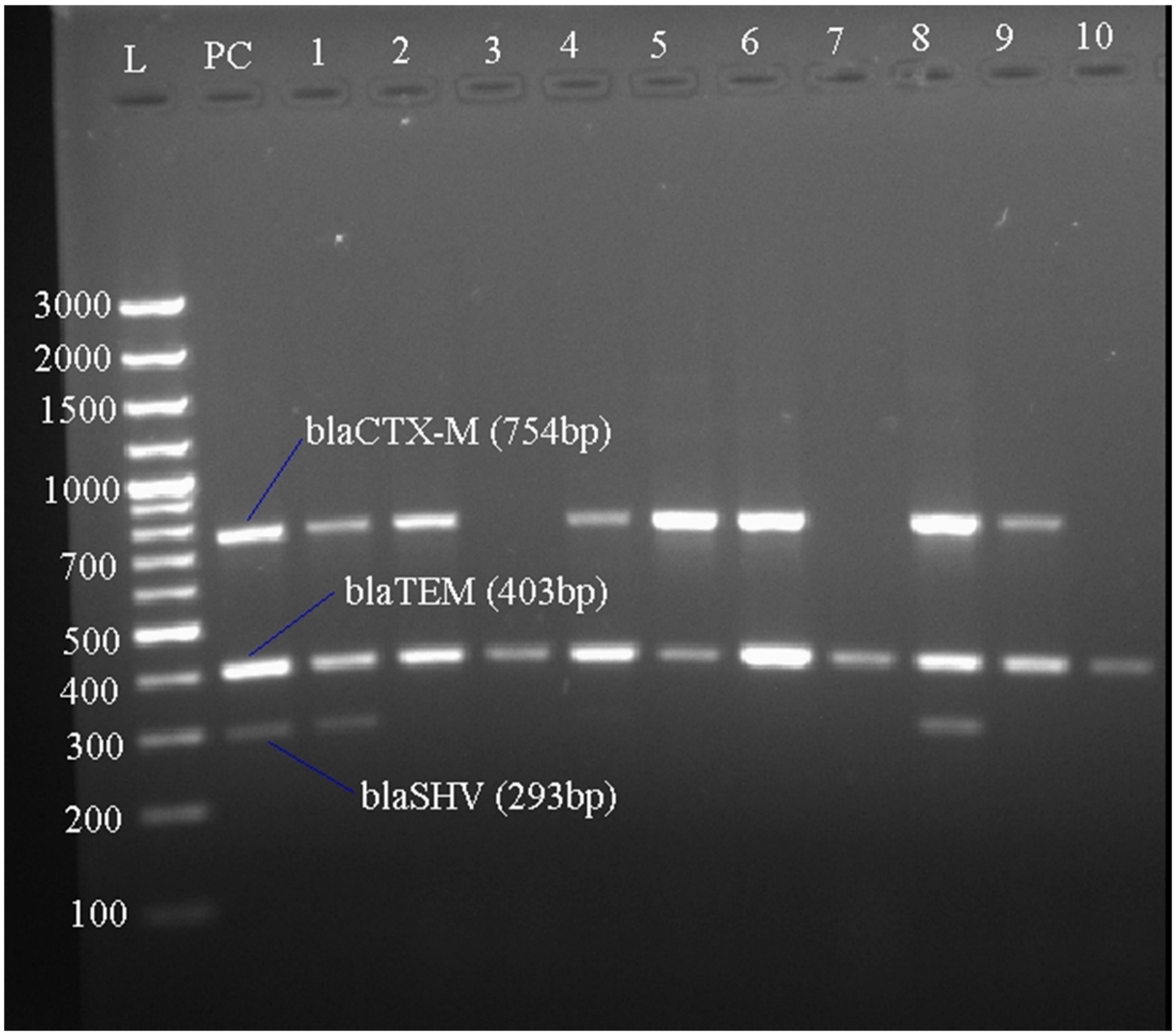
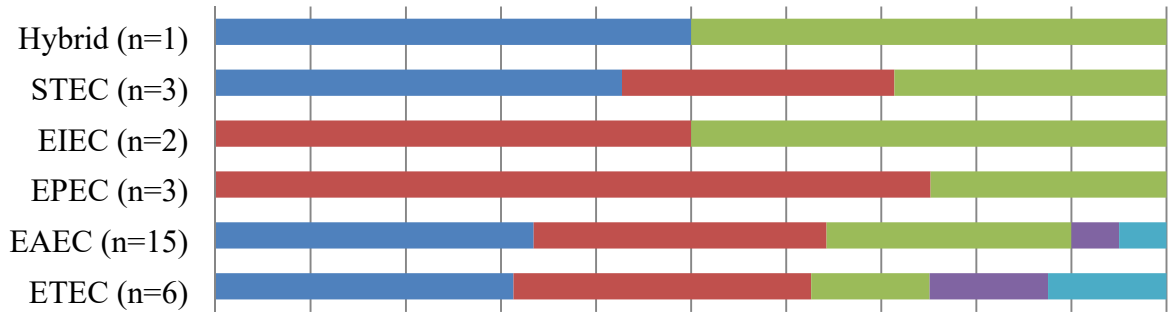


Figure 9. Gel image of the ß-lactamase genes of DEC pathotypes isolated from under -five children, Addis Ababa and Debre Berhan, Ethiopia 2020/21. Notes: Lane L, 100 kb+ DNA ladder (in bp, base pair); Lane PC, positive control ($bla_{CTX\text{-}M}$ *(754bp),* bla_{SHV}*(293bp) and* bla_{TEM} *(403bp)); Lanes 1and 8 are positive for* $bla_{CTX\text{-}M}$, bla_{TEM} *and* bla_{SHV}*; 2, 4, 5, 6, and 9 positive only for* $bla_{CTX\text{-}M}$ *and* bla_{TEM}*; and 3, 7, and 10 positive only for* bla_{TEM}.

4.2.3. Molecular characterization of ESBLs and Carbapenemase genes

ESBL genes were detected in all phenotypically ESBL-producing DEC pathotypes. The three common ß-lactamase genes including *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$, were detected. The predominant ß-lactamase genes detected were *bla*$_{TEM}$ (80%, 24/30), followed by *bla*$_{CTX-M}$ (73%, 22/30), and *bla*$_{SHV}$ (60%, 18/30). All the above three ß-lactamase genes were detected in 33% (10/30) of DEC in combination (Figure 10 and Table 9). Fifty percent (15/30) of the ß-lactamase genes were detected from EAEC, 20% (6/30), ETEC, 10% (3/30), EPEC, 10% (3/30), STEC, 6.7% (2/30), EIEC, and 3.3% (1/30) hybrid DEC pathotypes (Figure 10). Of the total ß-lactamase genes, 67% (20/30) were detected from DEC pathotypes isolated from Addis Ababa whereas 33% (10/30) were from DEC pathotypes isolated from Debre Berhan. Carbapenemase encoding genes, *bla*$_{NDM}$, and bla$_{OXA-48}$ were found to co-exist in 13.3% DEC pathotypes (4/30). *bla*$_{KPC}$ was not detected in any of the DEC pathotypes in the present study. The carbapenemase encoding genes were detected from DEC pathotypes isolated from children with age 25-59 months and living in Addis Ababa, and whose family were self-employed and had travel history.



	ETEC (n=6)	EAEC (n=15)	EPEC (n=3)	EIEC (n=2)	STEC (n=3)	Hybrid (n=1)
blaCTX-M	83%	87%	0%	0%	100%	100%
blaTEM	83%	80%	100%	100%	67%	0%
blaSHV	33%	67%	33%	100%	67%	100%
blaNDM	33%	13%	0%	0%	0%	0%
blaOXA-48	33%	13%	0%	0%	0%	0%

Figure 10. ß-lactamase genes detected in DEC pathotype isolated from under-five children in Addis Ababa and Debre Berhan, Ethiopia. Abbreviations: EPEC, Enteropathogenic Escherichia coli; ETEC, Enterotoxigenic Escherichia coli; EIEC, Enteroinvasive Escherichia coli; EAEC, Enteroaggregative Escherichia coli; STEC, Shiga-toxin producing Escherichia coli; DEAE, Diffusely adherent Escherichia coli

Table 9. ß-lactamase genes detected in DEC pathotype isolated from under-five children in Addis Ababa and Debre Berhan, Ethiopia.

Isolate ID	Pathotype	ESBL (or ß-lactamase) genes			Carbapenemase genes		Study area
		bla_{CTX-M}	bla_{TEM}	bla_{SHV}	bla_{NDM}	bla_{OXA-48}	
ET-1	ETEC	+	+	+	+	+	AA
ET-2	ETEC	+	+	-	+	+	
ET-3	ETEC	-	+	-	-	-	
ET-4	ETEC	+	+	-	-	-	
ET-5	ETEC	+	+	+	-	-	DB
ET-6	ETEC	+	-	-	-	-	
EA-1	EAEC	+	+	-	-	-	AA
EA-2	EAEC	+	+	+	-	-	
EA-3	EAEC	-	+	+	-	-	
EA-4	EAEC	+	+	+	-	-	
EA-5	EAEC	+	+	-	-	-	DB
EA-6	EAEC	+	+	+	-	-	
EA-7	EAEC	+	-	+	-	-	AA
EA-8	EAEC	+	-	+	-	-	DB
EA-9	EAEC	+	+	+	-	-	AA
EA-10	EAEC	+	+	+	-	-	
EA-11	EAEC	+	+	+	-	-	

EA-12	EAEC	+	-	-	+	+	
EA-13	EAEC	+	+	-	-	-	DB
EA-14	EAEC	-	+	+	+	+	AA
EA-15	EAEC	+	+	-	-	-	
EP-1	EPEC	-	+	-	-	-	AA
EP-2	EPEC	-	+	-	-	-	DB
EP-3	EPEC	-	+	+	-	-	AA
EI-1	EIEC	-	+	+	-	-	AA
EI-2	EIEC	-	+	+	-	-	DB
ST-1	STEC	+	-	-	-	-	DB
ST-2	STEC	+	+	+	-	-	AA
ST-3	STEC	+	+	+	-	-	DB
HB-1	Hybrid	+	-	+	-	-	AA

Abbreviations: EPEC, Enteropathogenic *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; EIEC, Enteroinvasive *Escherichia coli;* EAEC, Enteroaggregative *Escherichia coli;* STEC, Shiga-toxin producing *Escherichia coli;* DEAE, Diffusely adherent *Escherichia coli;* AA, Addis Ababa; DB, Debre Berhan

4.3. Whole Genome Sequence analysis of DEC

4.3.1. Genome analysis

Of the total 79 MDR strains, 30 were ESBL positive based on phenotypic analysis and were subjected to PCR for ß-lactamase (ESBL and carbapenemase) gene detection. From the remaining 49 MDR strains, randomly, a total of 28 DEC pathotypes (8 hybrids, 15 EAECs, and 5 ETECs) were subjected to WGS analysis. We renamed the isolates as 'EH', 'EA', and 'ET' following number to refer hybrid, aggregative and toxigenic DEC strains, respectively from Ethiopia.

The genomic analysis of the hybrid strains showed varied MLST, serotypes, and phylotypes. In the phylogenetic analysis, the hybrid strains were grouped into A (25%), B1 (25%), C (25%), and unknown (25%) phylogroup (Table 10). Only two hybrid strains were grouped in the same sequence type (ST 10). MGE (plasmid replicons or/and transposons) that were associated with VGs were found in 88% of the hybrid strains. The numbers of VGs associated with MGE were different. One hybrid strain (EAEC/ETEC) showed large number of VGs (12 virulence genes) that were associated with MGEs.

Table 10. ST, serotype, phylotypes, virulence genes and mobile genetic elements associated with virulence genes of the hybrid stains of the present study.

Strain ID	Strains	Study area	ST	Serotype	Phyloytpe	Virulence genes	Mobile genetic elements	
							Plasmid replicons	Transposons
EH_1	EPEC/EAEC	DB	ST 10	O71:H40	A	hlyF, ompT	IncFIB	-
EH_2	EPEC/ETEC	DB	ST 10	O13:H11	A	senB, astA	Col156	ISEic2
EH_3	EPEC/ETEC	DB	ST 517	H19	B1	toxB, espA, espF, ipfA, tir,eae	IncFll	ISEc38, MITEEc1
EH_4	EAEC/ETEC	AA	ST 23	O65:H12	C	espI, agg3A, agg3C, ORF4, agg3D, astA, agg3B, ORF3, aap, aar, aatA, aggR	-	ISEc30, ISSpu2
EH_5	EPEC/ETEC	AA	ST 155	O13	U	senB, eatA, traT, astA	Col156, IncB/O/K/Z	ISEc37, ISEic2
EH_6	EAEC/ETEC	AA	ST 568	O16:H18	C	aap, aatA, aar, cia, pic	Incl1	ISSpu2, ISEc38
EH_7	EAEC/ETEC	AA	ST 58	O58:H30	B1	traT, eatA, aap	IncFll(pCoo), IncFll(Phn7A8)	Tn2
EH_8	EAEC/ETEC	AA	ST 10512	O21:H21	U	-	-	-

Abbreviation: ST, Sequence type; AA, Addis Ababa; DB, Debre Berhan

All the EAEC strains subjected to WGS were MDR strains. The genome analysis of EAEC strains showed a different MLST, serotypes, and phylogenetic groups (Table 11). The EAEC strains were grouped in A (33%), B1 (13%), B2 (27%), D (27%) phylogenetic groups. Majority (87%, 13/15) were grouped in A, B2 and D phylotypes. Larger number of EAEC strains (60%, 9/15) contain MGEs (plasmid replicons or/and transposons) associated with ARGs. However, 40% (6/15) of EAEC strains do not contain MGE associated with ARGs. The MGE contains a varied number of ARGs. The genomic analysis of ETEC strains also showed a varied MLST and serotypes profiles (Table 12). All the ETEC strains contained at least one MGE, either plasmid replicons or/and transposons that are associated with ARGs (Table 12).

Table 11. ST, serotype, phylotypes, ARGs, and MGEs harboring ARGs of EAEC strains of the present study.

Strain ID	Study area	ST	Serotype	Phylo type	ARGs	Mobile genetic elements	
						Plasmid	Transposons
EA_001	AA	ST 2555	O153:H14	B2	blaTEM-1B, blaCTX-M-14b, sul2, aadA1, dfrA1, tet(D)	IncQ1	ISEc9, Tn7
EA_002	AA	ST 58	O58:H30	B1	blaTEM-1B, dfrA7, gyrA	IncFII	Tn2
EA_003	AA	ST 1861	O15:H5	A	blaTEM-1B, qnrS1, sul1, sul2, dfrA7, tet(A), catA1	-	Tn2
EA_004	AA	ST 69	O17:H18	D	blaTEM-1B, aph(6)-id, sul1, sul2, dfrA7, tet(A), tet(B), catA1, aadA1	IncQ1	-
EA_005	AA	ST 38	O153:H30	D	blaTEM-1B , blacCTX-M-14b, blaCTX-M15, blaNDM-5, blaOXA-1, tet(D), catB3, cmlA1, aac(6'), rmtB	-	-
EA_006	DB	ST 10	O69:H32	A	gyrA, tet (A), aadA1	-	-
EA_007	DB	ST 10	O125:H16	A	sul1, sul2, dfrA14, tet(A)	-	-
EA_008	DB	ST 154	O130:H10	A	blaTEM-1B, sul1, sul2, dfrA1, tet(A)	-	Tn2
EA_009	DB	ST 8746	O17:H18	D	blaTEM-1B, aph(6)-id, sul2, dfrA7, tet(A)	IncQ1	-
EA_010	DB	ST 449	O104:H12	B2	blaTEM-1B, blacCTX-M-14b, bla-CTX-M-27, dfrA7, gyrA, qnrS1, aac(6')	-	IS102
EA_011	DB	ST 4442	O7:H9	D	blaTEM-1B, sul1, sul2, catA1	-	-
EA_012	AA	ST 10512	O21:H21	A	blaTEM-1B, sul1, sul2, dfrA7, tet(A), mph(A)	-	-
EA_013	AA	ST 3749	O101:H48	B1	blaTEM-1B, sul1, sul2, dfrA7, qnrS13, tet(A)	-	ISKpn19
EA_014	DB	ST 10825	O11:H4	B2	blaTEM-1B, sul1, sul2, dfrA7, tet(A), mph(A)	IncQ1	-
EA_015	AA	ST 5614	H4:O27	B2	bla TEM-1B, bla CTX-M-15, blaSHV-106, qnrS1, sul2, dfrA8, tet (B), erm(B),	-	-

Abbreviation: ST, Sequence type; AA, Addis Ababa; DB, Debre Berhan

Table 12. ST, serotype, phylotypes, ESBL, ARGs, and mobile genetic elements associated with ARGs of ETEC stains of the present study.

