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Enterotoxins as a molecular marker of water quality

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ABSTRACT

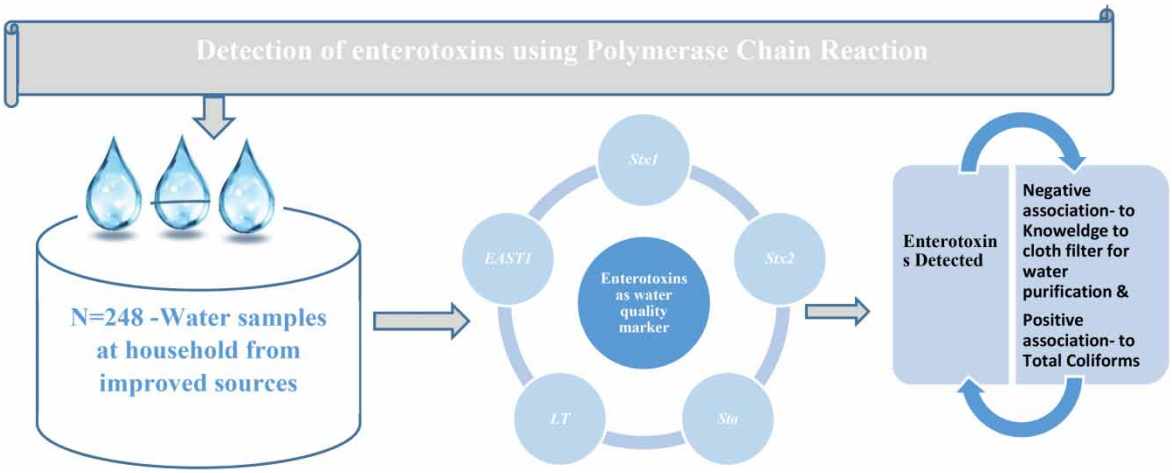
Safety of improved water supplies using enterotoxins as a molecular marker is evaluated. Water samples were collected from 248 households and tested for enterotoxins using polymerase chain reaction (PCR). The relationships between the presence of at least one enterotoxin and independent variables were investigated using Chi-square (χ^2), Fisher's exact test and binary logistic regression. Some 156 enterotoxin biomarkers were detected, 39% of samples had at least one, and 17% had multiple varieties. *EAST1* was detected in the highest proportion of samples (33%) and *Sta* in the lowest (2%). Shallow groundwater sources yielded 18% less enterotoxins than water from piped systems, a statistically significant result (P=0.031). A lower proportion of enterotoxins was detected in relation to those who did not know and use cloth filters than those with knowledge of them, and the negative association is statistically significant (P=0.017). It was shown that water samples in which total coliform (TC) colonies were detected were more likely to contain enterotoxins than those without (P=0.001). It is concluded that enterotoxin molecular markers can be used to monitor water safety.

Key words: enterotoxins, *Escherichia coli*, improved water, molecular marker, water quality

HIGHLIGHTS

- The quality of 'improved' water in Ethiopia was determined, which had not been done before.
- A high proportion of the water samples were shown to contain single or multiple enterotoxins.
- The type of water source, cloth filter water purification and TC were significantly associated with at least one enterotoxin detected.

GRAPHICAL ABSTRACT



ABBREVIATIONS

Abbreviation/acronym	Definition
AOR	Adjusted Odds Ratio
CFU	Colony Forming Unit
CI	Confidence Interval
DAEC	Diffusely Adherent *E. coli*
DEFF	Design Effect
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggregative *E. coli*
EAST1	Enteroaggregative *E. coli* heat-stable enterotoxin one
E. coli	*Escherichia coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
LT	Heat-liable
NTC	No Template Control
PCR	Polymerase Chain Reaction
Sta	Heat-stable
STEC	Shiga toxin-producing *E. coli*
stx1	Shiga-like toxin one
stx2	Shiga-like toxin two
TC*	Total Coliforms
TTC**	Thermotolerant Coliforms
uPVC	Unplasticized Polyvinyl Chloride
VTEC	Verocytotoxin *E. coli*
WHO	World Health Organization

WHO definitions:

* TC-'Total coliform' bacteria – includes a wide range of aerobic and facultatively anaerobic, gram-negative, non-spore-forming bacilli capable of growing in the presence of relatively high concentrations of bile salts, with the fermentation of lactose, and production of acid or aldehyde within 24 hours at 35–37 °C.

** TTC-'Thermotolerant Coliforms' – a subset of the total coliform group that can ferment lactose at higher temperatures (44–45 °C) and is considered the most suitable indicator of fecal contamination.

INTRODUCTION

The world's most vulnerable communities frequently drink contaminated water, which is linked to a variety of public health issues (WHO/UNICEF 2015; WHO 2017). Despite extensive construction of new, improved water supplies in recent decades, reports of diarrheal cases in South Wollo, Ethiopia, in 2016 and 2017 showed that 142,000 children under five and 124,000 people over five years old were infected in one year, when a decrease was expected (South Wollo 2017). Poor management of water at the source and the point of use has resulted in the consumption of water contaminated with enterotoxins, leading to sporadic and epidemic diarrhea (Rusin *et al.* 1997; WHO 2011; US-FDA 2012). The contamination level is higher in areas where open defecation is practiced and/or animal waste is poorly managed (Vannavong *et al.* 2018). Studies have confirmed that improved water supplies can be contaminated by human and animal waste, either at the water source or through household storage practices (Ercumen *et al.* 2017; Harada *et al.* 2018; Goma *et al.* 2019).

