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Molecular Detection and Antibiotic Susceptibility Pattern of Shiga Toxin-Producing *Escherichia coli* among Children with Diarrhea in Addis Ababa, Gondar, and Harar, Ethiopia

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ABSTRACT

Introduction: Shiga toxin-producing *Escherichia coli* (STEC) is a major cause of pediatric diarrhea globally, yet its burden, molecular characteristics, and antibiotic resistance patterns remain underexplored in low-resource settings like Ethiopia. This study aimed to assess the molecular detection and antibiotic susceptibility pattern of STEC among children with diarrhea in Ethiopia. **Methods**: A cross-sectional study was conducted between October 2021 and November 2022 in Ethiopia (Addis Ababa, Gondar, and Harar) among 568 children under 15 years of age. Socio-demographic and clinical data were collected using a standardized questionnaire and REDCap software. Stool samples were screened for STEC using ChromSTEC agar. Shiga toxin (*stx*) and intimin (*eae*) genes were detected using polymerase chain reaction (PCR). The antibiotic susceptibility profile was determined using the Phoenix M50 machine. Data analysis was performed using R version 4.3.2 for descriptive statistics and logistic regression analysis. **Results:** The overall prevalence of STEC among children with diarrhea was 12.15% [95% CI: 9.71-15.09], with the higher prevalence among children aged two to five years and those with bloody diarrhea. STEC isolates showed high antimicrobial resistance (AMR), with 46.38% showing multidrug resistance (MDR) and 2.90% classified as extensively drug-resistant (XDR). Additionally, 21.74% of STEC isolates were extended-spectrum beta-lactamase (ESBL) producers, including 12.50% of those harboring the *eae* gene. **Conclusions**: This study provides one of the first comprehensive assessments of STEC across pediatric age groups and multiple regions in Ethiopia, revealing high prevalence and antibiotic resistance with regional and seasonal variations. These findings highlight the urgent need for enhanced infection prevention and strengthened antimicrobial stewardship.

INTRODUCTION

Diarrheal diseases, mostly resulting from foodborne pathogens, cause 550 million illnesses and 230,000 deaths each year in low- and middle-income countries (LMICs) [1]. Infectious agents such as nontyphoidal *Salmonella* spp., STEC, and *Campylobacter* spp., present significant global health risks, causing severe diarrheal illness and potential long-term complications [2]. However, these etiologies are not well studied in Ethiopia, and the morbidity and mortality of diarrhea are significantly high where unimproved sanitation facilities and practices play an important role [3]. For instance, in Ethiopia, diarrheal disease was among the top five leading causes of mortality in 2015, with an age-standardized death rate of 88.6 per 100,000 population (95% UI: 59.4-127.1) [4], yet evidence on circulating pathogens remains fragmented. Routine testing mainly targets *Salmonella* and *Shigella*, often overlooking the burden of STEC. Although reports show a high prevalence of STEC in Sub-Saharan Africa, including Ethiopia, particularly among children, the regional burden is likely underestimated compared to the global

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burden, partly because of underreporting and diagnostic limitations [5].

The Shiga toxins (Stx), which are classified as Stx1 and Stx2, are crucial virulence factors responsible for the cytotoxicity of STEC in host cells [6]. These toxins are encoded by *stx1* and *stx2* genes, respectively, and disrupt host cell protein synthesis, causing cell death [7], bloody diarrhea, life-threatening conditions like hemolytic uremic syndrome (HUS), and central nervous system abnormalities [8]. The pathogenicity of STEC is further enhanced by an adherence factor (intimin), encoded by the *eae* gene, which facilitates intimate adherence to epithelial cells and the formation of attaching and effacing lesions [9,10]. Not all STEC strains harbor *eae* genes; STEC is defined by the presence of *stx1* and/or *stx2*, with approximately 400 known serotypes globally [11]. However, those with the *stx* genes (either *stx1*, *stx2*, or both) and *eae* are known as STEC harboring *eae* [10], and they are related to severe infections and complications [9, 10].

Although antibiotic treatment is discouraged in STEC infections because of the risk of enhanced toxin release and complications [12], some studies have reported that azithromycin may reduce such risks in prolonged infections [13, 14]. Antimicrobial resistance (AMR) remains a critical concern, with many STEC strains exhibiting resistance to commonly used antibiotics, including ampicillin, ciprofloxacin, and sulfamethoxazole-trimethoprim [15]. This resistance is largely driven by horizontal gene transfer and the proliferation of mobile genetic elements [10, 16].

Despite STEC's clinical significance, routine laboratory screening in Ethiopia remains limited, especially for non-O157 serotypes. Most local studies have focused on food and animal sources [17, 18] or *E. coli* O157 detection [19-21], with limited data from clinical cases. As a result, diarrheal illnesses are often treated empirically, potentially promoting AMR [22]. Furthermore, the role of seasonality and geographic variation in shaping STEC distribution is poorly understood, despite variations in hygiene, diet, and animal contact across Ethiopian regions [23, 24] and significant differences in climate and rainfall [25], which likely influence the prevalence and distribution of STEC and its virulence traits.

These gaps in clinical surveillance, regional and seasonal trends, and limited data on virulence factors necessitate a better understanding of STEC's burden in Ethiopian healthcare settings. Given this, the study aimed to generate evidence on the prevalence and AMR patterns of STEC and its associated virulence genes among children with diarrhea in Addis Ababa, Gondar, and Harar. This study prioritized regional and seasonal variations because of their relevance in informing targeted public health interventions and infection control strategies in areas with limited diagnosis and surveillance capacity. Specifically, the research was guided by two key questions: (1) What is the prevalence and seasonal distribution of STEC and STEC harboring the *eae* gene among children with diarrhea in Addis Ababa, Gondar, and Harar? and (2) Is there a variation in the distribution of virulence genes (*stx1*, *stx2*, and *eae*) among STEC isolates across these regions? Hence, understanding the burden and molecular characteristics of STEC infections in Ethiopia is essential to addressing gaps in diarrheal disease surveillance and control. Thus, the findings provide critical evidence to support public health decision-making in Ethiopia, particularly in strengthening diagnostic capacity, guiding antimicrobial stewardship, and informing region-specific prevention and control strategies for diarrheal diseases.