Strain ID	**Study area**	**ST**	**Serotype**	**Phylo type**	**ESBL**	**ARGs**	**Mobile genetic elements**	
							Plasmid replicons	Transposons
ET-(1)	AA	ST 88	O9:H19	C	-	bla TEM-1B, dfrA14, tet(A), mph(A)	IncQ1	ISKpn19
ET-(2)	AA	ST 88	O9:H9	C	-	bla TEM-1B, mph(A),qnrB1, dfrA5, dfrA14, sul2, tet(A)	-	IS6100
ET-(3)	DB	ST5039	O11:H4	A	-	bla TEM-1B, ,sul1, dfrA7, tet (A), mph(A)	Incq1	-
ET-(4)	AA	ST 9401	O8:H30	B1	+	bla TEM-1B, bla CTX-M-15, blaSHV-106, dfrA17, mph(A), sul1, aadA5	IncY	IS6100
ET-(6)	AA	ST 453	O23:H4	A	+	bla CTX-M-15, dfrA8, dfrA17, mph(A), sul1, aadA5, tet (A),	-	IS6100

Abbreviation: ST, Sequence type; AA, Addis Ababa; DB, Debre Berhan

4.3.2. Phylogenetic analysis

Genome wide-SNP is the best measures of phylogenetic diversity (192). Genome wide-SNP can discriminate closely related organisms, consider evolutionary signals originating along the length of entire genome, captures the complete variation within species or strains, and provide a strong phylogenetic signal (192). In the present study, SNP whole genome based phylogenetic analysis showed that the hybrid strains were grouped in to three major clusters (Figure 11a). Strain EH-5 and EH-8 were clustered independently. The other six strains were grouped in one major cluster. However, through time there was variation and they clustered differently. Among some clustered strains there is similarity in phylogenetic group. Hybrid strains grouped in unknown phylotype showed independent clustering whereas strains grouped in A, B1, and C relatively clustered together.

The EAEC strains were grouped into three major clusters (Figure 11b). In the final clustering stage, strains with the same phylogenetic groups were clustered together. EAEC strains clustered together showed varied source of sample area. However, some EAEC strains clustered together showed similar characteristics of phylogenetic group. Phylogenetic linked to metadata revealed that isolates did not cluster according to study area but according to phylogroup. Most EAEC strains that were clustered together were grouped in the same phylotype otherwise cluster independently.

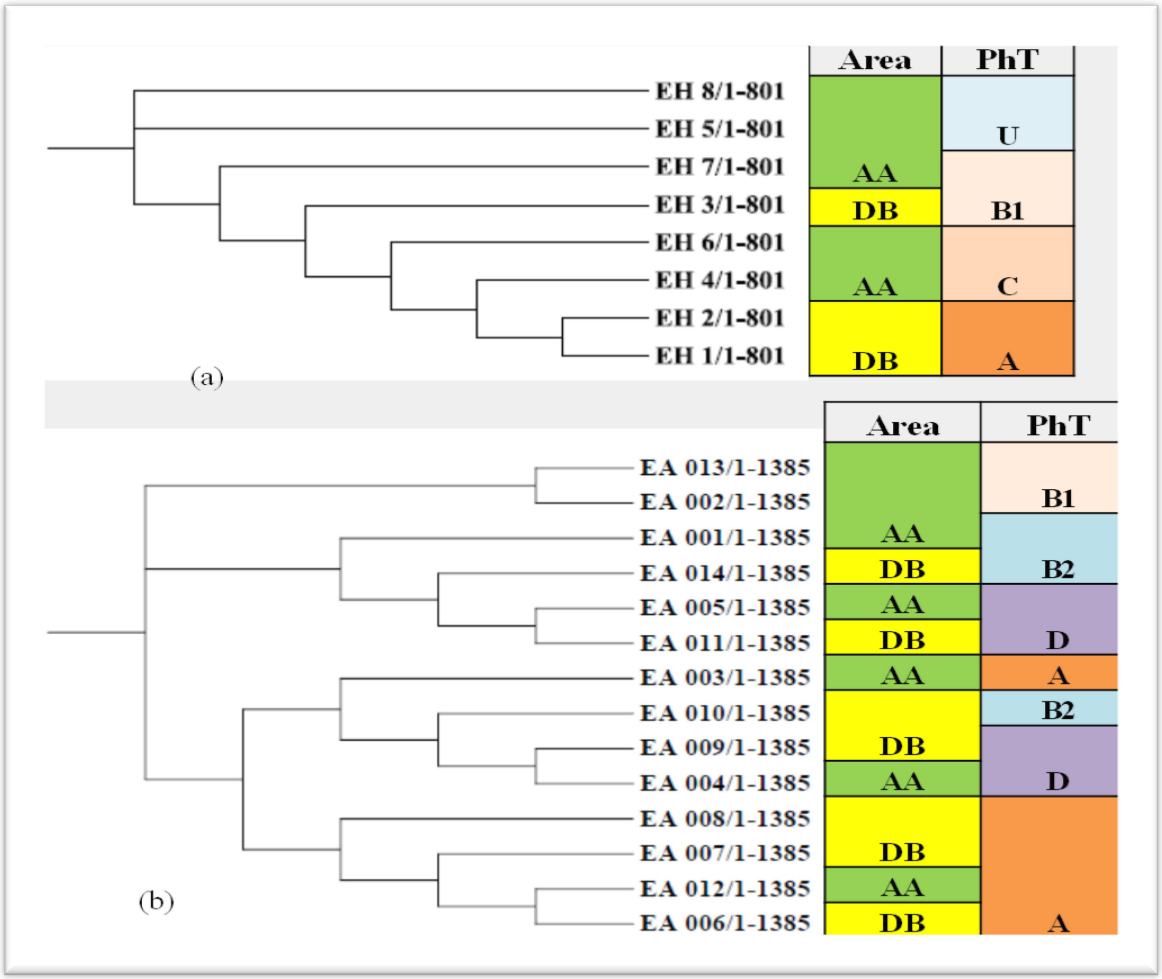


Figure 11. Phylogenetic tree of the hybrid strains (a) and EAEC strains (b) of the present study. The tree was constructed and annotated using interactive tree of life (ITOL). PhT, Phylotype; AA, Addis Ababa; DB, Debre Berhan.

The 8 hybrid strains and 14 EAEC strains were phylogenetically compared with other publicly available DEC pathotypes. The present hybrid strains were compared phylogenetically first with publicly available 10 hybrid strains (GenBank) and then together with 51 other DEC pathotypes from enterobase database (Figure 12). The hybrid strains with the 10 publically available were clustered in to three groups. EH-1 and EH-2 strains were clustered and show close relation with STEC/UPEC (ST 141, B2) hybrid strains. EH-4, EH-6 and EH-8 were clustered together with STEC/UPEC (ST 141, U/Cryptic) hybrid strains. The other 3 remaining strains (EH-3, EH-5, and EH-7) clustered with STEC/ETEC and EPEC/ETEC (four hybrids from GEMS study) hybrid strains.

When the present hybrid strains were compared with a total of 61 DEC pathotypes obtained from GenBank and enterobase databases, they showed a varied clustering with different strains isolated from varied origin. EH-3, EH-5, and EH-7 strains were clustered together with five different DEC pathotypes (2 STEC, 2 ETEC, and 1 EPEC) originated from different sources (human, animal, and food). EH-1 and EH-2 strains were clustered with a hybrid strains (STEC/UPEC) isolated from human in Germany. The remaining 3 hybrid strains (EH-4, EH-6 and EH-8) were clustered together with 44 different DEC pathotypes isolated from different sources and originated from different parts of the world. These 44 comparative DEC pathotypes (publicly available) were 9 hybrid strains, 13 EIEC, 10 STEC, 9 EPEC, 2 EAEC and 1 ETEC. Among the 9 hybrid strains, four were EPEC/ETEC, three were STEC/ETEC and two were STEC/UPEC. Of the 44 these DEC pathotypes, 26 were isolated from human, and other 11 from animals, 6 from food, and 1 from environment (water). Majority of the DEC pathotypes were from African/Asian countries (64%, 26/44), and the rest 36% (18/44) were from Europe, North and South America.

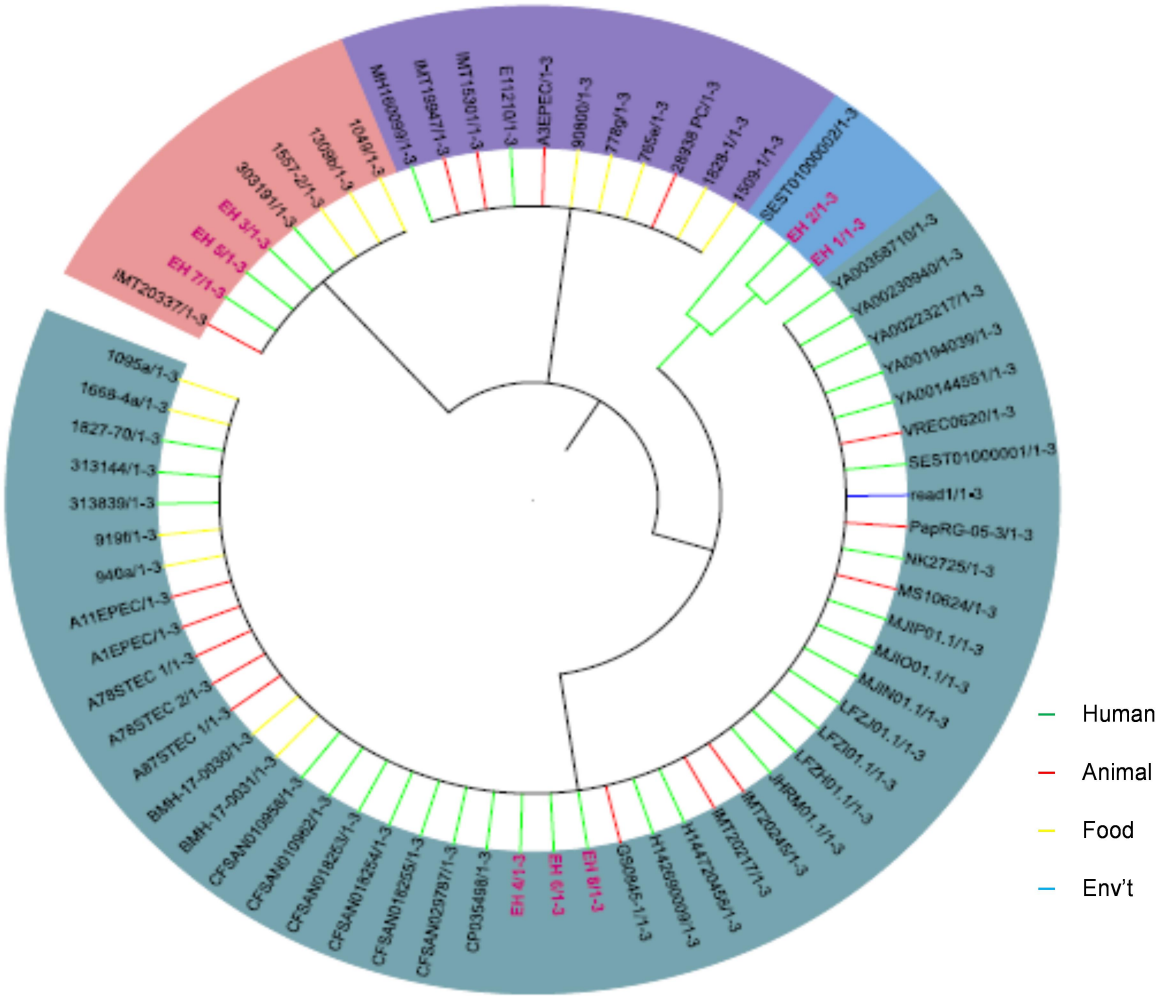


Figure 12. Circular phylogenomic tree that show the relationship between the hybrid isolates from this study and DEC isolates from publicly available databases. Taxa written in coloured font are the strains of the present study. The green line represent human origin, the red line represent animal origin, yellow line represent food origin and blue line represent environmental origin or source of the strains included this analysis.

In the EAEC strains, EA-001 strain shared a common ancestor with EPEC and STEC strains but clustered independently (Figure 13). EA-011 and EA-014 clustered together and share a recent ancestor with other STEC strains. Similarly, strain EA-013 and EA-002 were clustered together and share a recent common ancestor with other STEC strains. EA-008 was clustered with EIEC strain. EA-006 and EA-012 were independently clustered but shared a common ancestor with EPEC and STEC strains, respectively. EA-007 was clustered with STEC strains. EA-003 and EA-005 clustered together and share a recent common ancestor with

EPEC and EAEC strains. Similarly, EA-004, EA-009, and EA-010 shared a common ancestor with EAEC and EIEC strains.

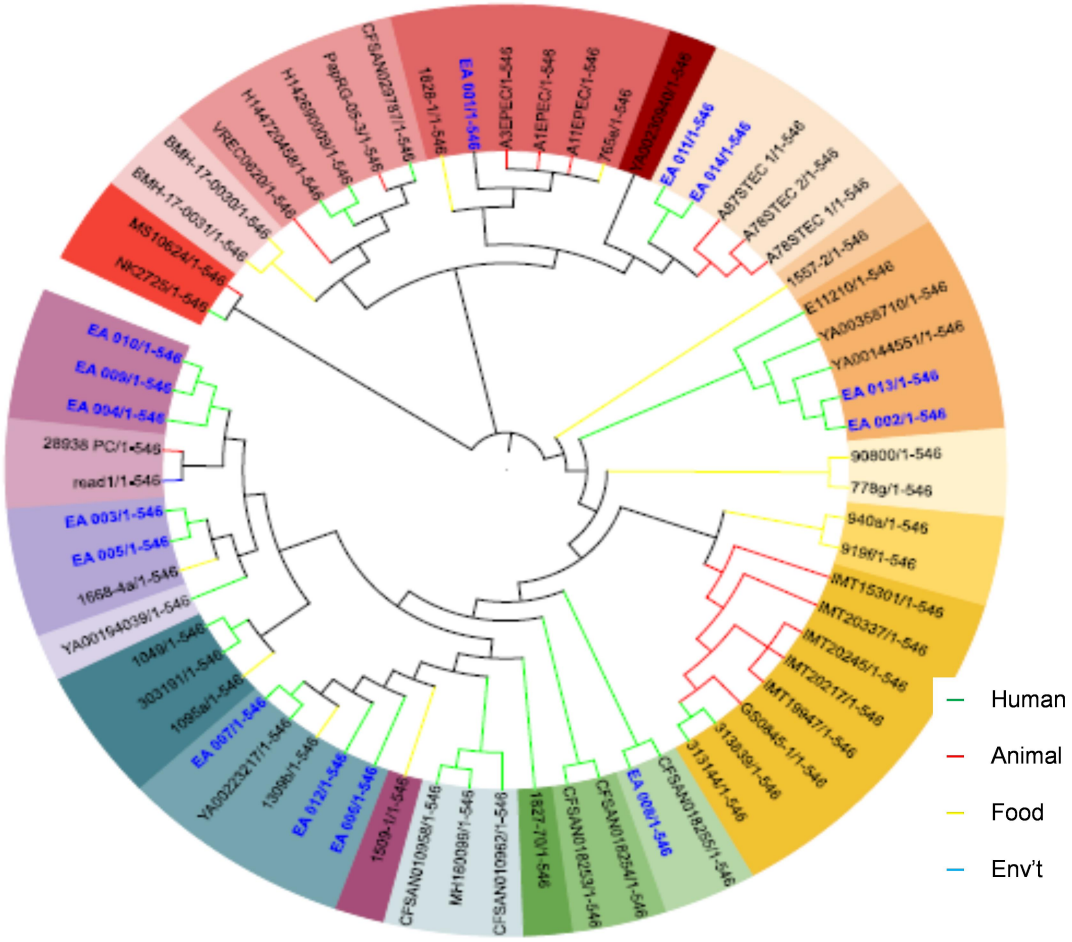


Figure 13. Circular phylogenomic tree that show the relationship between the EAEC isolates from this study and DEC isolates from publicly available databases. Taxa written in coloured font are the strains of the present study. The green line represent human origin, the red line represent animal origin, yellow line represent food origin and blue line environmental origin or source of the strains included this analysis.