Escherichia coli is an important enteric bacterial species found in the gut of many species. It is used as a biomarker of fecal contamination. Six waterborne *E. coli* pathotypes are associated with diarrhea:

enterohemorrhagic *E. coli* (EHEC), which mainly produce Shiga-like toxin 1 (*stx1*) and Shiga-like toxin 2 (*stx2*);
enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC), which produce heat-stable enterotoxin 1 *(EAST1)*;
enterotoxigenic *E. coli* (ETEC), which produces heat-stable (*Sta*) and heat-liable (*LT*) toxins (Hitchins *et al.* 2001; Nguyen *et al.* 2005);
enteroinvasive *E. coli* (EIEC), which carries the Ial (*ipa*) target gene; and, diffusely adherent *E. coli* (DAEC), which carries the target genes *F1845* and *daaC*, which are associated with diarrhea (Hitchins *et al.* 2001; US-FDA 2012).

These pathogenic *E. coli* cause diarrhea in humans as they carry toxins, adhesins, or intimin proteins that attach to intestinal cells (US-FDA 2012). The contamination source could be human or animal waste, which serve as *E. coli* reservoirs (Ahmed *et al.* 2020). Every year, bacteria carrying *stx1*, *stx2*, and *EAST1* genes account for 75% of diarrheal infections and 380,000 deaths worldwide, mainly among children (US-FDA 2012). Diarrheal enterotoxin diseases are increasingly associated with the development of resistivity to water treatment chemicals. For example, in Spain, *E. coli* carrying genes such as *stx1* and *stx2* have developed resistance to chlorine, which can result in persistent contamination either from improved water sources or in homes (Croxen *et al.* 2013).

Water from improved sources is generally considered pathogen-free and safe for use, both by users and providers (WHO/UNICEF 2015). The target of this study is the detection of molecular biomarkers in water from improved sources. Based on the recommendations of WHO/UNICEF and the Ethiopian Federal Democratic Republic, Ministry of Water, Irrigation and Energy, types of water sources are defined as:

Yard connections – water piped to the premises, or piped household connection inside the dwelling, plot or yard;
Piped connection to public stands – when water is connected to public taps;
On-spot springs – protected springs that deliver water to a collection chamber;
Shallow wells – drilled by machine, lined with unplasticized polyvinyl chloride (uPVC) or steel casing, and fitted with hand pumps; the diameter is usually 10 to 15 cm.
Hand dug wells – traditionally, groundwater is obtained from hand-dug wells between 5 and 20 m deep, at least 1 m diameter and fitted with a hand pump (FDR-MoWIE 2016; WHO/UNICEF 2017).

International standards define 'improved drinking water sources' as sources that, by the nature of their construction or through active intervention, are protected from external contamination, particularly with fecal matter (WHO 2011). This is in line with the 'safely managed' drinking water ladder (WHO/UNICEF 2017). Nevertheless, 'basic' and 'limited' drinking water ladders under the 'improved drinking water' take water quality indication as an option. Approximately one-third of the basic Ethiopian water services classified as 'improved water' do not provide potable water because they contain diarrheal pathogens such as *E. coli* (Gemeda *et al.* 2021). There are three categories in the WHO/UNICEF Joint Monitoring Program for Water Supply and Sanitation (JMP) ladder for household drinking water services: (1) 'safely managed' water sources are accessible, available on demand, and free of contamination; (2) 'basic' water sources do not meet the 'safely managed' criteria and require a round trip of 30 minutes or less, including queuing, to collect, and (3) 'limited' water sources do not meet any of the 'safely managed' criteria and require more than 30 minutes to collect (WHO/UNICEF 2017).

To date, no studies have been conducted in Ethiopia to detect enterotoxin *E. coli* pathogens in improved water using PCR techniques. Despite the increased use of improved drinking water supplies, people continue to report various types of diarrhea (South Wollo 2017). The quality of improved water sources was investigated in this study in the context of increased diarrhea reports by assessing the presence of *E. coli* enterotoxin genes as enterotoxin pathogen biomarkers in improved water supplies using PCR. By identifying enterotoxin genes – for example, *EAST1*, *stx1*, *stx2*, *LT* and *Sta* – in improved water supplies it is possible to improve water quality monitoring, to develop a water safety strategy, and to nurture an understanding of water hygiene at home (Ashtown Food Research Centre 2007).

METHODS

Study area

The study was carried out in South Wollo, Ethiopia. Population density is associated with probability of fecal contamination, and South Wollo was chosen due to its relatively high population density (nearly 170 people per km^2), with a population of more than 2 million (51% female) in an area of 17,000 km^2 (CSA Ethiopia & ICF 2016; Fakhr *et al.* 2016). Governments and non-governmental organizations in the area were intervening extensively, including constructing new and improved water supplies. The health facilities and woredas (districts) targeted in this study are shown in Figure 1. A woreda is an Ethiopian local government administrative division.