MATERIAL AND METHODS

Study area, design, and period

A cross-sectional epidemiological study was conducted between October 2021 and November 2022 at Yekatit-12 Hospital, University of Gondar Comprehensive Specialized Hospital, and Hiwot Fana Comprehensive Specialized Hospital in three geographically diverse cities of Ethiopia (Addis Ababa, Gondar, and Harar, respectively). Yekatit-12 Hospital is a specialized and teaching hospital located in Addis Ababa, representing the central part of Ethiopia, and serves around 5 million people. The University of Gondar Comprehensive Specialized Hospital is located in Gondar city, northwestern Ethiopia, and serves around 7 million people. Hiwot Fana Comprehensive Specialized Hospital is situated in the eastern part of Ethiopia and serves 5 to 6 million people (Figure 1).

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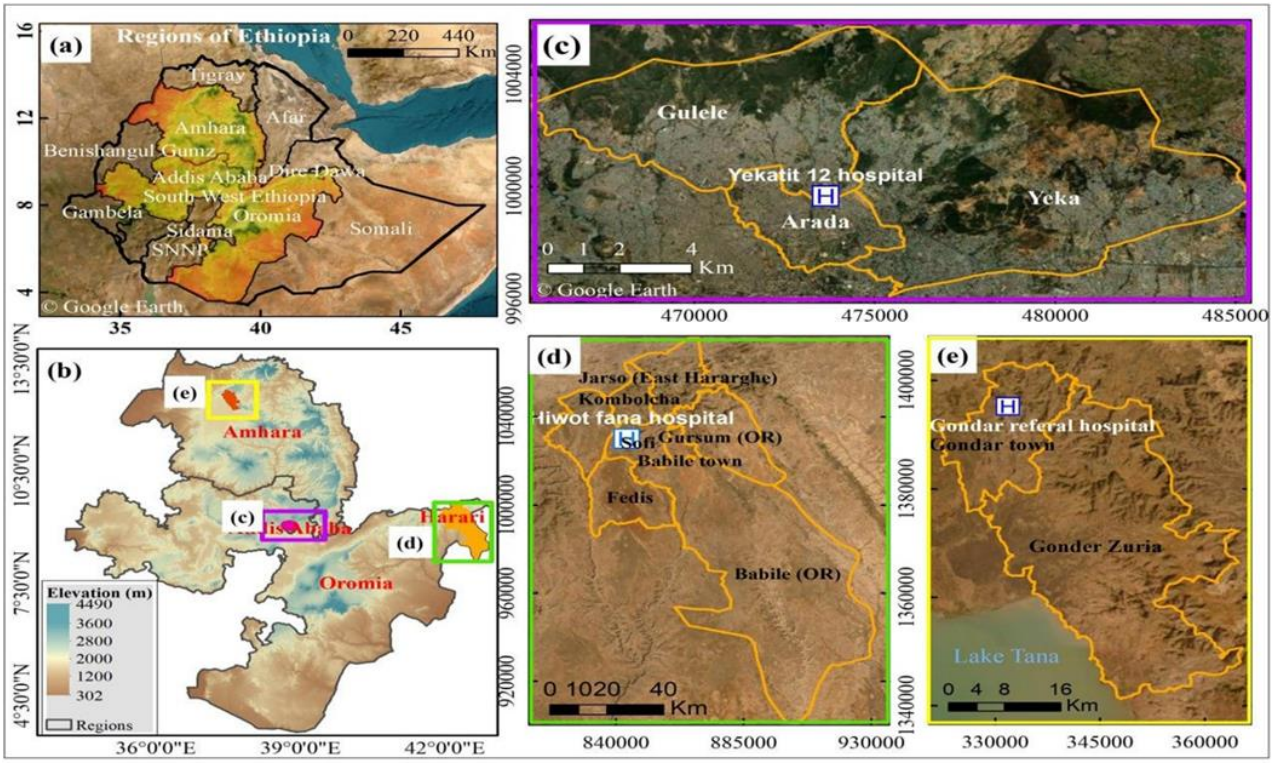

Fig. 1. Study areas of sample collection in Ethiopia, eastern Africa (a), from the three target regions (b), and the three hospitals, Yekatit-12 (c), Hiwot Fana Comprehensive Specialized Hospital (d), and University of Gondar Comprehensive Specialized Hospital (e), (Source of the administrative boundaries: Central Statistical Agency (CSA) of Ethiopia; background Google source data: Esri, Maxar, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community).

Data collection

Patients were enrolled in this study if they visited the three hospitals' clinical laboratories for the diagnosis of diarrheal illness. A total of 568 children under the age of 15 years were enrolled. Stool samples were collected using standardized microbiological techniques, and their parents/caregivers were interviewed using a standardized, structured questionnaire about their sociodemographic characteristics, study setting (urban/rural), region of their residence, and clinical information (Supplementary Material 1).

Sample collection, processing, and identification of STEC isolates

Stool samples were transported to the laboratory for microbiological analysis using standardized microbiological procedures, inoculated onto ChromSTEC agar (CHROMagar™, France), and incubated aerobically at 35±2ºC overnight. The growth of mauve colonies on the ChromSTEC agar [26], which is indicative of the tellurite-resistant nature of STEC, was considered presumptively STEC-positive. These presumptive isolates were confirmed by detecting the presence of the common virulence genes of STEC such as *stx1*, *stx2*, and/or *eae* genes using a commercially approved magnetic induction cycler quantitative polymerase chain reaction (qPCR) [27] using commercially available assays (primers, probes, master mix, and related supplies) [28].

Molecular confirmation

DNA was extracted from a pure colony using the boiling method as described previously [29]. Multiplex PCR testing was carried out using a previously designed assay protocol, primer sequences, and probes of the ISO/TS 13136 guideline [30], and the manufacturer's instructions for the PCR machine [27]. The isolates that were positive for *stx1* or *stx2* or both were defined as STEC. The STEC isolates confirmed positive for the *eae* gene were defined as STEC harboring the *eae* gene.

Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) was performed using the Phoenix M50 machine from BD (Becton Dickinson, USA) according to the manufacturer's instructions [31]. Each isolate was tested for penicillin, fluoroquinolones, tetracycline, folate pathway antagonists, aminoglycosides, cephalosporins, β-lactam/β-lactamase inhibitor combinations, carbapenems, monobactams, nitrofurantoin, fosfomycin, and polymyxins groups. The results interpretation was based on the breakpoints in the Clinical and Laboratory Standards Institute guidelines. Moreover, MDR was defined as acquired nonsusceptibility to at least one

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agent in three or more antimicrobial categories. XDR was defined as bacterial isolates that remain susceptible to only one or two antimicrobial categories [32]. Moreover, the BD Phoenix ESBL test was used to screen for ESBL with follow-up phenotypic confirmation using the double disc diffusion method [33] (Supplementary Material 2). Phenotypic confirmation of ESBL was performed using the double disc diffusion method [33], with a combined disc assay involving ceftazidime (30µg) versus ceftazidime-clavulanate (30/10µg), and cefotaxime (30µg) versus cefotaxime-clavulanate (30/10µg) discs. ESBL production was determined by an expansion of ≥ 5 mm in zone diameters for combined discs compared to ceftazidime and cefotaxime alone. *K. pneumoniae* (ATCC 700603) and *E. coli* (ATCC 25922) were used as the positive and negative controls testing for ESBL, respectively.