4.3.3. Virulence genes (VGs)

A total of 64 different VGs were identified from the hybrid strains of the present study. The VGs include genes that encode adherence factors, secretory system, toxins, regulators, effectors and many other factors. Among these, 14 genes were for adherence factors, 12 were for effectors, 5 were for iron uptake system, 10 were for toxins, 3 were for major regulators, and the rest 20 were others with different role during the pathogenicity of the strains. The VGs of the present hybrid strains were compared with other publicly available hybrid strains

(Figure 14). In the present study, 11-33 VGs, and 7-28 VGs in comparative strains were identified. Strains that contain 20 or more VGs (63%, 5/8) were among the present hybrid strains compared to the comparative hybrid strains (20%, 2/10). In some strains of the present study, higher numbers of virulence factors were identified. Some virulence factors were seen both in the present strains and in the comparative strains. Genes that encode adherence factors and toxins were identified more in the present study compared to the comparing strains. VGs that encode for other factors were commonly found both in the present and comparative strains.

The analysis of VG profile of EAEC strains found 70 VGs. Many of the VGs encode adherence factors, effectors, toxins, regulators and other factors. Among these, 21 genes were for adherence factors, 13 for effectors, 6 for iron uptake, 9 for toxins, 3 for major regulation, and 18 others that encode different factors with different role during pathogenesis. The VGs of the EAEC strains were compared one another (Figure 15). The strains contain 11-40 virulence genes. Some VGs were higher in some strains such as EA-005, EA-008 and EA-013 compared to others. Similar virulence genes (including that encode adherence factors, effectors, and major regulators) were seen commonly in some of the EAEC pathotypes of the present study.

Figure 14. Hierarchical clustering of 8 present study and 10 publicly available hybrid strains based on virulence factors. The dendrogram and associated heatmap based on the presence or absence of 90 virulence genes were constructed using GraphPad Prism 9.5. Red and light green colors indicate gene presence and gene absence, respectively.

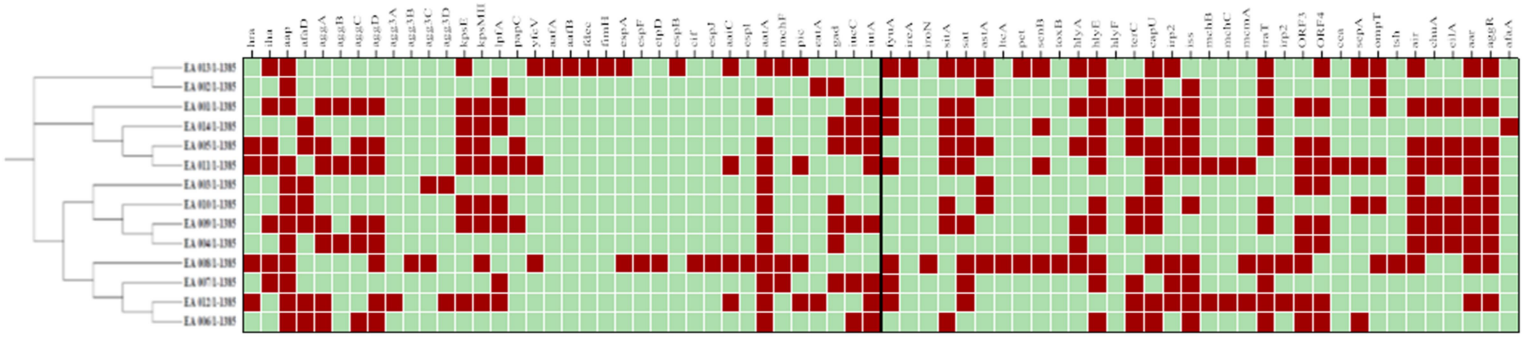


Figure 15. Hierarchical clustering of 14 EAEC strains based on virulence factors. The dendrogram and associated heatmap based on the presence or absence of 70 virulence genes were constructed using GraphPad Prism 9.5. Red and light green colors indicate gene presence and gene absence, respectively.

4.3.4. Antimicrobial resistance genes (ARGs)

Among the 28 DEC pathotypes subjected to WGS analysis, the hybrid strains were not MDR. The EAEC and ETEC strains were MDR strains. In the WGS analysis, a total of 29 variants of ARGs were identified from the present study (Table 13). The AMR genes include genes that encode for different classes of antimicrobials including ß-lactams, folate pathway antagonist, quinolone, tetracycline, macrolide, aminoglycoside and amphenicol. ARG that encode for resistance to ampicillin was detected in high number among the hybrid strains (88%, 7/8), EAEC (87%, 13/15), and ETEC (80%, 4/5). ESBL gene variants were identified from EAEC (Figure 16) and ETEC but not from the hybrid strains. Carbapenemase genes were also found in EAEC (7%, 1/15). In this study, ARGs that encode for resistance to folate pathway antagonist, quinolone, tetracycline, macrolide, aminoglycoside and amphenicol were higher in EAEC and ETEC compared to hybrid strains.

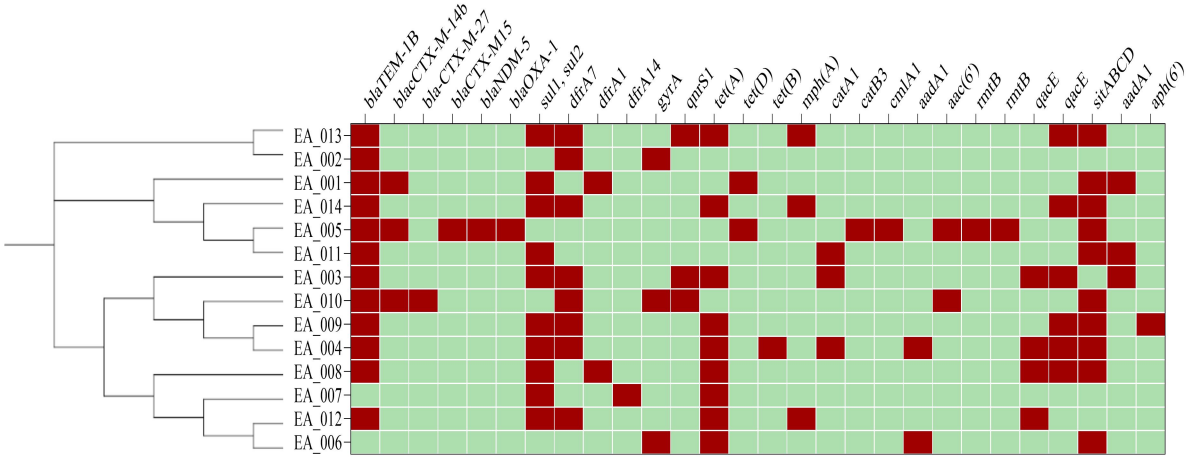


Figure 16. Hierarchical clustering of 14 EAEC strains based on antimicrobial resistance genes. The dendrogram was constructed using graph pad prism 9.4.

Different ß-lactamase gene variants were identified in the present study. Majority of the variant genes include blaTEM-1B (86%, 24/28) which were associated with resistance to ampicillin, amoxicillin, cephalothin or piperacillin. The second predominant ARG variant

was blaCTX-M-15 (14%, 4/28). The other ESBL and carbapenemase gene variants that cause resistance to wide ranges of AMR were identified in the present study (Table 13). In addition to the presence of potential ß-lactamase gene variants, six co-occurrences (two to five genes in a single isolate) of ß-lactamase genes were detected in the present study.

Table 13. Antimicrobial resistance genes (ARGs) profile of the present study (28 DEC pathotypes)

Class	Antimicrobials	ARGs	Hybrid (n=8)	EAEC (n=15)	ETEC (n=5)	Total (n=28)
ß-lactam	Ampicillin, amoxicillin, cephalothin or piperacillin	blaTEM-1B	7(88%)	13 (87%)	4(80%)	24 (86%)
	Cefoxitin, Ceftriaxone, Cefotaxime, Ceftazidime, Cefpime and Aztreonam	blaCTX-M-14b	0 (0%)	3 (20%)	0 (0%)	3 (11%)
		blaCTX-M15	0 (0%)	2(13%)	2 (40%)	4 (14%)
		bla-CTX-M-27	0 (0%)	1(7%)	0 (0%)	1 (4%)
		bla-SHV-106	0 (0%)	1(7%)	1 (20%)	2 (7%)
	Cefpime, impenem, ertapenem, and piperacillin+tazobactam	bla-NDM-5, bla-OXA-1	0 (0%)	1(7%)	0 (0%)	1 (4%)
Folate pathway antagonist	Sulfamethoxazole	sul1, sul2	4 (50%)	11 (73%)	5 (100%)	20 (71%)
	Trimethoprim	dfrA1 dfrA5, , dfrA7, dfrA8, dfrA14, dfrA17	6(75%)	12 (80%)	5 (100%)	23 (82%)
Quinolone	Nalidixic acid, Ciprofloxacin	gyrA, qnrS1, qnrB1	1 (13%)	5 (33%)	1 (20%)	7 (25%)
Tetracycline	Tetracycline, Doxycycline	tet(A), tet(B), tet(D)	6 (75%)	12 (80%)	4 (80%)	22 (79%)
Macrolide	Azithromycin, Erythromycin	mph(A)	1 (13%)	3 (20%)	5 (100%)	9 (32%)
Aminoglycoside	Streptomycin, Amikacin, Gentamicin	aadA1, aadA5, aac(6'), rmtB	2(25%)	8 (53%)	2 (40%)	12 (43%)
Amphenicol	Chloramphenicol	catA1, catB3, cmlA1	1 (13%)	4 (27%)	0(0%)	5 (18%)

4.3.5. Mobile genetic elements

In the WGS analysis of 28 DEC pathotypes, a total of 12 plasmid replicon types and 19 transposon types that harbour either ARGs or VGs were identified (Table 14, Annex 10). Two types of plasmid replicons (six IncQ1 and one IncY) were associated with ARGs. Seven transposon types (total 15) were associated with ARGs. A total of 19 plasmid replicons (10

types) and 21 transposons (12 types) were associated with VGs. Majority of the MGEs, 73% (19/26) plasmid replicons (83%, 10/12 type) and 58% (21/36) transposons (63%, 12/19 type) were involved in harbouring different virulence genes. The remaining 27% (7/26) plasmid replicons (17%, 2/12 type) and 42% (15/36) transposons (37%, 7/19 type) were involved in harbouring ARGs. Majority of the plasmid replicon types (73%) and transposons (58%) were associated with harbouring VGs in the present study.

The predominant plasmid replicon type in association with harbouring ARGs was the IncQ1 (86%, 6/7). In association with harbouring VGs, Col156 (21%, 4/19) and IncFll (pHN7A8) (21%, 4/19) were the predominant plasmid replicon type detected in the present study. Tn2 (33%, 5/15) and IS6100 (20%, 3/15) were the main transposon types associated with harbouring ARGs detected in the present study. The predominant transposon associated with harbouring VGs was ISSpu2 (29%, 6/21) in the present study.

Table 14. Mobile genetic elements (plasmid replicon type and transposon) that harbor ARGs and VGs in the *DEC pathotypes* of the present study.

MGE	**Types**	**Frequency (%)**	**ARGs/VGs**
Plasmid (n=7)	IncQ1	6 (86%)	blaTEM-1B, aph(6)-id, sul1, sul2, dfrA7, tet(A), tet(B), catA1, aadA1
	IncY	1 (14%)	bla CTX-M-15
Transposon (n=15)	IS102	1 (7%)	bla-CTX-M-27
	IS6100	3 (20%)	mph(A), dfrA17, sul1, aadA5
	ISEc9	1 (7%)	blaCTX-M-14b
	ISKpn19	2 (13%)	tet(A), qnrS13
	ISVsa3	1 (7%)	sul2
	Tn2	5 (33%)	bla TEM-1B, qnrS1, sul1, sul2, dfrA1, tet(A)
	Tn7	2 (13%)	blaTEM-1B, tet(D), , aadA1, dfrA1
Plasmid (n= 19)	Col156	4 (21%)	senB
	IncI1	1 (5%)	cia
	IncB/O/K/Z	1 (5%)	traT
	IncFIB	1 (5%)	hlyF, ompT
	IncFIB(pB171)	2 (11%)	afaD, aggC, aggA,, aggD, astA
	IncFIB (AP001918)	1 (5%)	capU, afaD, anr, aatA, sepA, traJ, ORF4, agg4D, aap, aggR
	IncFll	2 (11%)	toxB, aap
	IncFll(pCoo)	2(11%)	traT, eatA

	IncFll(pHN7A8)	4 (21%)	aap, traT
	IncFII(Prsb107)	1 (5%)	traT, anr
Transposon (n=21)	ISEc17	1 (5%)	aggC, aggD, aggA, aggB
	ISEc30	1 (5%)	espI, agg3A, agg3C, ORF4, agg3D, astA, agg3B, ORF3
	ISEc37	1 (5%)	eatA
	ISEc38	2 (10%)	pic
	ISEc43	1 (5%)	sat
	ISEc45	1 (5%)	iha
	ISEc48	1 (5%)	aafA, aar, aafD, aggR
	ISEic2	2 (10%)	astA
	ISRaq1	1 (5%)	astA
	ISSpu2	6 (29%)	aap, aar, aatA, aggR, ORF3, ORF4
	ISSso4	1 (5%)	etsC
	MITEEc1	3 (14%)	espA, espF, tir, IpfA, eae, terC, iss

Chapter five

5. Discussion

5.1. Occurrence of *E. coli* and other diarrhegenic bacteria

Diarrhea due to bacterial infection is often self-limiting and does not require identification of the etiological agent for patient management. However, in severe or prolonged cases, with symptoms consistent with invasive disease, or in patients with potential complications, the etiological agents need to be identified for effective treatment (38). In children under five years old, diarrheal disease is the second leading cause of death (91). Sadly, this is both preventable and treatable. The WHO with UNICEF (the United Nations International Children's Emergency Fund) developed the global integrated action plan to prevent and control pneumonia and diarrhea. The goal of the plan is to see in the reduction of death associated with these diseases. The target for diarrhea is to be less than 1 in 1000 live births by 2025. Among the interventions that are assumed to reduce the number of deaths is use of appropriate treatment in addition to the public health interventions. Epidemiological data to identify and track the causes of outbreaks of diarrheal diseases is important to plan and mobilize resource. It is with this in mind this PhD project was initiated. Periodic surveillance of the aetiology, the antimicrobial resistance pattern and burden of diarrhoeal disease is important. This PhD project focused on *E. coli*. In the project, the aetiology of the diarrhea, the AMR patterns as well as the molecular basis of the AMR were addressed.

The occurrence of *E. coli* isolates in the present study is higher than other previous reports (64, 74, 75). This increased occurrence of presumptive *E. coli* isolates in UFC with diarrhea may suggest the presence of medically important *E. coli* strains (DEC). Although the focus of this study is DEC we had the opportunity to isolate other bacteria, and this includes *Salmonella*, *Shigella,* and *Campylobacter.* However, the occurrence of *Salmonella*, *Shigella,*

and *Campylobacter jejuni* was low. Previous studies were comparative with the present finding for *Salmonella* (59, 84, 193) and *Shigella* (59, 61, 84), and *Campylobacter* (58). However, a slight difference was seen between the present study and other previous studies (59, 63, 84) for *Campylobacter*. The discrepancy may be due to variation in the study design (cross sectional versus systematic review), study population and detection method. Meta-analysis study estimates the pooled prevalence regardless of difference in local prevalence, unlike cross-sectional study. In the present study, only *Campylobacter jejuni* and *Campylobacter coli* were targeted, unlike other studies where they report at the genus level. The present finding supports the previous studies (59, 84) that occurrence of *Salmonella*, *Shigella*, and *Campylobacter* low variations (58, 61, 62, 193). However, *Shigella* and *Campylobacter* showed relatively a higher occurrence compared to *Salmonella* in this study.