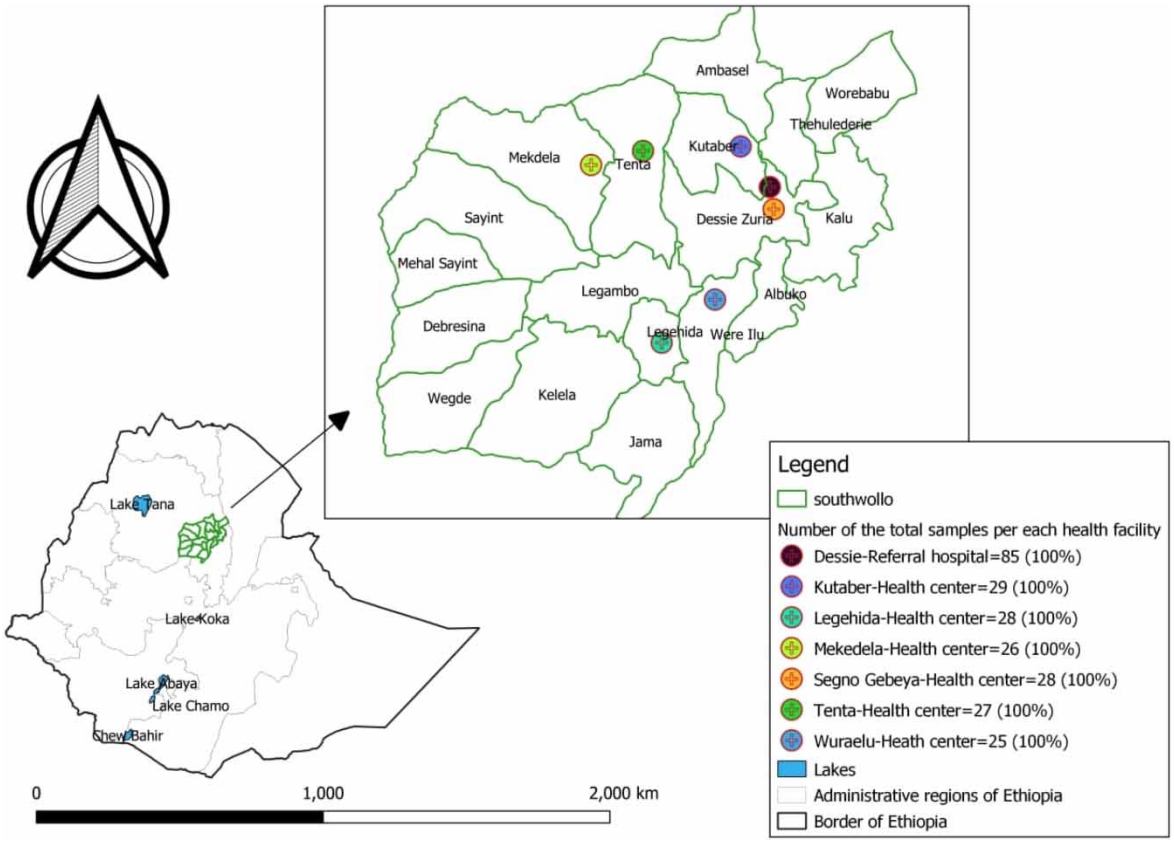


Figure 1 | Map of Ethiopia and South Wollo (developed from Ethiopian Administrative shape files 2013 using Quantum Geographic Information System (QGIS) Software version 3.2.1, 2020).

Sample size determination

Open source epidemiologic statistics for public health software (OPENEPI V. 3.01) (Sullivan *et al.* 2014) was used to determine representative sample size – Equation (1). A total of 248 households was chosen.

$$Sample\ size\ (n) = \frac{[DEFF{*}N_p(1-p)]}{\left[\frac{d^2}{Z^2_{1-\frac{\alpha}{2}}}{*}(N-1)+P{*}(1-P)\right]} \quad (1)$$

where, design effect (DEFF) is 1.5, population size (**N**) 1,001,490, prevalence rate of diarrhea in the area (**p**) 11%, and confidence limits (**d**) ($p<0.05$) with 95% confidence interval (CI) (**n**)=225 and considering a 10% non-response rate, the total sample size was 248.

DEFF was set to 1.5 even though the recommended value for simple random sampling is 1. This was done to minimize the effect of the tiered selection of the referral hospital out of the 3 hospitals in the woredas. The prevalence of diarrheal disease in the area was 11% (CSA Ethiopia & ICF 2016; South Wollo 2017). Sampling began with the random selection of districts (n=6 of 20 listed), which is about 30% of the total. The healthcare facilities were then grouped into two – hospitals and health centers. The three hospitals comprised two primary and one referral, the latter being included directly in the study because of its important position in the health system. Six of the eight health centers across the selected districts were selected randomly. The water sample size was determined based on the standard number of people served by health facilities. According to the Ministry of Health standard guideline, a hospital serves approximately 100,000 people and a health center approximately 25,000 (FMoH 2010). Diarrhea complaints treated in a health center or the referral hospital, were included in the study if they met the inclusion criteria (Additional file 1). Patients from six health centers account for 66% of respondents, while 34% came from the referral hospital. Diarrhea patients were interviewed subsequently in the healthcare facility. Additional interviews were conducted in the households and water samples were collected for analysis.

Water sampling and *E. coli* detection

300 mL water samples were collected from each household in non-reactive, 500 mL, sterilized polyethylene bottles. They were transported in ice-filled cold boxes to the regional branch of the Ethiopian Public Health Institute laboratory for analysis within four hours using membrane filtration techniques (ISO 2000; WHO 2011). Filter papers with bacterial colonies were transferred into 2 mL cryotubes and transported to the Armauer Hanssen Research Institute Laboratory in Addis Ababa, where they were stored at −20 °C to preserve the bacterial colonies in the filter for further analysis.

DNA extraction

The QIAamp® DNA Mini Kit (Qiagen, Germany) was used to extract total DNA from 183 cultures – there was no bacterial colony growth in 65 samples. 147 samples were determined for DNA, the other 36 were dropped because of poor quality and/or concentration. Cultured filter papers that showed full-size bacterial colonies were cut into small pieces and placed in 1.5 mL micro-centrifuge tubes for lysis, followed by extraction according to the manufacturer's instructions. Some 200 µl of bacterial DNA extracted from each bacterial isolate was stored at −20 °C for PCR analysis.

Detection of *E. coli* toxin genes

Five genes encoding enterotoxins were chosen for PCR-based detection. KAPA2G Hotstart master mix (Kapa Biosystems, Wilmington, Massachusetts (MA)) was used for PCR, with cycling conditions of initial denaturation at 95 °C for five minutes followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing temperature 60 °C (15 seconds), extension at 72 °C (15 seconds) and final extension at 72 °C (1 minute). Laboratory *E. coli* toxins *EAST1*, *stx1*, *stx2*, *Sta* and *LT* were used for positive control and molecular grade water as no-template controls (NTCs). The presence or absence of toxin genes was determined using a 2% agarose gel and electrophoresis of PCR products. PCR techniques are more effective than traditional laboratory methods in detecting and characterizing enterotoxins from *E. coli* in improved water supplies. The detection process is shown in Figure 2.