Data management and analysis

A standardized questionnaire was used to collect sociodemographic and clinical data electronically using REDCap software [34]. The analysis was performed using R version 4.3.2 software [35]. The dependent variables were STEC prevalence and STEC virulence genes (*stx1*, *stx2*, and *eae*), with independent variables including gender, age, study setting (urban/rural), study site, season, and clinical features. The year was divided into dry (October-February), short rainy (March-May), and long rainy seasons (June-September) [25]. Descriptive statistics were used to summarize the data, and STEC prevalence with 95% confidence intervals was calculated using binomial tests and proportion tests. Group differences in prevalence among study sites and seasons were evaluated using the chi-square test and two-sample proportion test where appropriate. The contingency table analysis showed the distribution of STEC and STEC harboring *eae* among various factors. Moreover, a proportion test was conducted to assess differences among groups for the hypothesized population showing significant differences between subgroups. A *P*-value <0.05 was considered significant, and the results were presented in tables and graphs. The sample size was calculated using a single proportion formula based on a previously reported STEC prevalence of 13.9% [36] with 80% power and 5% level of significance, aiming to ensure adequate estimation of overall prevalence. To account for potential non-response and loss due to unanalyzable stool samples, the calculated sample size of 552 was increased to 568. Although the initial objective was prevalence estimation, the final sample size was also adequate for comparative analysis across the three study sites. A post hoc power analysis assuming a small-to-moderate effect size (w=0.2) and alpha level of 0.05 showed a power of 99.3% for detecting statistically significant differences in prevalence across the three groups, confirming the sufficiency of the sample size for comparative purposes between the study sites.

Ethical consideration

Ethical approvals were obtained from the Ethiopian Public Health Institute (Protocol EPHI-IRB-311-2020); Yekatit-12 Hospital Medical College (Protocol 68/21); University of Gondar (Protocol V/P/RCS/05/101/2020); and Haramaya University (Protocol IHRERC/020/2021). Each of these ethical approvals was subsequently renewed annually by the respective institutions during the study period to ensure continued compliance during data collection. Written informed consent and parental permission were obtained from the parents or caregivers. Assent was also obtained from each participant between the ages of 12-14 years.

RESULTS

Demographic and clinical characteristics of the study population

The study enrolled a total of 568 children with age ranges from 2 months to 15 years of age with a mean age of 4.70 ± 3.60 years (SD). The majority (230/568 (40.49%)) were within the age group of 2-5 years, and the fewest were within the 12–15 years of age group, 38 (6.69%). Males were predominant, accounting for 329 (57.92%) of the participants. Moreover, most of the study participants were from urban settings, 505 (88.91%). In addition to having diarrhea, all the study participants had other accompanying symptoms such as abdominal cramps, 354 (62.32%), and undefined abdominal pain, 334 (58.80%) (Table 1). Regarding the nature of the stool samples, loose or watery diarrhea was the predominant stool type, 488 (85.92%). All variables included in the analysis had complete data; hence, no data imputation or exclusion due to missing values was necessary.

Prevalence of STEC

A total of 69/568 presumptive STEC isolates were identified, all testing positive for *stx*, resulting in an overall STEC prevalence of 12.15% [95% CI: 9.71-15.09]. Children in the age category of two to five years had the highest prevalence at 13.90% [95% CI: 10.03-18.99]. The prevalence was highest in Addis Ababa, 12.82% [95% CI: 8.45-18.97], and Gondar 12.56% [95% CI: 8.83-17.55], and lowest in Harar 11.11% [95% CI: 7.38-16.39] (Figure 2). The highest prevalence (13.70% [95% CI: 10.46-17.75; P = 0.01]) was detected during the dry season, followed by the long rainy season (13.01% [95% CI: 8.50-19.43]), while the short rainy season had the lowest (3.80% [95% CI: 1.30-10.58; P = 0.04]) (Figure 3).

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Table 1. Proportion of diarrheal cases positive for STEC (n=69) and STEC harboring *eae* (n=16) by sociodemographic and clinical characteristics (n=568) in Addis Ababa, Gondar and Harar, Ethiopia, from October 2021 to November 2022

Characteristics	Total number (%)	Number STEC positive (%) (n=69)	% of diarrheal cases that are STEC positive [95% CI]	Number STEC harboring *eae* (%) (n=16)	% of diarrheal cases that are STEC harboring *eae* positive [95% CI]
Age group (years)					
<2 years	96 (16.90)	10 (14.49)	10.42 [5.76-18.12]	5 (31.25)	5.21 [2.44-11.60]
2-5 years	230 (40.49)	32 (46.37)	13.91 [10.03-18.99]	2 (12.50)	0.87 [0.24-3.11]
5-12 years	204 (35.92)	23 (33.33)	11.27 [7.63-16.35]	8 (50.00)	3.92 [2.00-7.55]
12-15 years	38 (6.69)	4 (5.80)	10.53 [4.17-24.13]	1 (6.25)	2.63 [0.47-13.49]
Sex					
Male	329 (57.92)	39 (56.52)	11.85 [8.79-15.79]	12 (75.00)	3.65 [2.10-6.27]
Female	239 (42.08)	30 (43.48)	12.55 [8.94-17.35]	4 (25.00)	1.67 [0.65-4.22]
Study sites					
Addis Ababa	156 (27.46)	20 (28.99)	12.82 [8.45-18.97]	1 (6.25)	0.64 [0.01-3.54]
Gondar	223 (39.26)	28 (40.58)	12.56 [8.83-17.55]	11 (68.75)	4.93 [2.78-8.62]
Harar	189 (33.27)	21 (30.43)	11.11 [7.38-16.39]	4 (25.00)	2.12 [0.83-5.31]
Residence					
Urban	505 (88.91)	62 (89.86)	12.28 [9.70-15.43]	14 (87.50)	2.77 [1.66-4.60]
Rural	63 (11.09)	7 (10.14)	11.11 [5.49-21.20]	2 (12.50)	3.17 [0.90-10.86]
Season					
Dry	343 (60.39)	47 (68.12)	13.70 [10.46-17.75]	9 (56.25)	2.62 [1.39-4.91]
Short Rain	79 (13.91)	3 (4.35)	3.80 [1.30-10.58]	1 (6.25)	1.27 [0.22-6.83]
Long Rain	146 (25.70)	19 (27.54)	13.01 [8.50-19.43]	6 (37.50)	4.11 [1.90-8.68]
Types of Diarrhea					
Loose or watery diarrhea	488 (85.92)	62 (89.86)	12.70 [10.04-15.95]	14 (87.50)	2.87 [1.72-4.76]
Bloody diarrhea	53 (9.33)	7 (10.14)	13.21 [6.55-24.84]	3 (18.75)	5.66 [1.94-15.37]
Mucoid or mucus diarrhea	134 (23.59)	8 (11.60)	5.97 [3.06-11.34]	0 (0)	0 [0-0]
Signs and Symptoms					
Diarrhea	568 (100.00)	69 (100.00)	12.15 [9.71-15.09]	16 (100.00)	2.82 [1.74-4.53]
Vomiting	259 (45.60)	44 (63.77)	16.99 [12.91-22.04]	12 (75.00)	4.63 [2.67-7.92]
Nausea	104 (18.31)	39 (56.52)	37.5 [28.80-47.09]	11 (68.75)	10.58 [6.01-17.95]
Bloating	19 (3.35)	4 (5.80)	21.05 [8.51-43.33]	1 (6.25)	5.26 [0.94-24.64]
Fever	289 (50.88)	47 (68.12)	16.26 [12.46-20.96]	12 (75.00)	4.15 [2.39-7.12]
Abdominal Cramps	354 (62.32)	43 (62.32)	12.15 [9.14-15.96]	11 (68.75)	3.11 [1.74-5.48]
Undefined abdominal pain	334 (58.80)	46 (66.67)	13.77 [10.49-17.88]	12 (75.00)	3.59 [2.11-6.17]