5.1.1. Molecular epidemiology of DEC

DEC is one of the etiological agents for diarrheal diseases in UFC (26). In DC, DEC has contribution to more than 30% of acute diarrhea episodes in children (32). *E. coli* is an intestinal pathogen, extra-intestinal pathogens, or commensals (25, 105). Differentiating the DEC pathogen from commensal allows understanding the true occurrence of DEC pathotypes. The epidemiological data on DEC is limited in DC including Ethiopia, and the lack of the common LDMs (70) or limited applications of currently available LDMs in routine laboratories (32) contributed to the problem. In Ethiopia, the molecular epidemiology of DEC and its resistance profile is not well known. As DEC pathotypes are the leading causes of diarrhea associated with morbidity and mortality in UFC in DC, characterization of DEC was the primary objective of the present study.

DEC was isolated from 38.4% of UFC in the present study, and this is comparable to other reports, including 29% (42/144) from Bahir Dar (66) and 34.5% (38/110) from Wolaita Sodo (67) Ethiopia, 48% from Sudan (194), and 48.6% from Mozambique (195). However, a

higher occurrence of DEC was reported from other countries, Nigeria 73.8% (196), South Africa 82% (197), and Iran 90% (198). Other parts of the world reported a lower occurrence of DEC, China 7.9% (164), Colombia 9.8% (172), and India 17.4% (50). This discrepancy may be due to geographical differences associated with climate variability, different study population, water supply that could influence incidence of DEC, and other socio-economic and political factors. The high occurrence of DEC in many of the DC includes the present finding compared to other high-income countries showed the negative impact of DEC in these countries. As this is a preventable disease through access to safe drinking water, use of improved sanitation, handwashing with soap, good personal and food hygiene, and health education, the finding is the reflection of this practice in these countries (9). Identification of DEC pathotypes needs molecular techniques (to distinguish from commensal *E. coli* isolates) following traditional culture and biochemical tests. However, in Ethiopia, let alone molecular techniques like PCR assay in diagnostic laboratories, the traditional culture and biochemical tests are limited to few laboratories. In fact even some argue that the conventional methods (culture plus multiplex PCR) may underestimate the occurrence of the pathogens (199). Therefore, the present finding suggests the need of screening or LDM for DEC pathotypes in health facilities for correct patient management. The finding could initiate health personnel or other stakeholders for enhancement of laboratory diagnostic capacity such as culture and PCR assay to allow an immediate patient management at health facilities. It also helps in establishing active surveillance program and strengthening the IPC measures.

The occurrence of DEC in Addis Ababa and Debre Berhan was not statistically different ($p>0.005$) despite the demographic and geographic differences between the two areas, demonstrating the clinical importance of DEC in both study areas. This could be due to the presence of risk factors both areas, including pipe water supply interruptions in Addis Ababa (70) and contact with animals in Debre Berhan (72) that contributes to the transmission of *E.*

coli strains. DEC was detected in 40.7% of diarrheic and 28.2% of non-diarrheic UFC (p-0.020) in the present study. This finding agrees with reports from Nigeria (196), and Iran (198). The high occurrence of DEC among diarrheic cases compared to the healthy controls in the present study could show the clinical role of DEC in UFC in Ethiopia. In addition, the finding also shows the possible transmission of DEC pathotypes from asymptomatic individual (potential risk for immune-incompetents), and the clinical disease due to DEC varied depending on the immune status of the individual and the toxin variants of the specific DEC pathotypes (25). The presence of medically important strains in the study areas with significant association in diarrheic UFC could suggest the need of a careful diagnosis and treatment practice as well as attention in the IPC measures against DEC infection in Ethiopia.

EAEC (41.5%) was the predominant DEC pathotype identified in the present study and this was similar to the reports from Mozambique, with a higher level of 66.3% (195), Sudan, with 43% (194), and others (196, 200, 201). A recent report from Ethiopia (67) also found a similar finding with the present study. However, another recent report from Ethiopia (66) found low EAEC compared to other DEC pathotypes, in consistent to the present study. The absence of a well-defined working definition, its genetic heterogeneity (more genetic plasticity) (202) and difference in risk factors could contribute to varied occurrence of EAEC. EAEC causes persistent diarrhea in children in endemic regions (25). Persistent or chronic infections by EAEC can damage the intestinal epithelium and cause malnutrition in children in DC. EAEC alone or in hybrid strains (EAEC/EHEC) caused outbreaks in different areas of the world, even with fatal consequence (25). Thus, the presence with high occurrence of EAEC with emerging nature, persistent infections, and outbreaks characteristics could be considered as a potential health threat in UFC in the area. It needs diagnosis of the DEC pathotypes and appropriate patient management to prevent its burden in UFC with diarrhea.

The second most prevalent DEC pathotype was ETEC (21.3%) and was consistent with studies done in Nigeria (196), China (164), Sudan (194), and Iran (198). The recent report (66) from Ethiopia also support the present finding. However, there are reports with lower occurrence (172, 203) including a recent report from Ethiopia (67). Age category, case type, sample-taking methods, study period, and design may contribute to the discrepancy. The major virulence factors for ETEC are the LT and ST (25). The ST-positive ETEC is more frequently found in severe infections (25, 204). In the present study, the predominant is LT-positive ETEC. ETEC has two variants of LT, LT-I and LT-II (26) with further many sub-variants (25). Variable LT expression may have impacts on the severity of ETEC-associated disease (26). In one study, ETEC LT toxin has been associated with changing gene expression in brush border of intestinal epithelial cells and it showed affecting their absorption capacity (205). Due to this, ETEC infection has long term consequences including physical and cognitive stunting and delayed educational attainment (206). The presence of ETEC in high occurrence can be a potential threat to human infections in particular UFC, affecting their critical developmental milestones. One previous study found high (41%) stunting in UFC in Debre Berhan (207) and this could be not only due to nutritional deficiency but also due to persistent enteric infection such as ETEC-related infections in the area. In addition, ETEC infections were associated with increased fatality rate in previous large scale study (33). Thus, this finding could alarm public health stakeholders including policy makers and donors to generate surveillance data for better understanding on the distribution and magnitude of the burden, to use globally available interventions and to establish implementation strategy. And also it suggests the need of screening for ETEC including the toxin variants and timely treatment of ETEC-related infections to prevent mortality and long term consequences.

The present study only detected atypical EPEC, which lacked bfp and this is similar to a study in Norway (208), China (164), and recent studies in Ethiopia (66, 67). In studies done in India (209) and Saudi Arabia (210), they reported a changing trend of aEPEC over tEPEC in UFC with diarrhea. Unlike tEPEC (has only human reservoir), aEPEC has human and animal reservoir (25), and poor hygiene, poor sanitation, and improper food hygiene could contribute more to the occurrence of aEPEC. Atypical EPEC is highly heterogeneous group and closely related with some STEC strains (25). The strain is also associated with persistent diarrhea and inflammatory bowel diseases (211) and potential to cause outbreaks (25). One study showed that aEPEC clone emergence occurred through acquisition of distinct LEE subtype that are associated with distinct chromosomal backgrounds and insertion sites (212). Characterization of the LEE PAI variants of the strains present in the study area could allow knowing the types of aEPEC clones (or distinct LEE subtype). Thus, the presence of this aEPEC strains with heterogeneous or emerging nature and ability to cause persistent infections could create a huge burden on the children, and threat for occurrence of outbreaks in the study area.

The fourth predominate DEC pathotypes in the present study was EIEC (13%). Similar finding were reported from Iran (200), Mexico (213), and Burkina Faso (201). However, low occurrence of EIEC also reported in India (50), China (164), and in Mozambique (195). EIEC is identified after 50 years of *Shigella* discovery (26). EIEC are biochemically, genetically, and pathogenically closely related with *Shigella* (25). EIEC has few distinguishing characteristics from *Shigella* including its milder virulence (low virulence) and a higher infectious dose, ability to ferment mucate and utilize serine, xylose or sodium acetate, and low gene reduction rate (25, 214). Thus, the discrepancies of these reports and the present study may be due to the variation in discriminating *Shigella* and EIEC. The challenges to distinguish EIEC from *Shigella* made EIEC to be underestimated epidemiologically (25).

The low occurrence of STEC (1.6%, 3/183) in the present study is comparable with other reports (172, 203, 215). However, there are reports with higher STEC occurrence (17%) (66, 198) and with no detection of STEC (195). The discrepancy may be due to the difference to exposure to the risk factors, which include foreign travel, contact with ill people, farm animals or their environment, food consumption and exposure to untreated drinking water (216). STEC can cause diarrhea, haemorrhagic/bloody diarrhea, and HUS; progress to life-threatening HUS (25). And all the present STEC strains were EHEC (stx+ and eae+). Only one DAEC strains (0.6%, 1/183) was detected in the present study. A study done in Brazil revealed that DAEC strains were recovered in similar frequencies from diarrheic (16.5%) and asymptomatic children (19.6%), and more frequently from adults with diarrhea (18.9%) ($P < 0.01$) than from asymptomatic adults (4.2%) (217). The discrepancy may due to the number of target genes used for detection (afaE versus daaE/ daaF).

The newly emerging hybrid pathotypes of DEC were also detected in the present study. These hybrids include ETEC/EAEC, ETEC/EPEC, and EPEC/EAEC. The term hybrid pathotype refers to new combinations of virulence factors among the classic *E. coli* pathotypes (218). In 2015, an EPEC expressing the ETEC heat-labile toxin was observed in India (219). The same combinations of hybrid pathotypes to the present study were reported in a study done in South Africa (220). The hybrid strains in the present study were detected, 85% (11/13) from diarrheic and the remaining (15%) were from non-diarrheic. Recent reports from Ethiopia (66, 67) also indicated the occurrence of DEC hybrid pathotypes. Detection of such a combination of virulence factors in *E. coli* circulating in the community may lead to the occurrence of severe disease due to strains with higher pathogenic capacity (hyper-virulent strains) in the area. The *E. coli* genomic plasticity that results in the emergence of new hybrid strains or pathotypes could result in severe outbreaks (218). The detection of hybrid strains should be of public health concern that needs to be carefully monitored through

a SP. And laboratory diagnostic capacity that allow detection of the DEC pathotypes at the health-care facility level need for establishment of SP.

5.1.2. Factors associated with DEC acquisition

Socio-demographic factors associated with DEC outbreaks in UFC were season, family income, child-care, time of supplementary food starting, availability of domestic animals, and frequency of getting safe water. UFC were more likely to acquire DEC during the rainy season compared to the dry season in the present study. In agreement with this, studies conducted in China and Mexico reported that seasonal distribution revealed that DEC tended to occur during rainy season in children (164, 213). It has been reported that bacterial-caused diarrhea was high during rainy season and diarrhea due to viral pathogens was high during dry season (221). Low family income was another factor associated with increased DEC infections, most likely due to the lack of hygiene and sanitation facilities. Children who were cared for by their mothers were more protected than those who were cared for by others including grandmothers, close family members, and day care. This may be due to that the mother is more likely careful than other caregivers, potentially contributing to the incidence of DEC in the children. In addition, children whose mothers began supplemental food for their baby before 6 months were more likely to have DEC compared to those whose mothers started it after 12 months. It could be due to exposure to contaminated food through frequent contact during baby feeding. In addition, in some DEC pathotypes, like EPEC, infections are more associated in children age below 2 years and decrease with age due to loss of specific EPEC receptors and development of immunity. And this could more contribute to DEC infections in earlier age (before 6 months) compared to older one (after 12 months). The availability of domestic animals in the compound causes the children to be more likely to get DEC compared to those with no animals in the compound. STEC, ETEC, aEPEC, and EIEC are transmitted feco-orally to humans from animal reservoirs (25). Children who lived in the

family who got water in shift were more likely to get DEC compared to those children in families with daily water supply. A previous study in Ethiopia reported that periodically intermittent piped water supplies and point-of-use contamination of household stored water by *E. coli* were associated with acute diarrhea among UFC (70). In Ethiopia, most households in both urban (88%) and rural (92%) areas do not treat their water before drinking, and this likely increases the risk of DEC transmission (15).

In conclusion, the present study found low occurrence of *Salmonella, Shigella* and *Campylobacter jejuni* among UFC in Ethiopia. However, potential intestinal pathogenic *E. coli* for outbreaks or severe diseases were found with high occurrence and resistance profile in the study. All the six common pathotypes were found in the present study. DEC were found significantly associated with diarrhea and distributed across the study areas without significant difference. Strains that are commonly associated with high fatality rate, persistent infections, severe disease and outbreak, and that lead to long term consequences in UFC were found. Rainy season, child care by others (grandmothers, close family members, and day care), early beginning of supplement food, presence of animal in the compound and obtaining water in shift were identified contributing factors for DEC acquisition in the present study. The finding could be an evidence for initiating screening of DEC pathotypes in the health facilities, strengthening clinical laboratories capacity, initiate active surveillance programs, and monitoring the IPC measures against the potential bacterial pathogens.

5.2. Antimicrobial resistance (AMR) profile

5.2.1. AMR profile of DEC and other diarrheagenic bacteria

Most DEC infections are self-limiting and do not require intervention; however, those with severe, persistent, and invasive diseases due to EPEC, EIEC, ETEC, EAEC, and DAEC may require the use of antimicrobials (25). The Ethiopian guideline for treatment of diarrheal diseases recommends antimicrobials such as ciprofloxacin,

sulfamethoxazole+trimethoprim, and ceftriaxone (79). However, inappropriate antimicrobial utilization and management is a problem in Ethiopia (82). In the present study, DEC pathotypes were resistance to ampicillin (95.1%, 174/183), tetracycline (91.3%, 167/183), trimethoprim-sulfamethoxazole (42.6%, 78/183), ciprofloxacin (14.2%, 26/183), and ceftazidime and cefotaxime (16.4%, 30/183). DEC pathotypes were also found resistant to these commonly prescribed drugs in other studies, including in Mozambique (195), China (164), and South Africa (197). However, compared to other reports (164, 196, 197), in the present study, the rate of resistance to trimethoprim-sulfamethoxazole and ciprofloxacin were low. In addition, reports from recent studies in Ethiopia (66, 67) also found a varied resistance profile of DEC pathotypes against the different antimicrobials; some are consistent with the present study. That means, ciprofloxacin and trimethoprim-sulfamethoxazole relatively could be choices of treatment in Ethiopia following antimicrobial susceptibility tests. Nowadays, the emergences of resistant strains are becoming treat to our health (222, 223).

In Ethiopia, *E. coli* isolated from stool samples were found resistant to 3rd generation cephalosporin and carbapenem drugs (224, 225). However, there was no specific report on DEC pathotypes that showed resistance to these drugs in Ethiopia. The Ethiopian essential medicine list grouped the 3rd generation cephalosporin and carbapenem drugs as watch and reserve group antimicrobials, respectively (226). Watch group antimicrobials are most of the highest priority agents and widely used empiric treatment options for human medicine (226), and resistance will lead to use of reserve group antimicrobials. They are antimicrobials only prescribed for specific indications because they are at higher risk of bacterial resistance. Reserve group antimicrobials (called last-resort) are used when all alternatives have failed (226), and resistance to the last-resort antimicrobials lead to the death of the patient or no options for treatment. The presence of DEC pathotypes resistant to 3rd generation

cephalosporin and carbapenem drugs in the present study could show the extent of the problem in the areas. *E. coli* strains also could be an indicator to potential risk of transmission to and from human and animal populations, and the present finding inform about circulation of AMR in the human population (47, 48). Thus, protection and prioritization from misuse of these drugs have to be given attention at the right time.