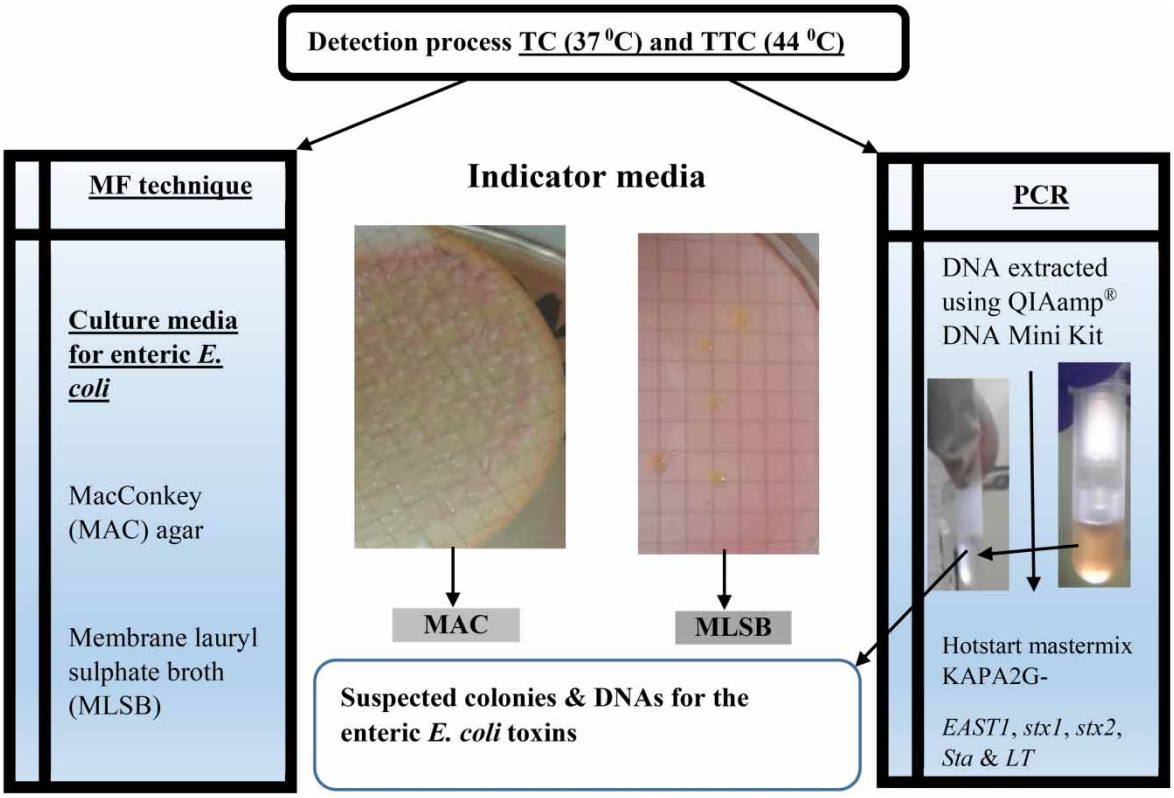


Figure 2 | Three steps in *E. coli* enterotoxin detection. Membrane filtration, culture at 37 and 44 °C using MacConkey agar and membrane lauryl sulphate broth, and PCR with gDNA extracted from samples.

Water supply and survey data collection

Water availability, type of water source, water handling, and the presence of human and/or animal waste are all factors that influence water contamination by enterotoxins and pathogens. This ultimately results in the morbidity and mortality associated with diarrheal diseases, and data on those factors were collected from people who presented diarrhea symptoms at the health facilities sampled. The interviews were conducted at their homes using KOBO Toolbox and KOBO Collect, on iPads and smartphones. Data collectors received one day's training on an adapted questionnaire used by trained healthcare professionals in healthcare facilities, as well as ethical research issues, and collection was monitored by two health experts and the principal investigator.

As noted in previous studies, the prevalence of bacterial pathogens in water samples was higher in the rainy season than the dry season. The risk of water contamination increases due to runoff that could transport bacteria into the water (Sidhu *et al.* 2013; Carlton *et al.* 2014; Kulinkina *et al.* 2016). Data and samples were collected one month before the end of the dry season and one month after the start of the rainy season to minimize the impact of seasonal variations such as temperature and rainfall on the detection level of toxin genes.

Data encoding and statistical analysis

Data were encoded using KOBO Toolbox and KOBO Collect on the iPad and smart phones. The data from KOBO Tool were transferred directly to SPSS software version 20 (IBM SPSS Statistics 20). Each household dataset contains a PCR detection of at least one enterotoxin in a sample – a categorical outcome variable (0=absent, 1=present) – the age (<5 years, 5 to 18 years and >18 years) and sex (F/M) of the respondents, the type of water source (yard connection, public stand, on-spot spring, shallow well, hand-dug well), household water ladder (safely managed, basic, limited), observed human and/or animal waste (yes/no), the respondents' knowledge and practice in household water purification methods (yes/no), TC and TTC detected (0 or 1 – maximum count in CFU/100 mL), and each of the five enterotoxins (*EAST1*, *stx1*, *stx2*, *Sta* and *LT*) detected (0=absent, 1=present).

The analysis was carried out using frequency distribution, and the Chi-square ($\chi 2$) and Fisher's exact tests. Variables with p-values ≤ 0.25 in the Chi-square and Fisher's exact test analyses were considered as candidates for multivariable logistic regression analysis. In the binary logistic regression model, variables with $p \leq 0.05$ were taken to be significantly associated with the outcome variable – the detection of at least one enterotoxin in a water sample. A 95% confidence interval was used to calculate the adjusted odds ratios (AOR) to assess the level of significance of the associations between variables.