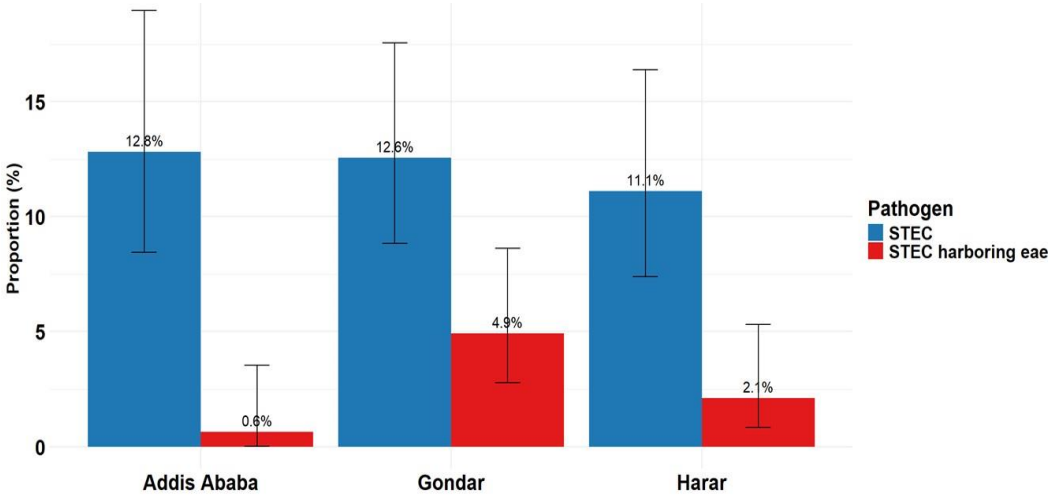

Fig. 2. Prevalence of STEC and STEC harboring *eae* among the study sites.

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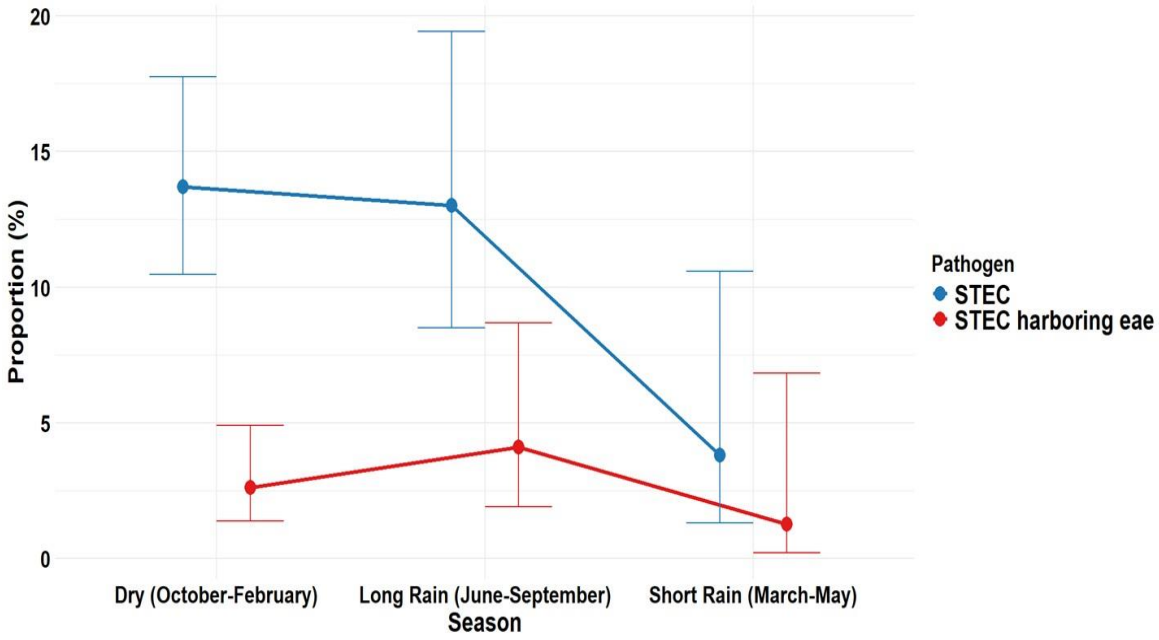

Fig. 3. Prevalence of STEC and STEC harboring *eae* by season.