DEC resistance to ceftazidime and cefotaxime were significantly higher in diarrheic children compared to non-diarrheic children (p=0.010) in the present study. The finding of the present study could be explained by the fact that HGT between pathogenic and commensal Enterobacterales can be enhanced by inflammatory diarrhea (227), thereby higher resistance among diarrheic groups. However, the controls used in the present study were small compared to the cases, and may need further matched case-control studies to conclude that DEC pathotypes resistant to 3rd generation cephalosporin are more prevalent in diarrheic than non-diarrheic children in Ethiopia..

Salmonella and *Shigella* cause a well-characterized spectrum of disease in humans (116) and need treatment. However, different studies reported resistant strains of *Salmonella* and *Shigella* to commonly used drugs in Ethiopia. A study done in Ambo town, Ethiopia (228) found a higher number of *Salmonella* and *Shigella* isolates that were resistant to ampicillin (88.9%), tetracycline (66.7%), cotrimoxazole (55.6%), and chloramphenicol (44.4%). In the present study, *Salmonella* and *Shigella* isolates were resistant to ampicillin (87%-100%), tetracycline (85%-88%), and cotrimoxazole (38%-63%), similar to the report from Ambo (228). However, 10%-25% resistance to ciprofloxacin was seen in the present study contrary to reports from Ambo where all isolates were sensitive to it (228). No *Salmonella* isolates was found to be resistant to third and fourth generation cephalosporin and carbapenem drugs in the present study. *Shigella* isolates were resistant to cefotaxime and ceftazidime (8%), and Meropenem and Ertapenem (3%). According to a systematic review and meta-analysis done

in Asia, the pooled prevalence rate of MDR and ESBL-producing *Shigella* strains was 68.7% and 23.9%, respectively (229). In the present study, MDR *Shigella*, more specifically ESBL- and carbapenemase-producing was identified in Ethiopia.

5.2.2. MDR, ESBLs, and Carbapenemases in DEC

The occurrence of MDR in the present study was 43.2% which is consistent with studies done in Ethiopia (67), China (164) and India (50). However, the MDR rate in the present study (43%) was lower than report from Iran (78.1%) (200) and another study from China (66.7%) (164). The inconsistency may be due to true variation, difference in contributing factors, variation in DEC pathotypes and geographical variation. However, the MDR characteristic of the DEC in the present study was found to be comparative with other reports (230, 231). Higher rate of MDR was found in EAEC (58%), ETEC (44%) and EIEC (30%) in the present study. Abbasi *et al* (200) also reported high rate of MDR in EAEC (82%), ETEC (67%) and EIEC (100%). ETEC and EAEC are associated as major cause of traveller's and chronic diarrhea (25). EIEC is also associated with shigellosis (25). When infection associated with EIEC, ETEC, and EAEC is severe or prolonged, treatment with antimicrobials is needed (25). However, the presence of MDR EIEC, ETEC, and EAEC strains could be challenging in the treatment of such strains using common and easily access antimicrobials in the study areas. Thus, the presences of MDR strains have to be considered during the treatment practice of enteric bacterial infections.

Globally, there is a serious and an urgent threat due to ESBL- and carbapenemase- producing Enterobacterales (49). High occurrence of ESBL-producing *E. coli* faecal carriage among children in one of the study area, Addis Ababa, was previously reported in line with the present finding (224). The presence of ESBL-producing DEC pathotypes in the gut may result not only in rapid spread of the resistance trait to non-resistant bacterial strains (154) but also will be a problem in patient management. In Ethiopia, mortality rate was associated with

infections caused by ESBL-producing Gram negative bacteria (232). Mandal *et al* reported occurrence of ESBLs among ETEC (18.32%), EPEC (10.9%), EAEC (6.8%), and EIEC (1.57%) from diarrheal children in India (222). In the present study the occurrence of ESBLs in ETEC, EPEC, and EIEC is comparative with Mandal *et al* report (222). However, in the present study among the DEC pathotypes isolated, 19.7% of EAEC and 15.3% of ETEC were ESBL producers; when compared with other DEC pathotypes, and the difference was statistically significant ($p<0.001$) in the present study. The occurrence of carbapenemase-producing strains was 2.2%, and found only in ETEC and EAEC strains. This low occurrence of carbapenemase-producing strains which was inconsistent with studies done in China (14.3%) (233) could show at least the emergence of carbapenemase DEC resistant strains circulating in Ethiopia. The ESBL- and carbapenemase- producing DEC pathotypes of the present study could suggest the existence of the problem, the need of clinical laboratory capacity and active SP that allow to have timely data on emergence of highly resistant strains. .

5.2.3. Molecular epidemiology of ß-lactamase genes in DEC

Nowadays, ESBL-encoding genes *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$ (except SHV-1), and *bla*$_{\text{TEM}}$ (except TEM-1 and TEM-2) are distributed in *E. coli* isolated from human (clinical and healthy carriers), including in this study, animals, food and environments (233). These common ESBL were also reported in *E. coli* strains isolated from sepsis patients in Ethiopia (85, 86). In agreement with those reports, the present study also found the ESBLs encoding genes (*bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$) from DEC pathotypes. The occurrence of ESBL-encoding genes detected in the present study were *bla*$_{\text{TEM}}$ (80%), *bla*$_{\text{CTX-M}}$ (73%) and *bla*$_{\text{SHV}}$ (60%). From a study in India, 86.1% of *bla*$_{\text{CTX-M}}$, 68% of *bla*$_{\text{SHV}}$, and 52% of *bla*$_{\text{TEM}}$ were detected from *E. coli* isolated from children with diarrhea (222) which is consistent with the present study. Other studies done in Iran (200) and Ghana (223) were also in line with the present

study. However, Monira *et al* (162) reported lower rates of these genes 39% $bla_{\text{CTX-M}}$, 26% bla_{TEM}, and 12% bla_{SHV} from children in Bangladesh compared to the present study. The discrepancy may be due to difference in study population (age and case type), sample size and detection methods. The present study was based on detection of the ß-lactamase genes by PCR assay and need sequencing for confirming some of the ß-lactamase gene variants detected in this study, specifically bla_{TEM}, and bla_{SHV}, are ESBL or not. In addition, other contributing factors such as variation in antimicrobial use, household hygiene practice, travel to high risk areas, and water source (224, 234) could contribute to the difference. However, more work is needed to conclude that the contribution of these risk factors (antimicrobial use, household hygiene practice, travel to high risk areas, and water source) for acquisition of ESBL-encoding genes in the present study areas. The present study showed ESBL-encoding genes circulating in the community and the need to understand contributing factors through large scale study for better understanding and measures to reduce the threat in the treatment practice.

In the present study the predominant ß-lactamase gene variant is bla_{TEM} which is contrary to other reports in Ethiopia from Enterobacterales in sepsis patients (85, 86) and other clinical disease (235) where $bla_{\text{CTX-M}}$ is more prevalent. The possible justification for the discrepancy may be, all the bla_{TEM} detected in the present study may not be ESBL variants (as TEM-1 or TEM-2 are not ESBL). DEC is the only strains of *E. coli* investigated in the present study whereas different strains that are members of Enterobacterales were involved in previous studies (85, 86). EAEC was the predominant MDR that showed higher occurrence of bla_{TEM} in the present study, and bla_{TEM} may be associated with resistance to antimicrobials in penicillin group; contribute to its high occurrence as it seen in the phenotypic analysis of this study. The types of DEC pathotypes identified predominantly in the study may contribute to the occurrence of the common ESBL variants. Therefore, the reason for dominance of

blaTEM over bla$_{CTX-M}$ seen in the present study, unlike most reports (85, 86, 156), may be due to the TEM-1 or TEM-2 variants or true variation (need further analysis). However, in agreement with the present finding, studies conducted in Iran from UFC (236) and in Ghana from diarrheic patients (223) found *bla*$_{TEM}$ as predominant ESBL gene. As all *bla*$_{TEM}$ are not ESBLs and only ESBL *bla*$_{TEM}$ variants have activity against cefotaxime (157), more molecular characterization work may be needed for better understanding on the occurrence of specific ESBL-encoding gene variants.

In the present study, *bla*$_{TEM}$ was detected in 83% (5/6) of ETEC, 87% (13/15) of EAEC, 67% (2/3) of STEC, and in all EPEC (3/3), and EIEC (2/2) strains. In the present study *bla*$_{CTX-M}$ was not detected from EPEC (0/3) and EIEC (0/2). Whereas *bla*$_{SHV}$ was detected from the two of the six ETEC and two of the two EIEC (2/2) in the present study contrary to previous report by Abbasi et *al* (200) where *bla*$_{SHV}$ in EIEC and ETEC were not detected. As the number of isolates is low it is difficult to comment on the discrepancy in the variation in acquisition of antimicrobial resistance determinants at different selection pressure in different area (237).

In Norway using data from 2007 to 2014 showed an increasing number of cases with carbapenemase producing enteric bacteria per year (238). The carbapenemase encoding genes, *bla*$_{NDM}$ and *bla*$_{OXA-48\text{-like}}$, are becoming the predominant carbapenemase variants detected in *E. coli* strains *(238).* In another study also carbapenemase genes such as *bla*$_{NDM-1}$ was identified in DEC pathotypes (215). In the present study, from phenotypically carbapenemase producing DEC pathotypes, *bla*$_{OXA-48}$ (13%) and *bla*$_{NDM}$ (13%) were detected at similar rates. Carbapenemase *bla*$_{OXA-48}$ was reported in different studies, these includes 31% in Egypt (239), 57% in Burkina Faso (240), 29% in Kenya (241), and 33% in Uganda (242). The higher occurrence in these studies compared to the present study may be due to the presence of high rate of emergence of carbapenemase-producing Enterobacterales in these

countries compared to Ethiopia (243). In addition, variation in the presence of risk factors contributing to the emergence of carbapenemase-producing bacterial strains (244) could contribute to the difference. In a studies done in China (164) and Egypt (239), bla_{NDM} was detected in DEC isolated from children. Both bla_{OXA-48} and bla_{NDM} hydrolyze penicillin and carbapenem, and bla_{NDM} hydrolyze cephalosporin and extended spectrum cephalosporin in addition (155). Detection of bla_{OXA-48} and bla_{NDM} in the present study could suggest the potential risk of dissemination of carbapenemase determinants in the areas.

In the present study 33% of the DEC pathotypes contain all the three common ESBL genes in combination (overlap) as it is reported by Nwafia *et al* in Nigeria (245). The co-existence was also seen for the carbapenemase genes in the present study. A study done in Egypt from clinical isolates of *Klebsiella pneumoniae* found that 48% co-harbored both bla_{NDM-1} and bla_{OXA-48} genes (181). The occurrence of β-lactamase genes in overlap (co-occurrence) in single strains suggests incidence of MDR DEC pathotypes, risk of dissemination of resistant determinants and emergence of resistance strains in the study areas.

Generally, high number of DEC pathotypes was resistant to commonly used antimicrobials and predominantly seen in EAEC and ETEC. The presence of MDR strains, including ESBL- and carbapenemase-producing DEC could be a challenge for treating severe or prolonged DEC related infections. Ethiopia developed and approved National AMR Surveillance Plan for laboratory-based AMR surveillance which was limited to few sentinel surveillance sites (246). The established surveillance systems was not enough in both access and pathogen targets. In the present study, the presence of resistance genes, in particular of ESBL and carbapenemase revealed the potential risk of wide spread of these resistant strains. These suggests the need of including the DEC pathotypes in the SP as one target organism and increasing sentinel surveillance sites for better monitoring. In 2021, Ethiopia revised the

AMR prevention and containment strategic plan to follow the one health approach (48). The plan has five strategic objectives including improve awareness, strengthening knowledge and evidence, enhance infection prevention and control, optimal utilization, and strengthening collaboration. On the other hand, in Ethiopia, diarrhea is among the leading health problem in UFC (15, 54, 55, 57) and based on the present study, DEC is found the major bacterial pathogens associated with diarrhea in UFC. The national IPC strategy and measure have to include DEC infection in UFC. In addition, the presence of different DEC pathotypes associated with diarrhea underline to have strict and evidence based treatment practices. Thus, the present finding will be a good input during the implementation of the plan in each strategic objective.

5.3. WGS analysis of DEC

5.3.1. Genome and phylogenetic analysis

The genomic diversity of DEC pathotypes in Ethiopia as well as in Africa remains largely unexplored compared to other regions (Europe, Asia, America, and Australia). The present study describes the genomic diversity of 28 human DEC pathotypes. Most strains (79%, 22/28) were compared with DEC pathotypes from different origin (different regions and sources). The study aimed to provide insight on the DEC pathotypes how they differ from other geographic origins.

The phylogenetic analysis has shown that *E. coli* is composed of four main phylogenetic groups (A, B1, B2, and D) (136) and the propensity to cause disease varies with its phylogenetic origins (138). Virulent extra-intestinal strains belong mainly to group B2 and, to a lesser extent, to group D (136). In the new phylogroup analysis, instead of triplex method (*chuA*, *yjaA* and TspE4.C2 genes), quadruplex method (*chuA*, *yjaA,* TspE4.C2 and arpA) allows classifying *E. coli* in to eight phylogroups, seven (A, B1, B2, C, D, E, F) belongs to *E. coli* and one (clade I) cryptic clade I (137, 138). Based on the quadruplex phylo-grouping

method 13% of *E. coli* isolates could be typed in C, E, F and clade I (137, 138). A study done in South Africa on DEC found predominant phylogroup B2 (30.4%, 24/79), followed by phylogroup B1 (22.8%, 18/79), phylogroups C and E (both 12.7%, 10/79), and 6% (5/79) of isolates were non-typable (247). Another study done in Mexico reported *E. coli* isolates from children with diarrhea were distributed in all phylogroups with the most frequent phylogroup A (42.9%) and B2 (15.7%) (248). Specifically, ETEC was found with phylogenetic groups A and B1 in one study (249) and EAEC grouped in phylogenetic group A, B1, B2, and D in another study (250). In the present study the hybrid strains were equally distributed in phylogroup A, B1, C, and the rest were non-typable. The EAEC strains of the present study were predominantly distributed in phylogroup A (33%), B2 (27%), and D (27%). The ETEC strains of the present study were also mainly grouped in A and C. Generally, the DEC pathotypes detected in this study fall almost in five phylogroups (A, B1, B2, C, and D) and this was comparable with studies done elsewhere in the world (247-250). The DEC pathotypes distributed in different phylogenetic groups observed in the present study showed their variation in phenotypic and genotypic characteristics, disease-causing ability and the disparate nature of DEC pathotypes circulating in the study area. A large scale WGS analysis may need for better understanding on their potential virulence genes content against their phylogenetic origin. Moreover, with cost reasons, PCR based follow up work targeting relevant genes, could allow better understanding on the occurrence of the DEC pathotypes.

In the present study, the MLST analysis showed DEC isolates belonged to a varied sequence types. ST 10 is one of the ST reported in the present study. ST10 *E. coli* strains are found in high frequency in different interface (human, animal, food, and environment) and belongs to pandemic sequence types (251, 252). It has a genome versatility nature that helps their pathogenicity, survival for long period, and transfer of genetic determinants (251). ST 10 was reported by Lee *et al* in STEC/ETEC hybrid strain (253) and by Yua *et al* in EAEC, EPEC,

and ETEC strains (254). Two ETEC strains of the present study were also ST 88. In agreement with this, ST 88 *E. coli* strain was found as predominant ST in a study that characterized *E. coli* from human, animal and food (255). ST 10 and ST 88 were also detected from patients and swine suffering from diarrhea in a study done in Korea (256). Previous study found a high level of sequence variations and genetic diversity in the population structure of DEC (254). Similarly, the present DEC pathotypes showed a varied serotype profile. Serotype variation could indicate difference in their virulence, disease causing characteristics, and sources of infection (133). The present sequence and serotype variations observed among the DEC pathotypes could be due to the small number of isolates included in the analysis. Otherwise, the sequence variations could support the possible occurrence of new clonal strains due to genetic plasticity nature of the organisms. The presence of strains with a superior capacity for survival and potential for spread of genetic determinants could alarm the health personnel to initiate active SP for monitoring emergence of potential pathogenic strains.