RESULTS AND DISCUSSION

Toxin genes detected in improved water in households

Of the 248 samples cultured, 74% (183) tested positive for TC bacteria and 40% (98/248) for TTC. Some 59% (147/248) samples were analyzed for enterotoxins and 156 *E. coli* enterotoxin biomarkers were detected. The prevalence of one or more molecular markers in improved water samples was 39% (96/248) (Table 1). This was much higher than in previous studies in Libya (2%; n=50 from drinking water from taps, wells, and reservoirs in urban settings in summer), and Australia (22%; n=22 from rainwater tanks in urban areas during rainfall events) (Ahmed *et al.* 2012; Ali *et al.* 2012). This higher prevalence could be attributed to any of several factors, including differences in sample size, type of water source and sampling point. The roof catchment rainwater harvesting system is also more exposed to contamination by flying animals like birds, whereas the water sources included in this study could be exposed to contamination from humans and any kinds of animals. On the other hand, the result from this study was slightly lower than that in Egypt (50%; n=300 from potable water of old service pipes in urban areas throughout the year) (Fakhr *et al.* 2016). Limited maintenance of the old water system and/or seasonal variation could account for the slightly increased prevalence of enterotoxins. The findings of previous studies show that seasonal variation could affect the prevalence of enterotoxins by an increase of 10 to 14% between the dry and wet seasons (Sidhu *et al.* 2013; Bhavnani *et al.* 2014).

In this study, 17% (41/248) of water samples revealed multiple *E. coli* enterotoxin genes, unlike a study in Libya that showed 0% (n=50) (Ali *et al.* 2012). The presence of multiple toxin genes in water samples through their bacterial strains would increase the infection risk, depending on the type, amount, and viability of the bacteria that carry them (WHO 2011; US-FDA 2012). The detection of large amounts of *E. coli* enterotoxin genes in water sources suggests significant levels of pathogenic *E. coli,* which may, therefore, be the cause of diarrheal disease in South Wollo. *EAST1,* the most prevalent enterotoxin gene, was detected in 33% (82/248) of samples (Table 1). *EAST1*

Table 1 | PCR and membrane filter technique test results of the enterotoxins and coliform counts

Detected enterotoxins[a]	PCR test results	Rate in % (n/N)
EAST1	Positive	33 (82/248)
	Negative	67 (166/248)
stx1	Positive	13 (33/248)
	Negative	87 (215/248)
stx2	Positive	9 (21/248)
	Negative	91 (227/248)
LT	Positive	6 (14/248)
	Negative	94 (234/248)
Sta	Positive	2 (6/248)
	Negative	98 (242/248)
Total enterotoxin markers	**Positive**	**156**
Single or more enterotoxins	Positive	39 (96/248)
	Negative	61 (152/248)
Two or more enterotoxins	Positive	17 (41/248)
	Negative	84 (207/248)
Grown coliform counts	**Membrane filter test results**	**Rate in % (n/N)**
Total coliforms in CFU/100 mL	Zero (0)	26 (65/248)
	1–220 count/s	74 (183/248)
Thermotolerant coliforms in CFU/100 mL	Zero (0)	61 (150/248)
	1–84 count/s	40 (98/248)
Total water sampled (N)		**100 (248/248)**

[a]Test results of *E. coli* enterotoxins for each type (*EAST1*, *stx1*, *stx2*, *LT* and *Sta*) of enterotoxins detected frequency in single or more toxins, multiple toxins detected, total coliforms (TC) in Colony Forming Unit (CFU)/100 mL and thermotolerant coliforms (TTC) in CFU/100 mL counts in improved water samples.

occurs in many *E. coli* bacterial strains or pathotypes (EAEC and EPEC), which could explain its high prevalence (Sidhu *et al.* 2013). Conversely, the high prevalence of *EAST1* could indicate continuous contamination by feces from untreated hosts (adults, children, and/or animals), since a greater number of gene copies or cells (1×10^6) is required to cause diarrhea than that of other genes – for example, *stx1* and *stx2* – which require only 10 to 500 copies/cells to induce this phenotype, thus reducing the chance to treat the host (USEPA 2010; WHO 2011).

The water samples evaluated from households were obtained from yard connections (44%=108/248), public stands (21%=53), protected on-spot springs (13%=33), protected shallow wells with pumps (12%=30) and protected dug wells with pumps (10%=24).

Toxin genes were detected in 88 of the piped connection samples (82%), of which 42% contained *EAST1*, 16% *stx1*, and 11% *stx2*. Enterotoxins were detected in 74% (39) public stands, of which 36% contained *EAST1*, 19% *stx1*, 13% *stx2*, and 6% *LT*. The highest *EAST1* and *LT* detections were in water from piped connections; *stx1* and *stx2* were detected more in public stands, and *Sta* in piped connections and protected hand-dug wells fitted with hand pumps (Table 2). This contradicts the findings in a previous study on developing countries that reported that

Table 2 | Detected toxin genes by water source types in South Wollo Zone of Ethiopia

Water source types[a]			Piped connection	Public stand	Protected on-spot spring	Protected shallow well	Protected dug well	Total
Water samples		% (n/N)	44 (108/248)	21 (53/248)	13 (33/248)	12 (30/248)	10 (24/248)	100 (248/248)
Molecular markers of toxin genes	*EAST1*	% (n/N)	42 (45/108)	36 (19/53)	21 (7/33)	13 (4/30)	29 (7/24)	33 (82/248)
	stx1	% (n/N)	16 (17/108)	19 (10/53)	3 (1/33)	3 (1/30)	17 (4/24)	13 (33/248)
	stx2	% (n/N)	11 (12/108)	13 (7/53)	0 (0/33)	3 (1/30)	4 (1/24)	8 (21/248)
	Sta	% (n/N)	4 (4/108)	0 (0/53)	3 (1/33)	0 (0/30)	4 (1/24)	2 (6/248)
	LT	% (n/N)	9 (10/108)	6 (3/53)	3 (1/33)	0 (0/30)	0 (0/24)	6 (14/248)
Total toxin genes in water samples		% (n/N)	81 (88/108)	74 (39/53)	30 (10/33)	20 (6/30)	54 (13/24)	63 (156/248)