The prevalence of STEC was the highest, 13.21% [95% CI: 6.55-24.84], among patients with bloody diarrhea, followed by those with symptoms of nausea, 37.5% [95% CI: 28.80-47.09]. STEC harboring *eae* had an overall prevalence of 2.82% [95% CI: 1.74-4.53], with the highest rates from Gondar, 4.93% [95% CI: 2.78-8.62], and among children under two years of age, 5.21% [95% CI: 2.44-11.60]. Rural areas had a higher prevalence of STEC harboring *eae*, 3.17% [95% CI: 0.90-10.86]**,** than urban areas, 2.77% [95% CI: 1.66-4.60]. The long rainy season also showed the highest prevalence of STEC harboring *eae*, 4.11% [95% CI: 1.90-8.68], compared with 2.62% [95% CI: 1.39-4.91] for the dry season and 1.27% [95% CI: 0.22-6.83] for the short rainy season. Patients with bloody diarrhea and nausea had 5.66% [95% CI: 1.94-15.37] and 10.58% [95% CI: 6.01-17.95] prevalence of STEC harboring *eae*, respectively (Table 1).

The distribution of three of the key virulence genes of STEC varied among the study sites, sociodemographic characteristics, and clinical presentations. The most frequently detected virulence gene was *stx1* in 57.97% (40/69) and *stx2* in 53.62% (37/69), with overlap due to co-carriage. Due to the overlapping gene carriage, the sum proportion of *stx1* and *stx2* exceed 100%. Distribution of the targeted virulence genes described below refers to the presence of that gene regardless of their combination with other virulence genes. For instance, the proportion of *stx1* was highest in Harar, 15 (37.50%), those of *stx2* and *eae* genes were highest in Gondar with a rate of 17 (45.95%) and 11 (68.75%), respectively (Figure 4). When considering the combination of *stx1* or *stx2* and the *eae* gene, the proportions were 6.25% (1/16) in Addis Ababa, 68.75% (11/16) in Gondar, and 25.00% (4/16) in Harar, with an overall proportion of 23.19% (16/69). Among the 10 isolates carrying both *stx1* and *stx2* genes, 10.00% (1/10) were from Addis Ababa, 30.00% (3/10) from Gondar, and 60.00% (6/10) from Harar.

Specifically, 29 isolates (42.03%) were positive for *stx1* only, and 20 isolates (28.96%) were positive for *stx2* only, and the remaining isolates carried a combination of two or three of the targeted virulence genes.

The overall proportion of isolates with both *stx1* and *stx2*,, was 14.49% (10/69), regardless of the presence or absence of *eae* gene. However, 5.80% (4/69) were STEC strains carrying both *stx1* and *stx2* genes only, without the *eae* gene. The *stx1* and *eae* combination was found in 12.50% (1/8) of isolates from Addis Ababa, 50.00% (4/8) from Gondar, and 37.50% (3/8) from Harar, yielding an overall proportion of 11.59% (8/69). The combination of *stx1*, *stx2* and *eae* was not detected in Addis Ababa but was present in 50.00% (3/6) of isolates from Gondar and Harar each, with an overall proportion of 8.69% (6/69). Lastly, the *stx2* and *eae* combination was not detected in Addis Ababa but was found in 71.43% (10/14) of isolates from Gondar and 28.57% (4/14) from Harar, with an overall proportion of 20.29% (14/69). These findings illustrate the regional variation in gene combinations, with Gondar showing the highest frequencies of *stx2* and *eae* combinations (Table 2). In addition, the proportion of virulence genes varied significantly across different sociodemographic groups. For instance, children aged two to five years exhibited a

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relatively high proportion of *stx1*, 8.26% [95% CI: 5.35-12.54], and *stx2*, 7.39% [95% CI: 4.67-11.52]. Moreover, Addis Ababa had the highest *eae* proportion, 7.10% [95% CI: 4.00-12.20], compared to Gondar and Harar.

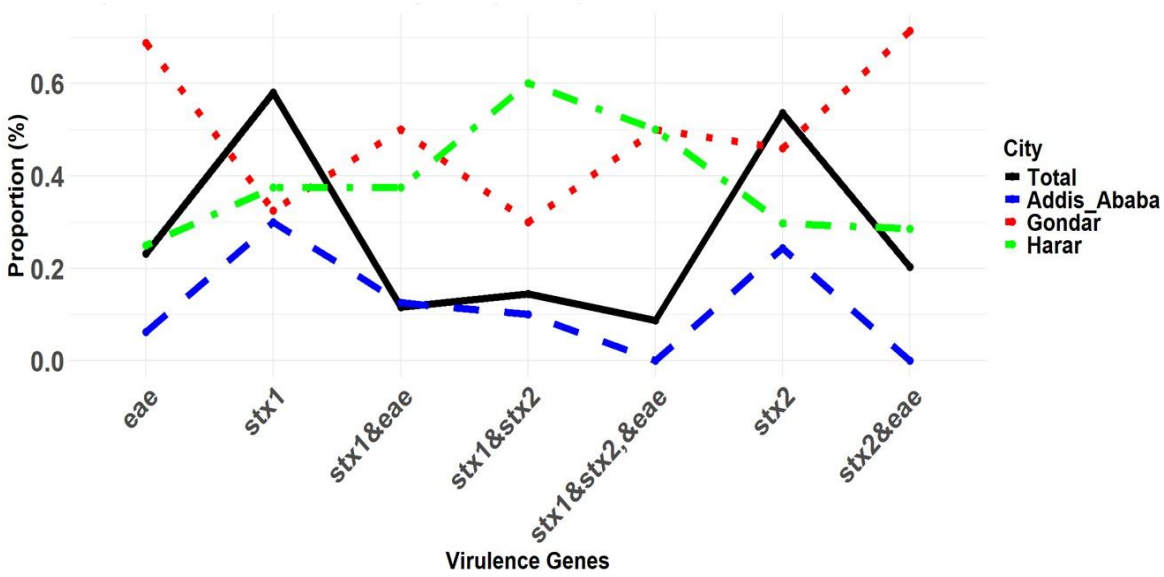

Fig. 4. The overall proportion of virulent genes, and their distribution among the study sites.

Antibiotic susceptibility pattern of STEC

The proportion of antibiotic-resistant STEC isolates showed notable variation across different antibiotics. Resistance was highest to ampicillin, 50 (72.46%) and amoxicillin-clavulanate 39 (56.52%), followed by trimethoprim-sulfamethoxazole 43 (62.32%) and cefuroxime 29 (42.03%). Moderate resistance levels were observed for ceftriaxone and levofloxacin, both at 26 (37.68%), and ciprofloxacin at 24 (34.78%). Resistance to aztreonam, cefepime, and cefazolin ranged between 21 (30.43%) and 28 (40.58%). In contrast, very low resistance was reported for colistin, ceftolozane-tazobactam, and fosfomycin, each below 2%, one STEC isolate was resistance for each. Other antibiotics such as piperacillin-tazobactam (5 STEC isolates), tigecycline (4 STEC isolates), and nitrofurantoin (7 STEC isolates) had resistance rates, that is below 12%. For carbapenems (imipenem, ertapenem, and meropenem), resistance was consistently low, 2 to 3 STEC isolates were resistant, *i.e.*, under 5%, highlighting their effectiveness against STEC isolates (Figure 5). However, there was regional variability in STEC isolates with respect to the proportions of their resistance (Figure 6).