E. coli hybrids that are medically important have emerged, and enlarged the currently recognized set of DEC pathotypes (87). The phylogenetic analysis of the present hybrid strains showed a varied clustering among themselves. EH-5 (EPEC/ETEC) and EH-8 (EAEC/ETEC) were clustered independently and they carried different MGE associated with VGs, MLST, and serotype, but both were isolated from Addis Ababa and phylogenetically they were non-typable. EH-1 and EH-2 were closely related, the same ST and phylogroup, and from same study area. However, when the hybrid strains are phylogenetically analysed with other comparative strains, they clustered with different strains originated from different sources. Two hybrid strains (EPEC/EAEC and EPEC/ETEC) were clustered with a hybrid strain (STEC/UPEC) isolated from human in Germany. *E. coli* outbreak caused by an EAEC/STEC hybrid strain had occurred in 2011 in Germany (257). EPEC/ETEC hybrids

were found in humans (219). The other three strains isolated in this study clustered with five DEC pathotypes originated from animal (STEC, O26: NM), human (two ETEC), and food (EPEC and STEC). The strains were identified in different countries including Germany, Paraguay, and Australia. The other three remaining hybrid strains (3 EAEC/ETEC) were clustered together with 44 different strains originated from animal sources (11 strains), food (6 strains), human (26 strains), and environment or water (1 strain). The types of the comparative DEC pathotypes were 9 EPEC, 10 STEC, 9 hybrids, 13 EIEC, 2 EAEC, and 1 ETEC. The hybrid strains were two STEC/UPEC hybrids, four EPEC/ETEC hybrids, and three STEC/ETEC hybrids. Hybrid *E. coli* pathotypes are not restricted to mixes of DEC pathotype but hybridization occurs between DEC pathotype and extra-intestinal pathotype such as STEC/UPEC hybrids that cause both diarrhea and UTI (87). The phylogenetic analysis showed that the present hybrid strains have potential to circulate in the different interface (human, animal, food and environment), genetically varied and are comparative phylogenetically with other DEC pathotypes originated from elsewhere in the world.

The present EAEC strains were closely related with 19 different DEC pathotypes originated from animals (seven strains), humans (six strains), food (five strains) and environment (one strain). Majority of the comparative strains which were closely clustered with the present EAEC strains were from Asian/African countries (64%), and the rest were from Europe, Australia and South America (32%). EAEC is an emerging pathogen identified predominantly from human, animal and food (25, 258). Regardless of the contribution of geographical location, patient age, socioeconomic status, and variation in method of detection to the occurrence of EAEC (26), majority of the present study were phylogenetically closely related with strains originated from Asian/African countries (where diarrhea is high burden). Many studies reported EAEC in Asian/African countries including Japan, India, Mali, Libya, and Sub Saharan Africa (25). Over the last two decades the global mobility have increased

and most importantly this between African countries and Afro-Asia (https://www.migrationdataportal.org/regional-data-overview/eastern-africa). Our finding can also be explained in this regard. The high frequency of EAEC described in the present study, along with its phylogenetic closeness with different DEC pathotypes, originated from different sources in different geographical areas, and high MDR rate, and other factors call for being vigilant about DEC in the study area and the region. In other word, EAEC is emerging pathogens distributed globally and have potential to cause severe outbreaks (25).

5.3.2. Virulence genes (VGs)

The gene gain and loss mediated by MGE (e.g. transposons and plasmids) afford pathogenic *E. coli* to emerge with new pathotypes (25). With the current understanding, hybrid *E. coli* strains are characterized by the presence of VGs that are characteristic of more than one *E. coli* pathotypes (87). The EPEC/EAEC hybrid strain contains 23 VGs including VGs from EPEC and EAEC. In the present study, three EPEC/ETEC hybrid strains containing 11-20 VGs were identified. All these hybrid strains contain *eae*, *tir*, *espA*, *espF*, and *ltcA* VGs. The Heat-labile enterotoxin (LT) is one of a definitive characteristic of ETEC (25, 26). LT A subunit which is encoded by *ltca* gene was found in all the three EPEC/ETEC hybrid strains of the present study. The EAEC/ETEC hybrid strains of the present study contain *agg3A*, *agg3D*, *aatA* and *astA* (in 3 strains), and *aggR*, *aap*, *aar*, and *aaiC* (in 3 strains) which are VGs associated with EAEC. Heat-stabile enterotoxin ST-Ia (in 1 strain), LT (in 4 strains), and enterotoxigenic *E. coli* (ETEC) autotransporter A (in 3 strains) which are VGs associated with ETEC were identified. In 88% of the hybrid strains, different VGs were found associated with MGE. The hybrid strains containing VGs involved in the clinical disease could have potential implication in more severe disease (218).

The virulence profile of the DEC pathotypes was varied in the present study. The major VGs played in adherence, biofilm formation, and toxins production were identified in the present

study. Vital virulence factors of EAEC played in adherence include *aggR (*transcriptional activator of virulence genes), AAF/I–AAF/IV genes (aggregative adherence fimbriae), and *aap* (antiaggregation protein dispersin) (25). In EAEC, aggregative adherence fimbriae (AAFs) facilitate adherence to the intestinal mucosa (25). The AAFs (on pAA virulence plasmid) includes chaperone (encoded by *agg3D*), long fimbriae (encoded by *agg3A*), minor pilin subunit (encoded by *agg3B*), and pore-forming usher (encoded by *agg3C*) and the expression of AAFs is regulated by AggR, (a major virulence regulator in EAEC) (25, 26, 259). Other VGs include *aap* (encode dispersin), *aatA* (plasmid carried gene), *aaiC* (chromosomal carried gene), and *aar* (aggR-activated regulator) are associated with EAEC pathogenicity and among the most commonly used target for EAEC identification (26, 259). In the present study, 13 AAF subunits (afaD, aggA, aggB, aggC, aggD, agg3A, agg3B, agg3C, agg3D, aafA, aafB, aafC and aafD) were identified. The main VGs of EAEC strains involved in biofilm formation include AAF, *shf*, *yafK, fis, aatA, set1A,* and *aggR* genes (260). In the present study, *shf*, *yafK,* and *set1A* were not detected but others *(AAF, aatA, and aggR)* were found. The genetic plasticity of the organisms may contribute to the discrepancy (261). Key VGs of EAEC involved in toxin production are *astA (*EAST1), *pet (*plasmid-encoded toxin), *sepA (shigella extracellular protein), sat (*secreted autotransporter toxin), *set (shigella* enterotoxin 1), and *pic (*protein involved in colonization) (260). Almost all the toxin genes (except *set*) were found in the present study. In some of the present EAEC strains, additional putative VGs previously reported in extra-intestinal pathogenic *E. coli* (ExPEC) were found (262, 263). These VGs were *kps* (polysialic acid transport protein), *yfcV* (fibrial protein), *papC* (outer membrane usher P fimbriae), *fyuA* (siderophore receptor), *fimH* (type 1fimbriae), *hly* (hemolysin), and *chuA* (outer membrane hemin receptor). The presence of VGs associated with ExPEC could suggest the possible occurrence of EAEC/ExPEC hybrid strains in the area. The present study revealed the presence of highly virulent strains in the study area.

5.3.3. Antimicrobial resistance genes (ARGs)

The antimicrobial prescription in Ethiopia follows the category of antimicrobials, access, watch and reserve antimicrobial groups in Ethiopian essential medicine list (226) and antimicrobials resistance to these groups is a threat in the country. ARGs associated with resistance of common antimicrobials were higher in the present study. The ARGs, blaTEM-1B (associated with resistance to ampicillin, amoxicillin, cephalothin or piperacillin) and tet(A) variants (for resistance to tetracycline, doxycycline) were identified in 86% (24/28), and 76% (22/28), respectively. The high level of blaTEM-1B detected in this WGS analysis could support the predominant blaTEM identified in the PCR-based detection in the present study. Other ARGs associated with commonly used antimicrobials (sulfamethoxazole, trimethoprim, ciprofloxacin, azithromycin, amikacin, gentamicin, and chloramphenicol) were found in the present study. These include sul1 and sul2 (71%, 20/28), dfrA variants (75%, 21/28), gyrA, qnrS1, and qnrB1 (25%, 7/28), mph (A) (32%, 9/28), aadA variants and rmtB (43%, 12/28), and catA variants (18%, 5/28). The high proportion of sul1 and sul2, and dfrA variants genes contributed to resistance to folate pathway antagonist and aminoglycoside class of antimicrobials and MDR strains in the present study. CTX-M-14b, CTX-M-15 and CTX-27 were found in the present study and they were also reported from extra-intestinal *E. coli* strains in a previous study done in Ethiopia (235). Carbapenemase encoding genes, bla-NDM-5 and bla-OXA-1 were found only in single isolate and could indicate the low occurrence of it. However, the presence of strains with potential ARGs with co-occurrence of ß-lactamase genes could alter the epidemiology of resistant DEC pathotypes in the area. Thus, the detection of different ARG variants, including ESBL- and carbapenemase-encoding gene variants, and their co-occurrence calls for high attention to have sentinel surveillance program.

5.3.4. Mobile genetic elements (MGEs)

Microbial evolution (change in genome content over time) in pathogenic bacteria is mainly by acquisition of virulence determinants through HGT (261). The acquisition of new virulence determinants through MGE (261, 264) can lead to the emergence of new pathotypes (261). The MGE including plasmids and transposons in the *E. coli* strains can carry ARGs and/or VGs (265). Plasmid replicon types and transposons were determined from WGS subjected DEC pathotypes of the present study.

Plasmids can be typed using conjugation based incompatibility test (Inc typing) or replicon sequence analysis (265, 266). In the present study, plasmid replicon types were determined using plasmidFinder databases that have known plasmid replicon sequences (plasmid types). The plasmid replicon types found in the present study are almost Inc. group (except Col156) with IncF predominant. Majority of the plasmid type associated with VGs in *E. coli* strains are grouped in IncF (267) which is supported by the present finding. The virulence factors associated with the plasmid replicon types in the present study include VGs for adherence, toxins, regulator, and transport proteins. In addition, Col156 which is associated with carrying ExPEC VGs (267) was detected in the present study and this could contribute to occurrence of hybrid strains from the classic DEC pathotypes and ExPEC. A study in China found plasmid replicon types, IncFII (55%, 6/11), and IncFIB (73%, 8/11) from carbapenemase producing *E. coli* strains (215). IncI1 and Col156 were found in the present study, and both are associated with MDR, and colistin resistant (267). IncQ1 which is associated with broad-host-range plasmids and contribute to MDR and IncY which is a phage-like plasmids (267) were also found in the present study. The presence of such potential MGE with a pool of genetic determinants could play crucial role in microbial evolution and severe diseases (265) and this can alter the epidemiology of virulent strains significantly in the area.

Transposable elements are DNA sequences in the bacterial genome (transposons) and able to transfer among different locations within the genome (265). A total of 21 transposons (12 types) were associated with VGs in the present study and except MITEEc1 all were IS elements. Several types of transposable elements contribute to the acquisition of resistance to different class of antimicrobials in *E. coli* (268). Various transposable elements (e.g. Tn3, Tn5, Tn7, Tn9, and Tn10) carrying ARGs found in *E coli* (265). In the present study, seven types of transposons were involved in harbouring ARGs. Two were Tn2 and Tn7 and the rest were IS elements. Tn2 is associated with blaTEM genes that encode extended-spectrum ß-lactamases, and predominant in clinical isolates (264). The present study revealed the potential of DEC pathotypes that could be characterized by rich in MGE associated with ARG and VGs. The presence of strains with potential MGE would be a threat for emergence of new pathotypes that are MDR, high dissemination of virulent and resistant determinants among bacterial community, particularly in gut.

Chapter six

6. Conclusion and recommendation

6.1. Conclusion

All six common medically important DEC pathotypes were found in Ethiopia. Strains that are commonly associated with high fatality rate, persistent infections, severe disease and outbreak, and that lead to long term consequences in UFC were found. In addition, contributing factors for the acquisition of DEC such as low family income and poor childcare were identified. Epidemiologically, DEC pathotypes are more prevalent compared to other common bacterial agents of diarrhea in UFC in Ethiopia.

High resistance to commonly used antimicrobials and an increasing number of MDR DEC such as ESBLs and carbapenemase-producing strains reduces the potential treatment options for DEC infections. DEC with MDR profile, and ß-lactamase and carbapenemase encoding genes that are associated with serious and an urgent global threat were detected in the present study. Co-existence of β-lactamase genes could suggest the severity of the problem. The presence of MDR, ß-lactamase including carbapenemase producing DEC in the gut of UFC could reveal the presence of risk for emergence of MDR bacterial strains in the area.

The DEC pathotypes of the present study subjected to WGS analysis showed existence of different phylogenetic groups with varied ST, and serotype patterns. The strains also showed a diverse association with different DEC pathotypes of different origin. High VGs and ARGs were found and many of the determinant genes were associated with MGEs. The presence of MDR, including ESBL and carbapenemase-producing DEC in the gut of UFC could reveal the presence of risk for emergence of MDR bacterial strains in the area. The presence of genetically diverse DEC pathotypes (in sequence type, serotype, phylotype, VGs, and ARGs) could support the possible occurrence of new pathotypes, hyper-virulent and hybrid strains

not only within the classic DEC pathotypes but also with extra-intestinal pathogenic strains in different interface include human, animal, food and environment.

Generally, DEC has the potential to be a big concern in UFC in Ethiopia. Therefore, public health personnel have to understand the presence of virulent and MDR DEC pathotypes among UFC. It needs urgent attention for timely controlling the dissemination of such hyper-virulent and MDR strains that have potential to cause severe outbreaks in the area.

6.2. Recommendation

Based on our findings we recommend the following:

- **To clinicians (treatment perspective)**
  1) To understand the need of diagnosis or screening of DEC isolates in health facilities for right time treatment to avoid long term consequences and fatality in UFC.
  2) To consider the circulating DEC pathotypes among diarrheic UFC that the case could be due to the treatable DEC pathotypes (EAEC, ETEC, EIEC and DAEC) or untreatable (STEC) so that the right treatment will benefit the patient and protect the wrong treatment that worsen the disease.
  3) To consider the high level of resistant EAEC, ETEC, and EIEC strains present in the area (may be request antimicrobial susceptibility test) and use the right drugs.
  4) *E. coli* is considered as an indicator microorganism for AMR level in enteric bacterial pathogens and the present AMR profile of DEC pathotypes could indicate the level of circulating AMR pathogens in the community. Clinicians are recommended to take in to account the possible presence of MDR, ESBL- and carbapenemase producing gram negative bacterial pathogens in the study area.
- **To public health stakeholders** (policy makers, donors, scientific community) to do the following are recommended

1) To improve and increase access and diagnostic capacity of clinical laboratories that allows detection of DEC pathotypes such as culture and PCR assays
2) Strengthening IPC practices.
3) Establishing and expanding enteric pathogen surveillance program. Generating surveillance data could help to monitor the occurrence of emerging DEC pathogens and outbreaks, and allow better understanding on the distribution and magnitude of the burden.
4) Implementing antimicrobials stewardship and preparedness for outbreak response.
5) Improving water access, sanitation condition, hygiene practice, clinical care, and socio-economic status. The presence of pathogen determinant traits (ARGs and VGs associated with MGE) is a threat for dissemination of such pathogens and early prevention through intervention against the risk factors associated with acquisition of the pathogens will help preventing the transmission.
6) Finally, the finding could support the Ethiopia revised AMR Prevention and Containment Strategic Plan (53) through providing evidence for awareness and education of AMR, and IPC measures. In addition, the focus area of the National IPC Policy includes education and training, healthcare associated infections surveillance, and research (76). The present finding would be an excellent input during the implementation of the plans in all the strategic objectives and focus areas.

- **To researchers** to do the following are recommended

Little is known on the genetic background of the DEC pathotypes. We recommend conducting further research for better understanding on the genetic background of each DEC pathotypes (using large scale WGS data) and the epidemiology and transmission dynamics in different interfaces (preferably with one health approach).