[a]Water source types categorized under 'improved water source types' in the drinking water ladder of the Joint Monitoring Program (JMP), by WHO/UNICEF category, that are included in this study in South Wollo, Ethiopia.

piped supplies had significantly lower *E. coli* contamination than non-piped water, due to residual chlorine (Shields *et al.* 2015). Other studies reported that Shiga toxin-producing bacteria are resistant to antibiotics and disinfectants, which could explain the detection of *stx1* and *stx2* in piped connections and public stands (Allué-Guardia *et al.* 2014; Ahmed *et al.* 2020). In this study, only 11% (28) of samples contained the recommended residual amount of chlorine. Possible reasons for the development of resistance to disinfectants could be under-dosing and/or mis-application of disinfectants in piped systems. Both *Stx1* and *Stx2* can return to lysogenize *E. coli* after some treatments due to its ability to survive various inactivation conditions (Allué-Guardia *et al.* 2014).

Samples from piped connections to households had the highest number of different toxin genes, in contrast to the findings of a study by WHO (2011). Significant amounts of enterotoxins were detected in basic household piped water supplies – 68% (106/156). The enterotoxins detection with the water ladder category; water samples under the 'limited' category reported 23% (36) and the safely managed category in the water ladder reported 9% (14) contamination with enterotoxins (Table 3). This contradicts the findings in Ethiopia and other developing countries that water from a piped system was less likely to be contaminated and cause diarrhea (Shields *et al.* 2015; Soboksa *et al.* 2020). As in this study, different amounts of *EAST1*, *stx1*, *stx2*, *ST* and *LT* (33, 20, 7, 13 and 26%, respectively), were detected in a piped water supply system in Egypt (Fakhr *et al.* 2016). The finding here shows that, even if water is from improved sources with good quality construction, there is a strong possibility of enterotoxin contamination before consumption during collection, storage, and/or use. This could arise from poor systems maintenance or household water handling, including storage (Shaheed *et al.* 2014). Inadequate household water treatment practices were reported. For example, only 16% of respondents reported that they applied chlorine in their drinking water. The presence of STEC in 22% of samples in this study matched that in southern Brazil, also 22% (n=210, groundwater for human consumption in rural areas) (da Silva *et al.* 2019). This could be linked to groundwater contamination related to land use in Brazil – for example, livestock and domestic septic systems – and in Ethiopia to the use of unimproved pit latrines and open defecation (approximately 21% of respondents reported having no access to latrines).

Table 3 | Test result of Enterotoxin molecular marker by household water ladder

	Molecular markers PCR result – % (n/N[a])					
Improved household water ladder	*EAST1* Positive	*stx1* Positive	*stx2* Positive	*Sta* Positive	*LT* Positive	Total
Safely managed	10 (8/82)	9 (3/33)	5 (1/21)	0 (0/6)	14 (2/14)	9 (14/156)
Basic	63 (52/82)	73 (24/33)	86 (18/21)	67 (4/6)	57 (8/14)	68 (106/156)
Limited	27 (22/82)	18 (6/33)	9 (2/21)	33 (2/6)	29 (4/14)	23 (36/156)
Total	100% (82)	100% (33)	100% (21)	100% (6)	100% (14)	100% (156)

[a]PCR enterotoxin test results of enterotoxins in improved household water by in accordance with the drinking water ladder. (NOTE – where n –is the detection frequency per the water ladder and N – is the total number of enterotoxins detected of the types concerned.

Sociodemographic and risk factors of contamination

In this study, 117 (47%) of respondents were female (Table 4). A linearity test for the respondents age and enterotoxin genes detection in improved water was predicted and the plot of linearity with a continuous variable age and residual errors is presented in Figure 3. As can be seen, the observations follow the horizontal line flow, and, as respondent age increases, toxin gene detection increases or vice versa. Of the diarrheal cases, 66% (163) were from six health centers and 34% (85) from the referral hospital. Of the diarrhea cases themselves, 55% (136) were adults (>18 years), but the highest prevalence – 41% (24) – of enterotoxins was reported in young people (5 to 18 years) (Table 4). This supports previous results that young peoples' susceptibility to *E. coli* enterotoxins is higher than that of adults in settings where hygiene is poor as natural immunity increases with age. It could also be due to diversity of individual awareness and cleanliness, and children's behavior (Qadri *et al.* 2005).

Water treatment – public knowledge and practice

The interviews were used to assess respondents' knowledge of water treatment for drinking. 90% (222) of respondents reported knowing about boiling water for treatment. Furthermore, 56% (138), were familiar with settling,

Table 4 | Participants' responses on socio-demographic and water contamination risk factors

Factor	Response	Frequency (n)	Proportion (%)
Sex	Female	117	47
	Male	131	53
Age	<5 years	54	22
	5–18 years	58	23
	>18	136	55
Type of health facility	Health center	163	66
	Hospital	85	34
Name of health facility	Desie-Referral hospital	85	34
	Kutaber-Health centre	29	12
	Segno Gebeya-Health centre	28	11
	Wuraelu-Heath centre	25	10
	Legehida-Health centre	28	11
	Tenta-Health centre	27	11
	Mekedela-Health centre	26	11
Animal waste observed[a]	No	106	43
	Yes	142	57
Human waste observed [a]	No	134	54
	Yes	114	46

[a]Animal and human waste observed near households or water sources.