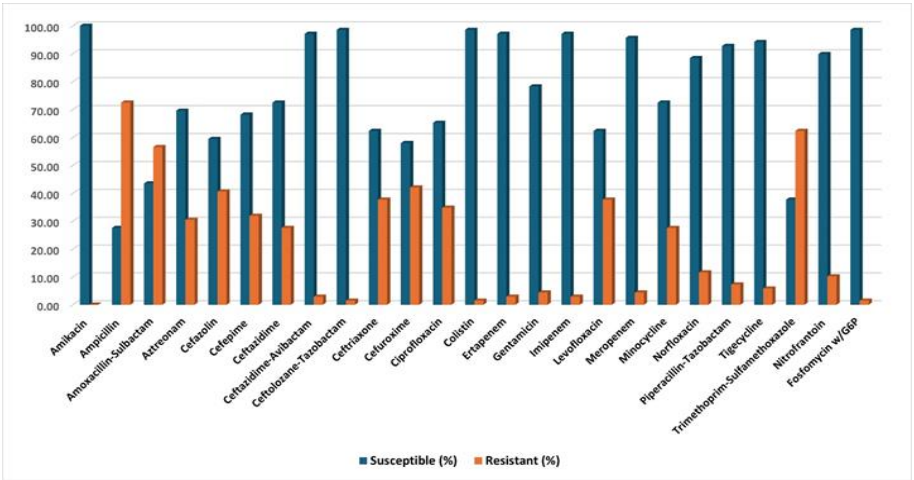

Fig. 5. An overall antibiotic susceptibility pattern of STEC isolated from the three study sites.

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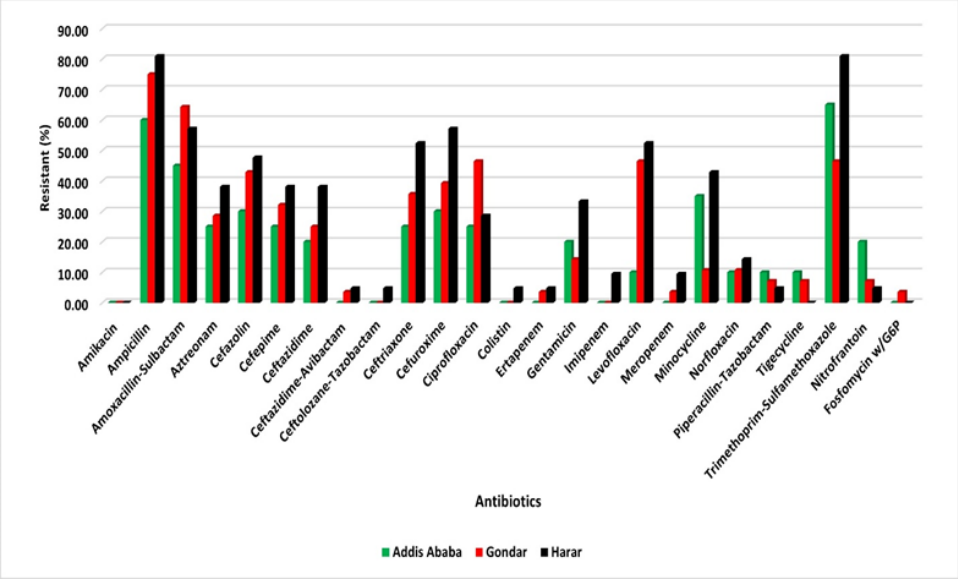

Fig. 6. The proportion of antibiotic-resistant STEC isolated from all three study sites.

Among the 69 STEC isolates, 15 (21.74%) were ESBL producers with notable variations among the three study sites (Figure 7a). Harar exhibited the highest proportion of ESBL isolates, 7 (10.14%), while Addis Ababa showed the lowest, 2 (2.90%). Moreover, the XDR rate was 2 (2.90%), with both cases from Harar. The overall MDR rate was 32 (46.38%) (Figure 7b) with Harar having the highest, 13 (40.63%), and Addis Ababa having the lowest, 7 (21.88%) rates.

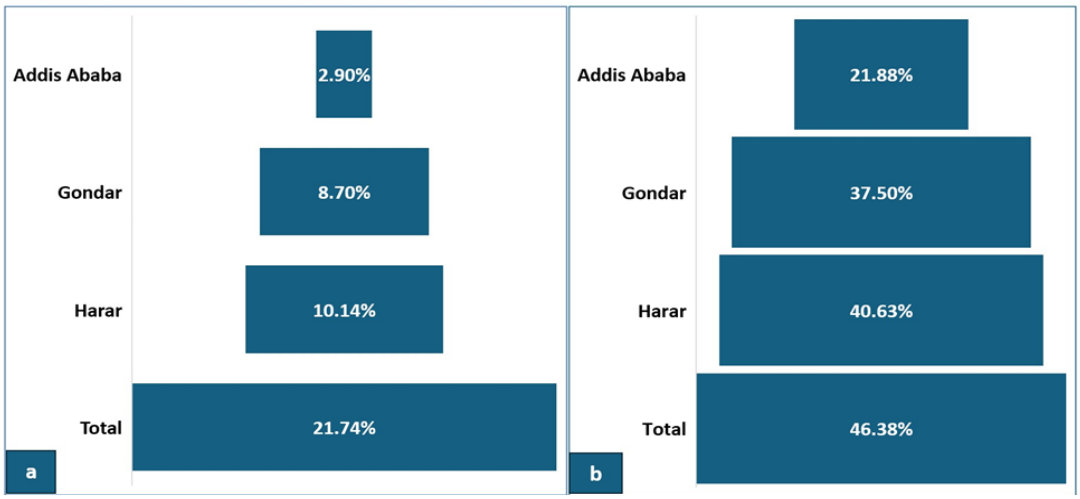

Fig. 7. The overall proportions of extended-spectrum beta-lactamase (a), and multidrug resistance (b), and their distribution among study sites.