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Annexes

Annex 1. Procedure for stool sample collection

a) Prepare Cary Blair transport medium (and alkaline peptone water) and placing it on ice packs (or in the refrigerator).
b) Wear gloves when collecting and handling the specimen
c) Collect stool from patients (UFC), aseptically
d) Then, using two small wooden sticks transfer at least one small teaspoon (5 g or 5 ml) of faeces (containing blood, mucus or pus) to the transport media
e) Immediately insert the swab into transport medium.
f) Break off the top portion of the stick that was in contact with the gloved fingers.
g) Repeat steps the above steps for an additional sterile swab.
h) Adhere specimen label to the container or write on adhesive tape

Annex 2. Procedure for bacterial isolation and identification using culture method

1) Do enrichment using Selenite F enrichment broth (1-2 gm of stool sample or 10% by volume) for *Salmonella*.
2) Prepare faecal suspension by taking the wooded sticks (cotton swabs) from the transport media containing the stool sample and rinsed thoroughly in 1 ml of saline. For the liquid (or enriched) stool sample, saline was not used.
3) Inoculated the stool sample onto MacConkey and Xylose-Lysine-deoxycholate (XLD) media, and incubated at 37 °C for 18 -24 hours for the isolation of *E. coli, Shigella* and *Salmonella*
4) When cholera suspected several loopfuls of the peptone water culture (taken from the surface), inoculate on thiosulphate citrate bile-salt sucrose (TCBS) agar and incubated aerobically at 35–37 °C overnight
5) Subculture on the following media by streaking a loopful of colonies suspension (with saline):
    - MacConkey agar, Xylose–lysine–deoxycholate agar, and blood agar, and incubate at 37 °C for 18-24 hours
6) Do biochemical tests using the pure colonies
    - Oxidase test, triple sugar iron agar, urease test, lysine, motility, indole , manitol, and Simon's citrate for *E. coli, Shigella* and *Salmonella* (See Annex 3)
    - Oxidase test and salt tolerance Vibrio isolates.
7) Interpret the biochemical test results

Annex 3 Procedure for biochemical tests

The following biochemical tests were done: oxidase test, triple sugar iron agar, urea, lysine, motility, indole, manitol, and Simon's citrate

1. Oxidase test (Cytochrome oxidase test)

Procedure of oxidase test using filter paper

a) Take a filter paper soaked with the oxidase reagent
b) Moisten the paper with a sterile distilled water
c) Pick the colony to be tested with wooden loop and smear in the filter paper
d) Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds
e) *E. coli* is negative *V. cholera* is positive

2. Triple sugar iron Agar (TSI)

Procedure for inoculation of TSI

a) Use a straight wire to inoculate TSI medium
b) First stabbing the butt and then streaking the slope in a zig-zag pattern
c) Incubate at 37 $^{\circ}$C overnight, left loose the tube tops
d) Examine the slant and the butt (fermentation, gas, alkaline or acid, H2S production)
e) *E. coli* is lactose fermenter (few strains are non-lactose fermenter) and *Shigella* and *Salmonella* are non-lactose fermenter.

3. Urease test

Procedure for urease test

a) Inoculate the test organism in a test tube containing 3 ml sterile Christensen's modified urea broth.

b) Incubate at 35–37 °C for 3–12 hours

c) Look for a pink colour in the medium

d) *E. coli*, *Shigella* and *Salmonella* are urease negative

4. Lysine decarboxylase test

Test procedure

a) Transfer inoculum from a pure culture aseptically to a sterile tube of lysine decarboxylase broth

b) Incubate the inoculated tube at 35-37 °C for 24 hours

c) Observe a change from purple to yellow

d) The final results are then obtained by observing the tube at 48 hours

e) Change back to purple from yellow indicates a positive test for lysine decarboxylase

f) Failure to turn yellow at 24 hours or to revert back to purple at 48 hours indicates a negative result.

g) *E. coli* is lysine decarboxylase variable, *Shigella* is negative, and *Salmonella* is positive

5. Motility

a) Inoculate (a single stab) the bacterial suspension in to Motility agar with a straight inoculating needle should

b) Incubate overnight at 35 –37 °C.

c) Examine and motility is indicated by the presence of diffuse growth (appearing as clouding of the medium) away from the line of inoculation.

d) Non-motile organisms do not grow out from the line of inoculation.

e) *E. coli* is motile (but few exception), *Shigella* is non-motile, and *Salmonella* is motile

6. Indole test

Procedures for detecting indole using tryptone water

a) Inoculate the test organism in a test tube containing 3 ml of sterile tryptone water.

b) Incubate at 35–37 °C for up to 48 hours.

c) Test for indole by adding 0.5 ml of Kovac's reagent. Shake gently.

d) Examine for a red colour in the surface layer within 10 minutes.

e) *E. coli* is indole positive, *Shigella* and *Salmonella* are indole negative

7. Simon's citrate (citrate utilization test)

Test procedure for citrate method using Simmon's citrate agar

a) Prepare slopes of the medium in a test tube.

b) Using a sterile straight wire, first streak the slope with a saline suspension of the test organism and then stab the butt.

c) Incubate at 35 °C for 48 hours.

d) Look for a bright blue colour in the medium.

e) *E. coli* and *Shigella* are citrate negative, and *Salmonella* is citrate variable

8. Mannitol fermentation test

Test procedures for mannitol fermentation using phenol red mannitol broth media

a) Transfer a pure colonies to phenol red mannitol broth

b) Incubate at 35-37 °C for 24 hours

c) A colour change from red to yellow is positive

Annex 4. DNA extraction procedures (stool sample and culture)

Pellet preparation for extraction (stool sample)

a) Suspend 20 mg thawed stool sample in 5.0 mL phosphate-buffered saline (PBS; pH 7.2).
b) After vortexing, centrifuge the mixture at 500 rpm for 4 min
c) Take the supernatant to new 5 ml sterile tube
d) Resuspeneded the pallet in 5 ml PBS buffer and centrifuge it at 500 rpm for 4 min and repeat twice
e) Centrifuged all the supernatants at 9000 rpm for 5 min
f) Discard the supernatant, take the cell pellet, and resuspend in molecular grade water for extraction processing.

DNA extraction by TE boils extraction method (stool sample)

a) Suspended the cell pellet in 200 μL TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA]
b) Mix the mixture briefly mixed on a vortex mixer
c) Boil the suspension at 94 °C for 10 min in dry block incubator
d) Place in a freezer at -20 °C for 10 min and then place 1 min at room temperature
e) Then centrifuge at 14 000 *g* for 5 min
f) Take a 100 μL aliquot of the supernatant and transfer to a sterile tube and stored at-20 °C until PCR testing

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DNA extraction by boiling method (bacterial colonies)

a) An overnight liquid culture (Tryptic Soya Broth) suspension of bacterial isolates
b) Boiled at 94 °C for 10 min in a dry block incubator (Thermo-fisher scientific, California)
c) Placed in a freezer at −20°C for 10 minutes
d) Then placed at room temperature for one minute and centrifuged at 14,000g for 5 min.
e) Finally, 100µL of the supernatant was transferred into nuclease free Eppendorf tube and stored at −20°C until use for PCR assay
f) The purity of the extracted DNA was assessed by Nanodrop.

Reference

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Annex 5. Bacterial pathogen detection using target genes by PCR assay

Virulence Genes detection from culture (DEC pathotypes)

a) Subculture on Tryptic Soya Agar and incubate overnight at 37 ℃

b) Transfer 3-4 colonies to Tryptic Soya Broth (TSB) and incubate overnight at 37 ℃

c) Transfer 1 mL of bacterial suspension to sterile Eppendorf tube

d) Extract the DNA by boiling method (see Annex 4)

e) Assess the quality using Nanodrop (OD 260/280 ≥ 1.8-2.0 and an OD 260/230 ≥ 1,8-2,2)

f) Prepare master mix (in PCR master mix room)

- Label the PCR tube correctly
- Determine the sample volume and total reaction volume as manufacturer instructions (or optimize using positive control strains)
- Add 10 µl Platinum™ II Hot-Start PCR Master Mix (2X)
- Add 0.4 µl forward primer and 0.4 µl reverse primer for each primer.
    - If target genes are 3, add 1.2 µl of forward primer mix (0.4 µl each primer), 1.2 µl of reverse primer mix (0.4 µl each primer)

g) Add 1 µl of template DNA to the master mix, and molecular grade water to the total reaction volume (20 µl)

h) Set the thermocycling conditions, name the program, and run the PCR machine

- An initial denaturation of 94 °C for 2 min, followed by 35 cycles of 92 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 30 sec and a final extension at 72 °C for 5 min in a PCR machine

i) Run agarose gel electrophoresis (see annex

Virulence Genes detection from stool sample (*Campylobacter*)

a) Prepare pellet from thawed stool sample

b) Extract DNA using TE boil extraction method

c) Assess the quality using Nanodrop (OD 260/280 ≥ 1.8-2.0 and an OD 260/230 ≥ 1,8-2,2)

d) Prepare master mix (in PCR master mix room)

   - 12.5 µl Platinum™ II Hot-Start PCR Master Mix (2X), 0.4 µl forward primer and 0.4 µl reverse primer for each primer

e) Add 1 µl of template DNA to the master mix, and molecular grade water to the total reaction volume (25 µl)

f) Set the thermocycling conditions, name the program, and run the PCR machine

   - One cycle of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min.

g) Run agarose gel electrophoresis (See Annex 6)

Annex 6. Procedure for Agarose gel electrophoresis

a) Measure agarose powder (1.7%, 2.04 gm for 120 ml 1X TAE buffer) and mix with 1X TAE buffer in flask

b) Dissolve the agarose powder by boiling the solution in microwave. Mix the by swirling the flask.

c) Cool agarose to 60 ℃ and add ethidium bromide on it

d) Prepare the gel casting tray: seal the ends of the gel-casting tray with the rubber end cups, place the well template (comb) appropriately

e) Poor the cooled agarose solution into the prepared gel-casting tray, let sit at room temperature for 20-30 min, until it has completely solidified.

f) Remove the end caps and comb (take particular care when removing comb)

Loading Samples and Running an Agarose Gel

g) Once solidified, place the gel (on the tray) into electrophoresis chamber. Completely cover the gel with 1X TBE buffer

h) Add loading buffer (dye) to each of your DNA samples.

i) Carefully load a molecular weight ladder into the first lane of the gel.

j) Carefully load your controls, samples into the additional wells of the gel in consecutive order

k) Place the safety cover; check the gel is properly oriented.

l) Run the gel at 120 V for 1hours

m) Remove the gel and casting tray from the electrophoresis chamber and proceed to visualization using UV light trans illuminator.

Annex 7. Antimicrobial susceptibility test (Disc diffusion method)

Preparation of inoculum

a) Prepare a pure culture by streaking onto a non-inhibitory agar medium (blood agar, brain heart infusion agar) and incubate at 35 °C overnight
b) Select 4-5 pure colonies with an inoculating needle (loop), and transfer the growth to a tube of sterile saline or non-selective broth (*e.g.*, tryptone soy broth) and vortex thoroughly.
c) Compare the bacterial suspension to the 0.5 McFarland turbidity standard.

Inoculation procedure

a) From the prepared bacterial suspension and using a sterile swab, inoculate a plate of Mueller Hinton agar.
b) With the petri dish lid in place, allow 3–5 minutes (*no longer than 15 minutes*) for the surface of the agar to dry.
c) Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate.
d) Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35 °C for 16–18 hours
e) After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent.
f) Using a ruler on the underside of the plate measure the diameter of each zone of inhibition in mm.

Annex 8. DNA extraction procedures (Quick-DNA™ Miniprep Plus Kit)

Reagent preparation

- Add 1,040 µl Proteinase K storage Buffer to each Proteinase K (20 mg) tube prior to use. The final concentration of Proteinase K is ~20 mg/ml. store at -20 °C after mixing.

Sample processing

- Resuspend cultured cell or *E. coli* pellets using DNA elusion buffer or an isotonic buffer (e.g. PBS): $<1*10^6$ cells in 100 µl or $1\text{-}5*10^6$ cells in 200 µl.

Procedure

a) Add up to 200 µl sample to a microcentrifuge tube and add 200 µl BioFluid and Cell Buffer (red), and 20 µl Proteinase K. Note: For inputs <200 µl biological fluid, proportionally decrease BioFluid and Cell Buffer (red), Proteinase K, and Genomic Binding Buffer.

b) Mix thoroughly or vortexes 10-15 seconds and then incubate the tube at 55 °C for 10 minutes.

c) Add 1 volume Genomic Binding Buffer to the digested sample. Mix thoroughly or vortex 10-15 seconds. Example, add 420 µl Genomic Binding Buffer to the 420 µl digested sample.

d) Transfer the mixture to a Zymo-Spin™ IIC-XLR Column in a Collection tube. Centrifuge at ≥ 12, 000 g for 1 minute. Discard the collection tube with the flow through.

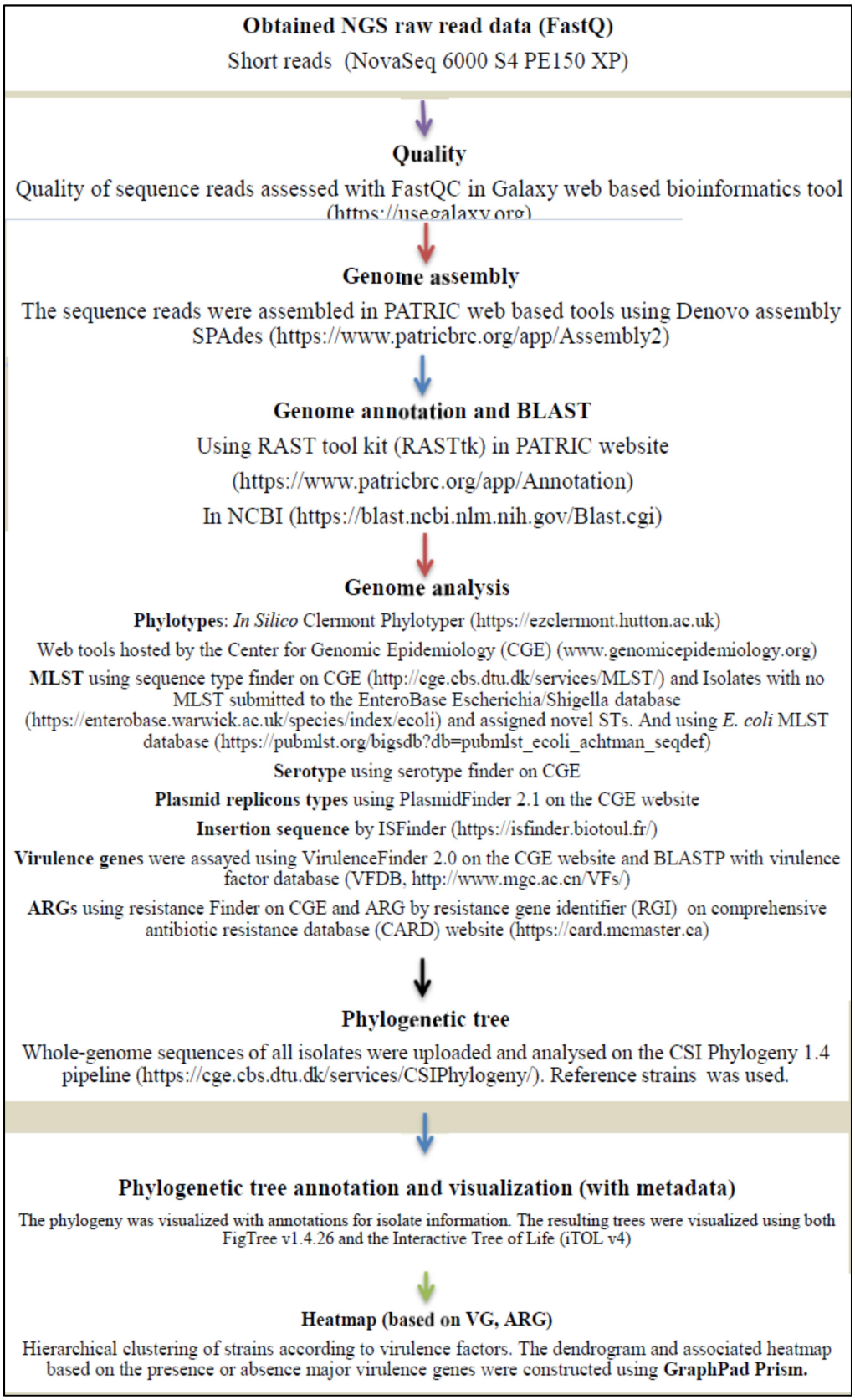
e) Add 400 µl DNA Pre-Wash Buffer to spin column in a new Collection Tube. Centrifuge at ≥ 12, 000 g for 1 minute. Empty the collection tube.

f) Add 700 µl g-DNA Wash Buffer to the spin column. Centrifuge at ≥ 12, 000 g for 1 minute. Empty the collection tube.

g) Add 200 µl g-DNA Wash Buffer to the spin column. Centrifuge at ≥ 12, 000 g for 1 minute. Discard the collection tube with the flow through.

h) Transfer the spin column to a clean microcentrifuge tube. Add ≥ 50 µl DNA Elusion Buffer or water. Directly on the matrix. Incubate for 5 minutes at room temperature, then centrifuge at maximum speed for 1 minute to elute the DNA. The eluted DNA can be used immediately for molecular based applications or stored at ≤ -20 °C for future use.