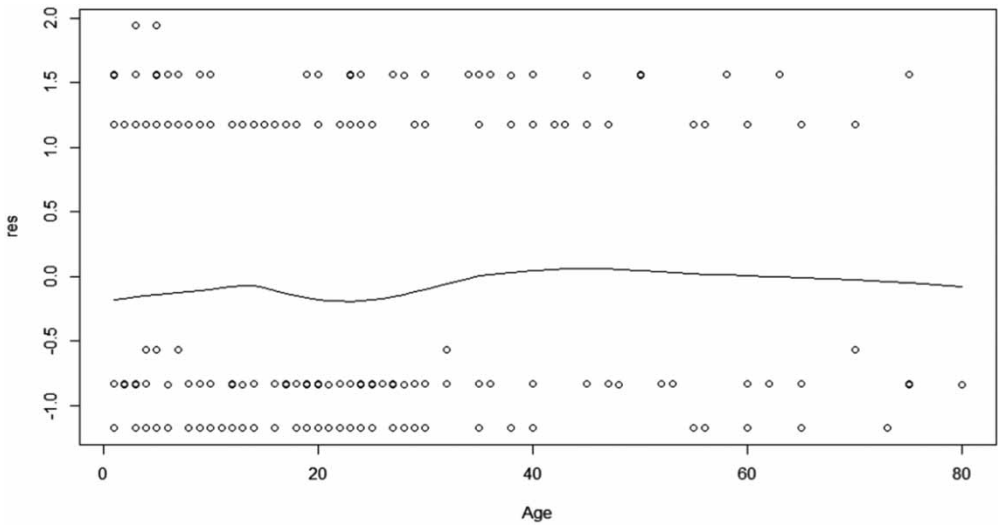


Figure 3 | Linearity test of age and toxin gene detection in the samples.

46% (114) with chlorine disinfection, and 41% (102) with cloth filters. Respondents did not know, however, about solar water disinfection methods (Table 5).

There was significant difference between the number of participants, 46% (114), who knew about the use of chlorine for disinfection and those, 16% (40), who actually practiced it. This could be due either to lack of chlorine in local markets or the participants' negative perception toward chlorine use for water treatment – people can think, for instance, that chlorine will change water's taste and odor (Mitro *et al.* 2019). Some 44% (108) of respondents used cloth filters for drinking water treatment, while 42% (105) used settling, 25% (61) boiling, and 16% (40) chlorine. Solar disinfection, however, was used by none of the respondents (Table 5). A study in Nigeria reported that the commonest treatment method there was the addition of alum – 43% (n=368) (Miner *et al.* 2015). It was also reported that those with knowledge of water treatment practiced at least one method in their household.

Association between *E. coli* enterotoxin genes and contamination risk factors

The univariable Chi-square (χ^2) and Fisher's exact tests showed that water source type (P=0.001), using a cloth filter for water treatment (P=0.004), animal (P=0.044) and human waste (P=0.006) observed near homes and/or

Table 5 | Respondents' knowledge and practice of household water treatment methods

	Knowledge			Practice		
Methods[a]	Yes-% (n/N)	No-% (n/N)	Total-% (n/N)	Yes-% (n/N)	No-% (n/N)	Total-% (n/N)
Cloth filter	41 (102)	59 (146)	100 (248)	44 (108)	56 (140)	100 (248)
Settling	56 (138)	44 (110)	100 (248)	42 (105)	58 (143)	100 (248)
Boiling	90 (222)	10 (26)	100 (248)	25 (61)	75 (187)	100 (248)
Chlorine	46 (114)	54 (134)	100 (248)	16 (40)	84 (208)	100 (248)
Solar disinfection	1 (3)	99 (245)	100 (248)	0 (0)	100 (248)	100 (248)

Note – the total number of respondents, N, was 248.

[a]The household level water treatment methods included in this study were cloth filters – the pouring of water through clean cloths into a clean container, settling – allowing water to settle before use, boiling – heating water to 100 °C to kill pathogens, chlorine – addition of a disinfectant (chlorine) to water to a standard dosing rate, and solar disinfection – using solar heat to kill microorganisms.

water sources, and TC (P=0.001) were all statistically significantly associated with the detection of at least one water enterotoxin. Water source type, use of a cloth filter and TC were also statistically significant variables with the detection of at least one enterotoxin using multivariable logistic regression. The results also showed that the use of a shallow well reduced the chances of finding enterotoxins in the water by 18%, compared to the use of piped systems connected to yards (P=0.031).

A lower proportion of enterotoxins was detected in relation to those who did not know and use cloth filters than those with knowledge of them, and the negative association is statistically significant (AOR=0.44; 95% CI, 0.23 to 0.86; P=0.017). This is due to the low proportion of respondents, 41 and 44%, respectively, who know about and practice cloth filter treatment. Incorrect cloth filter use could also result in bacterial growth in sediment trapped in the cloth or the water storage container could be contaminated after filtration (Suzuki *et al.* 2020). A study in Bangladesh showed that a simple nylon filter reduced bacterial counts by 48%, a two-layer cloth filter reduced them by 40%, and a two-layer cloth filter with *Moringa oleifera* seed, which caused coagulation, by 99% (Colwell *et al.* 2003; Suzuki *et al.* 2020).

In this study it was shown that water samples in which TC colonies were detected were more likely to contain enterotoxins (AOR=4.49; 95% CI, 1.81–11.15) than those without TC colonies (P=0.001). The same is not true for TTC. This suggests that the TC detected was highly likely to include pathogenic enteric bacteria. In a 40-year study, Wu *et al.* (2011) found that pathogen detection was related to TC counts, suggesting that TC are related to pathogens. This is similar to the concept of *E. coli* whole, pan, and core genomes; of 15,741 gene families, only six *E. coli* strains are human and animal feces-related pathogens (Lukjancenko *et al.* 2010). TTC counts were not significantly associated (P=0.185) with the presence of one or more enterotoxin molecular markers, although enterotoxins were detected in a high proportion of the samples. This supports a previous study showing that 95% of TTC are *E. coli*, but only 8% are pathogenic (WHO 1996). Furthermore, there was no association between TTC, or *E. coli* or virulent *E. coli* genes in a study in Australia comparing known virulence genes associated with *E. coli* strains with freshwater sites during the dry and wet seasons (Masters *et al.* 2011).