DISCUSSION

Diarrheal diseases remain a critical public health challenge in Ethiopia, particularly among children under 5 years of age, contributing to significant morbidity and mortality. Despite strides in health interventions, diarrhea persists as a leading cause of death, accounting for 23% of under-5 mortality and contributing to 44% of stunting [37]. While bacterial pathogens such as *Campylobacter*, nontyphoidal *Salmonella*, *Shigella* spp., and diarrheagenic *Escherichia coli* (DEC) are known to be major causes, the specific burden of STEC remains underexplored [38, 39]. STEC has been identified as a key diarrheagenic pathogen in children across Africa, including Ethiopia, but comprehensive data comparing its prevalence with other diarrheagenic bacteria in the Ethiopian context are scarce [39]. This evidence gap hinders a clear understanding of the relative contribution of STEC to diarrheal cases in Ethiopia, making it challenging to rank its burden relative to other pathogens. Addressing this knowledge gap is crucial for tailoring effective public health interventions and prioritizing pathogen-specific strategies.

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Moreover, a life-threatening complication beyond diarrhea is a well-known consequence of STEC infections [3]. The limited number of studies conducted in Ethiopia mainly emphasized the confirmation of STEC using latex agglutination [20], biochemical tests, and PCR [19, 40] but did not further show the distribution of the key virulence genes. Hence, despite its severity, STEC is often overlooked in clinical settings, resulting in sporadic low-incidence reports and an unclear actual burden. In this regard, the current study provided the most updated evidence on the burden of STEC across pediatric age groups and wider geographic areas in Ethiopia, identified the proportion of the three key virulence genes, and determined the antibiotic susceptibility pattern of STEC isolates.

The overall STEC prevalence in our study was 12.15%, revealing a significant yet under-recognized burden among children in Ethiopia, with notable differences from previous studies that ranged from 7.7% [41] in Gondar, and 8.1% in Addis Ababa [42] to 30.3% in Harar [43]. The differences in STEC prevalence across studies can be attributed to variations in methodology, such as the genes targeted (*stx1* versus *stx2*), which may lead to underestimation when only one gene is included. Additionally, studies relying solely on culture methods or biochemical tests without molecular confirmation may overestimate the prevalence. These methodological differences highlight the need for comprehensive molecular approaches for accurate prevalence estimation.

Moreover, there are hundreds of serotypes of STEC harboring *eae*, among which the top five serotypes that cause human illness are O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 [10]. Of these, STEC O157 is the main cause of foodborne illnesses, and the focus of the majority of the literature, although more recently, cases and outbreaks from non-O157 strains have been rising [44,45]. However, most of the previous studies conducted in Ethiopia targeted genes that can be detected only from STEC O157:H7, which is only a single subset from among hundreds of other pathogenic serotypes such as STEC harboring *eae* that are linked to serious illnesses and outbreaks worldwide [45]. Hence, the strains that are currently known to cause outbreaks and severe illnesses worldwide were not well represented in the previous studies in Ethiopia, which as a result undermined the overall burden of STEC harboring *eae* in the country.

In the current study, however, 23.19% of the STEC isolates were STEC harboring *eae*, with an overall 2.82% (16/568) prevalence among all diarrheagenic study participants. In this connection, a notable difference was observed also among the three regions where the prevalence ranged from 0.64% in Addis Ababa, which was the lowest of all the three sites, to the highest in Gondar with a prevalence of 4.93%. These differences align with findings from prior studies conducted in their respective geographic areas [19-21, 40, 46]. The variability pattern of the prevalence of STEC harboring *eae* between the three study sites in our study is comparable to the reports from previous studies in the respective geographic areas. However, there are notable variations between the findings from this study and those from the previous studies regarding the overall STEC harboring *eae* carriage rates, where the prevalence from the previous studies was much lower than our findings. The possible reason for the variation might be that the target populations in the previous studies differed; for example, only children under 5 years of age were considered in the Zelelie *et al*. study [40], and they might not be at risk of exposure to risky food types such as raw meat, milk, and vegetables. Moreover, the inclusion of patients of all age groups including adults [21], who have a likely increased risk of exposure to high-risk foods such as raw meat, raw vegetables, and raw milk, might have been responsible for the higher prevalence reported in the latter study. Furthermore, the recruitment of patients who had cattle in their households [19] might have increased the likelihood of exposure to STEC harboring the *eae* gene, possibly explaining their higher estimates, unlike our cases where only 18 out of the 568 participants had cattle in their household. The role of livestock as reservoirs for STEC is well-documented. In Ethiopia, a study found a 14.4% detection rate of STEC O157:H7, one of the serotypes of the STEC harboring *eae* **,** from cattle, indicating a potential zoonotic transmission route [19]. This is consistent with global data showing that cattle are significant carriers of STEC and STEC harboring *eae*, contributing to environmental contamination and human exposure [47]. Furthermore, the use of the latex agglutination method without a confirmatory test [20] may have been responsible for overestimating the prevalence in the other studies, as this method may be prone to cross-reactivity with antigens from non-STEC strains, which may cause a remarkable reduction in specificity.

On the other hand, our findings on the prevalence of STEC in Ethiopia align with global patterns observed in both low- and high-income countries. For instance, in Argentina, the incidence of STEC in under 5 years of age children is approximately 12 per 100,000 population, highlighting a significant burden in this age group [48]. In France, surveillance of STEC was conducted through voluntary clinical and microbiological monitoring of HUS in children under 15 years of age. Annual incidence rates for pediatric STEC-HUS have remained relatively high, ranging from 0.6 to 1.5 cases per 100,000 population, with rates exceeding 4 cases per 100,000 in children under 3 years [49]. The primary serogroups identified in these cases were O26, O80, and O157; notably, there has been an increase in cases associated with serogroups O26 and O80 in recent years, coinciding with a decrease in O157 cases [49]. Hence, the higher STEC detection rates in LMICs, such as Argentina and

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Ethiopia, may be due to factors like limited access to clean water, inadequate sanitation, and differences in dietary practices. In contrast, high-income countries like France might benefit more from their robust public health infrastructure and stricter food safety regulations, which may contribute to lower reported incidence rates.

The prevalence of STEC and STEC harboring *eae* varied by season, where dry and long rainy seasons showed the highest prevalence. Dry conditions may promote the concentration of pathogens in water sources or increase contamination from animals, while long rainy seasons may cause flooding that contributes to contamination [19, 46]. In support of this, other studies conducted elsewhere have shown that certain environmental conditions can influence the incidence of STEC infections. For example, a summary of reported outbreaks due to *E. coli* O157 infections in the United States during 2003–2012 indicated that environmental factors could play a role in the transmission dynamics of STEC harboring *eae* [50]. Similarly, in Australia, research has highlighted the impact of environmental conditions on the epidemiology of STEC. A study on the epidemiology of STEC in Australia discussed how environmental factors, including seasonal variations, could affect the prevalence and transmission of STEC infections [51]. These international findings underscore the importance of considering environmental and seasonal factors in the surveillance and control of STEC infections.