Annex 9. Work flow for WGS analysis



Annex 10. MGE of the present DEC pathotypes

Isolate ID	Study area	Plasmid	Transposon	ARGs	VGs
Hybrid strains					
EH_1	DB	IncFIB	ISVsa3	sul2	hlyF, ompT
EH_2	DB	Col156	ISEic2, Tn7	aadA, dfrA1	senB, astA
EH_3	DB	IncFll	MITEEc1, ISEc38, Tn2	tet(A), blaTEM-1B	toxB, espA, espF, ipfA, tir,eae
EH_4	AA		ISEc30, ISSpu2		espI, agg3A, agg3C, ORF4, agg3D, astA, agg3B, ORF3, aap, aar, aatA, aggR
EH_5	AA	Col156, IncB/O/ K/Z	ISEc37, ISEic2		senB, eatA, traT, astA
EH_6	AA	Incl1	ISSpu2, Tn2	qnrS1, blaTEM-1B	aap, aatA, aar, cia, pic
EH_7	AA	IncFll(p Coo), IncFll(p HN7A8)	Tn2	blaTEM-1B	traT, eatA, aap
EH_8	AA				
EAEC strains					
EA_001	AA	IncQ1	ISEc9, Tn7, ISSpu2	blaCTX-M-14b, sul2, aadA1, dfrA1, tet(D)	aar, aggR, aap
EA_002	AA	IncFII, IncFII (pCoo) IncFII (pHN7A 8)	Tn2	blaTEM-1B	trap, eatA, aap
EA_003	AA		Tn2, ISSpu2, ISEc38	blaTEM-1B, qnrS1, sul1, sul2,	aar, aatA1, aap, pic
EA_004	AA	IncQ1, IncFII (pRSB10 7)	ISEc17, ISEc45	aph(6)-id, sul1, tet(A), tet(B), catA1,	aggC, aggD, aggA, aggB, anr, traT, iha
EA_005	AA	IncFII (pB171)	ISSpu2		afaD, aggC, aggA, aggD, astA, aap, aggR, aar, aatA, ORF3, ORF4
EA_006	DB	IncFIB (AP0019 18), IncFII (pB171)	MITEEc1		capU, afaD, anr, aatA, sepA, traJ, ORF4, agg4D, aap, aggR, iss
EA_007	DB	-			
EA_008	DB		Tn2, ISSso4	blaTEM-1B, dfrA1	etsC
EA_009	DB	IncQ1	ISEc43,	aph(6)-id, sul2	sat, terC,

			MITEEc1		
EA_010	DB		IS102, IS6100	bla-CTX-M-27, mph(A)	
EA_011	DB	Col156	ISSpu2		aar, senB
EA_012	AA	-			
EA_013	AA		ISKpn19, ISEc48, ISRaq1	tet(A), qnrS13	aafA, aar, aafD, aggR, astA
EA_014	DB	IncQ1, IncFll(pHN7A8), Col156		sul2	traT, senB
EA_015	AA				
ETEC strains					
ET-(1)	AA	IcnQ1	ISKpn19	sul2, tet(A)	
ET-(2)	AA		IS6100	mph(A)	
ET-(3)	DB	IncQ1, IncFll(pHN7A8), Col156		sul1	traT, senB
ET-(4)	AA	IncY	IS6100	bla CTX-M-15, dfrA17, mph(A), sul1, aadA5	
ET-(6)	AA		IS6100	dfrA17, mph(A), sul1, aadA5	

Curriculum Vitae

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- MSc in Medical Microbiology
- PhD candidate in Addis Ababa University

Professional Work Experience

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- Currently work in Debere Berhan University as instructor and researcher
- Participated in different leadership

Training and certificate

- Certificate for successfully completed the three module training courses on TB operational research and the conduct of TB operational research in team
- Certificate of health research method and ethics training by the Ethiopian Public Health Association
- Certificate of scientific paper presentation by Ethiopian Public Health Association, 2011
- Certificate of scientific paper presentation by Ethiopian Public Health Association, 2016
- Certificate of Higher Diploma License
- Certificate for external examination in Wollo University

Published research papers

1) **Tizazu Zenebe,** Tadesse Eguale, Zelalem Desalegn, Daniel Beshah, Solomon Gebre-Selassie, et al. Distribution of ß-Lactamase Genes Among Multidrug-Resistant and Extended-Spectrum ß-Lactamase-Producing Diarrheagenic Escherichia coli from Under-

Five Children in Ethiopia. Infection and Drug Resistance 2023:16 7041–7054.

2) **Tizazu Zenebe Zelelie,** Tadesse Eguale, Berhanu Yitayew, Dessalegn Abeje, Ashenafi Alemu, Aminu Seman, *et al*. Molecular epidemiology and antimicrobial susceptibility of diarrheagenic *Escherichia coli* isolated from children under age five with and without diarrhea in Central Ethiopia. PLoS ONE . 2023: 18(7): e0288517
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On-going publications

- Epidemiology of *Shigella*, *Salmonella* and *Campylobacter* among diarrheic children in central Ethiopia
- Enteroaggregative *Escherichia coli* (EAEC) isolated from under-five children with diarrhea in

Ethiopia: Genomic and phylogenetic analysis

- Multidrug-resistant profile and prevalence of extended spectrum ß-lactamase and carbapenemase producing enterobacteriaceae in under- five children in Debre Berhan and Addis Ababa, Ethiopia
- Genomic and phylogenetic analysis of hybrid strains of diarrhagenic *E. coli* from diarrheic under-five children in Ethiopia
- Burden of diarrhea and other infections in under-five children in Ethiopia: a retrospective study

Membership

- Member of Ethiopian Public Health Association (EPHA)
- Member of American Society of Microbiology (ASM)

Skills

- Language: Excellent listening, speaking, reading and writing skills in Amharic and English
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Sample list of published papers

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PLoS One. 2023 Jul 14;18(7):e0288517. doi: 10.1371/journal.pone.0288517. eCollection 2023.

Molecular epidemiology and antimicrobial susceptibility of diarrheagenic Escherichia coli isolated from children under age five with and without diarrhea in Central Ethiopia

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PMID: 37450423 PMCID: PMC10348587 DOI: 10.1371/journal.pone.0288517

Free PMC article

Abstract

Background: Diarrhea is a serious health problem in children, with the highest mortality rate in sub-Saharan Africa. Diarrheagenic Escherichia coli (DEC) is among the major bacterial causes of diarrhea in children under age five. The present study aims to determine molecular epidemiology and antimicrobial resistance profiles of DEC and identify contributing factors for acquisition among children under age five in Central Ethiopia.

Methods: A health facility-centered cross-sectional study was conducted in Addis Ababa and Debre Berhan, Ethiopia, from December 2020 to August 2021. A total of 476 specimens, 391 from diarrheic and 85 from non-diarrheic children under age five were collected. Bacterial isolation and identification, antimicrobial susceptibility, and pathotype determination using polymerase chain reaction (PCR) were done.

Results: Of the 476 specimens analyzed, 89.9% (428/476) were positive for E. coli, of which 183 were positive for one or more genes coding DEC pathotypes. The overall prevalence of the DEC pathotype was 38.2% (183/476). The predominant DEC pathotype was enteroaggregative E. coli (EAEC) (41.5%, 76/183), followed by enterotoxigenic E. coli (21.3%, 39/183), enteropathogenic E. coli (15.3%, 28/183), enteroinvasive E. coli (12.6%, 23/183), hybrid strains (7.1%, 13/183), Shiga toxin-producing E. coli (1.6%, 3/183), and diffusely-adherent E. coli (0.6%, 1/183). DEC was detected in 40.7% (159/391) of diarrheic and 28.2% (24/85) in non-diarrheic children (p = 0.020). The majority of the DEC pathotypes were resistant to ampicillin (95.1%, 174/183) and tetracycline (91.3%, 167/183). A higher rate of resistance to trimethoprim-sulfamethoxazole (58%, 44/76), ciprofloxacin (22%, 17/76), ceftazidime and cefotaxime (20%, 15/76) was seen among EAEC pathotypes. Multidrug resistance (MDR) was detected in 43.2% (79/183) of the pathotypes, whereas extended spectrum ß-lactamase and carbapenemase producers were 16.4% (30/183) and 2.2% (4/183), respectively.

Conclusion: All six common DEC pathotypes that have the potential to cause severe diarrheal outbreaks were found in children in the study area; the dominant one being EAEC with a high rate of MDR.

https://pubmed.ncbi.nlm.nih.gov/37450423/

Infect Drug Resist. 2023 Nov 6:16:7041-7054. doi: 10.2147/IDR.S432743. eCollection 2023.

Distribution of ß-Lactamase Genes Among Multidrug-Resistant and Extended-Spectrum ß-Lactamase-Producing Diarrheagenic *Escherichia coli* from Under-Five Children in Ethiopia

Tizazu Zenebe [1 2], Tadesse Eguale [3 4], Zelalem Desalegn [1], Daniel Beshah [5], Solomon Gebre-Selassie [1], Adane Mihret [1 6], Tamrat Abebe [1]

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PMID: 37954506 PMCID: PMC10637226 DOI: 10.2147/IDR.S432743

Free PMC article

Abstract

Purpose: *Escherichia coli* strains that produce extended-spectrum ß-lactamase (ESBL) and carbapenemase are among the major threats to global health. The objective of the present study was to determine the distribution of ß-lactamase genes among multidrug-resistant (MDR) and ESBL-producing Diarrheagenic *E. coli* (DEC) pathotypes isolated from under-five children in Ethiopia.

Patients and methods: A cross-sectional study was conducted in Addis Ababa and Debre Berhan, Ethiopia. It was a health-facility-based study and conducted between December 2020 and August 2021. A total of 476 under-five children participated in the study. DEC pathotypes were detected by conventional Polymerase Chain Reaction (PCR) assay. After evaluating the antimicrobial susceptibility profile of the DEC strains by disk diffusion method, confirmation test was done for ESBL and carbapenemase production. ß-lactamase encoding genes were identified from phenotypically ESBLs and carbapenemase positive DEC strains using PCR assay.

Results: In total, 183 DEC pathotypes were isolated from the 476 under-five children. Seventy-nine (43%, 79/183) MDR-DEC pathotypes were identified. MDR was common among enteroaggregative *E. coli* (EAEC) (58%, 44/76), followed by enterotoxigenic *E. coli* (ETEC) (44%, 17/39)) and enteroinvasive *E. coli* (EIEC) (30%, 7/23). Phenotypically, a total of 30 MDR-DEC pathotypes (16.4%, 30/183) were tested positive for ESBLs. Few ETEC (5.1%, 2/39) and EAEC (2.6%, 2/76) were carbapenemase producers. The predominant β-lactamase genes identified was bla_{TEM} (80%, 24/30) followed by $bla_{CTX\text{-}M}$ (73%, 22/30), bla_{SHV} (60%, 18/30), bla_{NDM} (13%, 4/30), and $bla_{OXA\text{-}48}$ (13%, 4/30). Majority of the ß-lactamase encoding genes were detected in EAEC (50%) and ETEC (20%). Co-existence of different β-lactamase genes was found in the present study.

Conclusion: The bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{SHV}, bla_{NDM}, and $bla_{OXA\text{-}48}$, that are associated with serious and urgent threats globally, were detected in diarrheagenic *E. coli* isolates from under-five children in Ethiopia. This study also revealed the coexistence of the β-lactamase genes.

Keywords: Diarrheagenic E. coil; ESBL; Ethiopia; carbapenemase; multidrug resistance; under-five children; ß-lactamase.

https://pubmed.ncbi.nlm.nih.gov/37954506/

Zenebe *et al. Ann Clin Microbiol Antimicrob* (2020) 19:61
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and Antimicrobi

REVIEW Open Acc

Prevalence of *Campylobacter* species in human, animal and food of animal origin and their antimicrobial susceptibility in Ethiopi a systematic review and meta-analysis

Tizazu Zenebe[1*], Niguse Zegeye[2] and Tadesse Eguale[3]

Abstract

Background: *Campylobacter jejuni* and *Campylobacter coli* accounts for most cases of human gastrointestinal infec tions. The infection occurs through ingestion of contaminated food or water, and direct contact with feces of infect animal or human. Regardless of few local reports of *Campylobacter* and its antimicrobial susceptibility profile, there no comprehensive data that show the burden of *Campylobacter* infection at national level in Ethiopia. This systemic review and meta-analysis aimed to determine the pooled prevalence of *Campylobacter* and its resistance patterns i Ethiopia from different sources.

Method: A comprehensive literature search of PubMed, Google scholar, Science direct and Google engine search was conducted for studies published from 2000 to July 30, 2020 on prevalence and antimicrobial susceptibility of *Campylobacter* in human, animal and food. The study was conducted according to the Preferred Reporting Items fo Systematic Reviews and Meta-Analysis (PRISMA) Checklist. Data from articles was extracted using a standardized da extraction format. The quality of the studies was assessed based on the Newcastle–Ottawa scale. The Q test and I^2 t statistic were used to test heterogeneity across studies. The Pooled estimate of prevalence of *Campylobacter* spe- cies and its antimicrobial susceptibility profile was computed by a random effects model using STATA 16.0 software Results were presented in forest plot, tables, funnel plot and figures with 95% confidence interval.

Results: A total of 291 articles were retrieved initially. The pooled prevalence of *Campylobacter species* from differer sources was 10.2% (95% CI 3.79, 16.51). In this meta-analysis, the lowest prevalence was 6.0% whereas the highest prevalence was 72.7%. In the sub-group analysis, the pooled prevalence was similar in Amhara and Oromia region, higher in Gambella and lower in Sidama. Prevalence of *Campylobacter* was higher in animals (14.6%) compared to humans (9%). The pooled antimicrobial resistance rates of *Campylobacter species* to different antimicrobials ranged from 2.9–100%. Overall, higher rate of resistance was to cephalothin (67.2%), gentamicin (67.2%), and trimethoprim- sulfamethoxazole (33.3%) in *Campylobacter* isolates from all sources. In isolates from human, resistance to cephaloth was 83% followed by amoxicillin (80%), amoxicillin-clavulnate (36%), trimethoprim-sulfamethpxazole (32%), clindan cin (31%) and ceftriaxone (28%). On the other hand, higher rate of resistance to penicillin (100%), cephalothin (60%) ciprofloxacin (71.2%), and trimethoprim-sulfamethoxazole (39%) was recorded in isolates from animals.

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Declaration

I undersigned declare that this PhD Thesis is my original work and has not been presented for a degree in any other University, and all sources of material used for the Thesis has been duly acknowledged.

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   Signature: _____ Date: _____

2. Dr. Adane Mihiret (PhD, Associate Professor)

   Signature:  Date: _____

3. Dr. Tadesse Eguale (PhD, Associate Professor)

   Signature:  Date: _____