In this study, human and animal wastes observed near homes and improved water sources were not associated statistically with enterotoxin detection in water samples, which may be due to greater influence by other variables (Table 6). Whereas 142 respondents – more than half – reported animal waste and 114 (46%) human waste were observed within 10 m of houses or water sources (Table 4). This result contradicts those in studies that confirm that human and animal waste are equally important contaminants affecting water quality (Ercumen *et al.* 2017; Harada *et al.* 2018; Goma *et al.* 2019). A study in Egypt found a link between *E. coli* toxin genes and cattle waste (Tahamtan *et al.* 2010; Goma *et al.* 2019; Ahmed *et al.* 2020). This variation might arise from animal waste storage, which reduces the detection of bacterial enterotoxin genes gradually in rural and peri-urban areas where animal waste is a common fertilizer and household energy source (Avery *et al.* 2005).

When categorizing water sources using the drinking water ladder for this study, it was difficult to know whether the sources were free of fecal matter and prior chemical contamination, as there were no water quality data when the data and samples were collected. The free residual chlorine results were, therefore, used to determine whether water sources were managed safely as recommended by WHO/UNICEF (WHO/UNICEF 2017). Because of this, the number grouped in the 'safely managed' category may not be accurate.

Table 6 | Distribution and likelihood of occurrence of the toxins across different risk factors using Chi-square and Fisher's exact tests, and logistic regression

Distribution of occurrence of one or more toxin/s across factors (Chi-square test)							Likelihood of occurrence of toxins across risk factors (logistic regression)	
Variable	**Category**	**Tested (n)**	**Positive**	**Prevalence (%)**	**X^2**	***p*-value**	**AOR [95% CI]**	***p*-value**
Sex	Female	117	47	40	0.19	0.376		
	Male	131	49	37				
Age	Very young <5	54	19	35	0.46	0.794		
	Child 5–18	58	24	41				
	Adult >18	136	53	39				
Water source	PS-yard	108	55	51	19.66	0.001*	Ref.	
	Dug well	24	8	33			2.50 [0.92, 6.76]	0.071
	Shallow well	30	4	13			4.18 [1.13, 15.42]	0.031**
	On-spot spring	33	7	21			2.52 [0.83, 7.66]	0.103
	PS-public stand	53	22	42			2.03 [0.92, 4.49]	0.078
Water ladder	Safely managed	28	11	39	1.726			
	Basic	149	62	42		0.422		
	Limited	71	23	32				
Settling	No	110	46	42	0.805	0.222*	1.54 [0.75, 3.14]	0.232
	Yes	138	50	36			Ref	
Chlorine	No	134	57	43	1.800	0.113*	1.14 [0.55, 2.35]	0.712
	Yes	114	39	34			Ref	
Boiling	No	26	11	42	0.158	0.422		
	Yes	222	85	38				
Cloth filter	No	146	67	46	7.715	0.004*	Ref.	0.017*
	Yes	102	29	28			0.44 [0.23, 0.86]	
Solar disinfectant	No	245	95	39	0.037	0.667		
	Yes	3	1	33				
Water treatment practice	No	37	10	27	2.502	0.079*	1.34 [0.64, 2.81]	0.437
	Yes	211	86	41			Ref	
Storage-clean	No	37	10	27	2.50	0.114*	2.37 [1.00, 5.61]	0.111
	Yes	211	86	41			Ref	
Observed animal waste	No	106	48	45	3.372	0.044*	Ref	0.759
	Yes	142	48	34			1.89 [0.44, 1.79]	
Observed human waste	No	134	32	46	7.020	0.006*	Ref.	0.264
	Yes	114	34	30			1.47 [0.74, 2.89]	
Total coliforms	Zero (0)	65	10	15	20.20	0.001*	Ref.	0.001**
	1–220***	183	86	47			4.49 [1.81, 11.15]	
Thermotolerant coliforms	Zero (0)	150	53	35	1.824	0.112*	Ref	0.163
	1–84***	98	43	44			0.62 [0.32, 1.20]	

*p-values ≤0.25 in a Chi-square or Fisher's test are considered a cut-off point to be included in the next regression analysis.
**Significant variables with p-value ≤0.05 in the multivariable logistic regression. Water source type and total coliform counts were positively associated with at least one detected water enterotoxin. Knowledge of cloth filters was negatively associated with at least one detected water enterotoxins.
***Results of total coliform and thermotolerant coliforms in colony forming unit (CFU) count per 100 mL of water sample.

CONCLUSIONS

One or more *E. coli* enterotoxin genes were detected in 39% of samples taken from improved water supplies at the household level. The gene encoding *EAST1* was the most prevalent detected, while *Sta* was the least prevalent. Water source type, use of a cloth filter for water treatment and TC were all associated significantly with the detection of at least one enterotoxin molecular marker in improved water. Molecular markers for the *E. coli* enterotoxins targeted can be used for regular water safety monitoring and developing water safety strategies.

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CONFLICT OF INTEREST STATEMENT

The authors are not affiliated with or involved with any organisation or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this paper. Please address queries about Conflicts of Interest to the journal office: editorial@iwap.co.uk.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol for this study was approved by the College of Natural and Computational Science Institution (CNS-IRB), Addis Ababa University Review Board (CNSDO/729/10/2018) dated July 24, 2018. The data obtained were approved by the Ethiopian Institute of Water Resources, Addis Ababa University, and local kebele leaders of the study target areas. To protect the dignity, rights, and welfare of study participants, confidentiality of information was respected, and standards and operational guidance for the ethics review of health-related research with human participants of WHO were followed.

CONSENT FOR PUBLICATION

The authors guarantee that the contribution to the work has not been previously published elsewhere. The authors grant the Publisher the sole and exclusive license of the full copyright in the contribution, which license the Publisher hereby accepts.

AUTHORS' CONTRIBUTIONS

STG led the research from experimental design to drafting the manuscript. AFD and SRG draft and experimental design of the manuscripts, JJ designed and reviewed the manuscript, WMW review and lab guidance, and MNH reviewed and supported the laboratory work. All authors reviewed and approved the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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