The molecular detection of virulence genes in our study revealed *stx1* (57.97%) as the predominant gene in STEC isolates, followed by *stx2* (53.62%) and *eae* (23.19%). These virulence factors have significant public health implications. The *stx1* and *stx2* genes encode Stx that are central to the pathogenicity of STEC and are associated with severe clinical outcomes such as HUS. The detection of *eae* in this study, although less frequent, underscores the need for its inclusion in the overall STEC surveillance; STEC harboring *eae* are sub-types, and as it encodes intimin, a protein critical for intestinal epithelial adherence and damage, thus contributing to the severity of infections. Similar to ours, studies from other countries such as Kenya also reported comparable detection rates of these virulence factors where *stx1* was detected in 52.9%, *stx2* in 29.4%, and *eae* in 20.5% of isolates [52]. In addition, an Iranian study found *stx1* and *stx2* genes in 60% and 40% STEC isolates, respectively, with no detection of the *eae* gene [53]. Although many studies in Ethiopia targeted mainly *stx1* and *stx2* [41, 42], it should be noted that detection of the *eae* gene deserves attention in any future STEC and STEC harboring *eae* studies because the intimin protein plays a crucial role in causing significant gastrointestinal damage and, hence, severe clinical outcome [54].

This study revealed alarmingly high levels of antimicrobial resistance among STEC isolates, with rates ranging from 62.3% for trimethoprim-sulfamethoxazole to 72.5% for ampicillin, significantly undermining the effectiveness of commonly used antibiotics and complicating treatment outcomes. These results align with findings from other Ethiopian studies [19, 36], though regional variations were observed. Notably, resistance was also detected against critical, last-resort antibiotics, including 2.90% to 4.35% for carbapenems, 2.90% for ceftazidime-avibactam, and 1.45% for ceftolozane-tazobactam. Additionally, 21.74% of isolates were ESBL producers, posing further therapeutic challenges. Compared to reports from countries like Egypt, where only 3.0% of isolates showed carbapenem resistance and 7% produced ESBLs [55], the resistance levels in our study are significantly higher. MDR was observed in 46.38% of isolates using a stringent definition of resistance to three or more antibiotic classes, a rate comparable to other Ethiopian studies [19] but lower than the 88.2% reported from Bahir Dar, which applied a less stringent definition in which resistance to two or more groups of antibiotics was considered as MDR in their studies [36]. Our findings are also consistent with reports from countries such as India and Egypt [55, 56], where limited regulation and poor sanitation contribute to the spread of resistant strains. Furthermore, 2.90% of isolates exhibited XDR profiles, underscoring the potential for severe clinical and public health consequences. The antibiotic categories to which the XDR isolates were resistant included penicillins, fluoroquinolones, tetracyclines, folate pathway antagonists, aminoglycosides, cephalosporins, β-lactam/β-lactamase inhibitor combinations, monobactams, nitrofurantoin, and fosfomycin. In Ethiopia, widespread access to antibiotics without prescription and limited public awareness about the risks of misuse likely contribute to the emergence of resistance. Additionally, the growing use of antibiotics in animal farming, especially in rural areas, may facilitate the transmission of resistant bacteria to human populations [57].

The present study had significant strengths due to it covered a wide range of geographic areas encompassing northwestern, central, and eastern parts of Ethiopia; it included pediatric age groups; investigated the three important circulating virulence genes; and determined antimicrobial resistance tests against 25 antibiotics among the STEC isolates. On the other hand, some limitations of this study included: First, one of the study's limitations is the use of chromogenic agar, which may miss the diagnosis of some STEC serotypes and hence underestimate the total prevalence of STEC. Second, due to the availability of limited resources, molecular characterization of additional virulence genes such as *hlyA* genes, resistance genes such as *bla* genes, and extensive characterization of STEC serotypes such as O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 using whole genome sequencing (WGS) were not performed. Third, the inconsistent medical record-keeping practices in Ethiopia represented another

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limitation in this study, precluding evaluation of clinical outcomes related to illness severity, particularly in relation to severe forms of the disease such as HUS. This limits our understanding of the public health impacts of these strains in the study sites. Fourth, the low numbers of positive cases of STEC per different factors limit us from further exploring the possible risk factors associated with STEC infection. Fifth, the study population was limited to those experiencing diarrhea and submitting a stool sample to the hospital clinical laboratories; hence, our estimates cannot be interpreted as prevalence in the general population. This is because individuals who are experiencing diarrhea and seek medical care are more likely to have an active infection and severe symptoms, which can lead to a higher detection rate of pathogens. Sixth, the absence of a control group of non-diarrheal or healthy children in this study might restrict our ability to establish associations between STEC infection and diarrheal illness and may limit the generalizability of the findings to the broader pediatric population. Seventh, the limited response rates to the environmental and behavioral components of the questionnaire constrained our ability to explore potential associations between these factors and the observed seasonal variations in the prevalence of STEC and STEC harboring *eae*.

In conclusion, a substantial prevalence (12.15%) of STEC isolates including STEC harboring *eae* was detected in diarrhea-affected children. Age, geographic location, and seasons were significantly associated with the STEC detection rate in the study sites. The majority of these isolates harbored *stx1* alone or in combination with *stx2* or *eae*. The highest proportion of these genes was observed among children with bloody diarrhea and nausea. Moreover, a significantly high proportion of ESBL producers, as well as notable number of MDR and XDR isolates, were found in this study. Therefore, future research should incorporate advanced molecular tools such as WGS to further characterize resistance mechanisms, track transmission dynamics, and inform more effective prevention and control strategies. In future studies, laboratory diagnosis aimed at identifying bacterial etiologies responsible for diarrheal diseases needs to carefully consider STEC due to the potential for severe complications, such as HUS. Hence, strengthening diagnostic capacities and promoting evidence-based treatment guidelines are critical steps in mitigating risks and improving patient outcomes. In addition, to have a complete picture of STEC's impact on human health, future studies on *E. coli* need to consider investigating not only from environmental and dietary sources but also from clinical settings.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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DATA AVAILABILITY

The data for this study are accessible upon requests, from the Ethiopian Public Health Institute's national data management centers.

AUTHORS' CONTRIBUTIONS

AMT: Methodology, Project administration, Data curation, Investigation, Formal analysis, Software, Visualization, Writing – original draft preparation. WEA: Supervision, Data curation, Validation, Formal analysis, Writing – review and editing. AAK: Supervision, Methodology, Writing – review and editing. ET: Methodology, Writing – review and editing. DB: Methodology, Writing – review and editing.

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