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 ($\chi^2$), 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 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

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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; Widiasih 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-labile (LT) toxins (Hitchins et al. 2001; Weintraub 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; on-spot springs – public taps located in the yard; 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 – e.g., EAST1, stx1, stx2, LT and Sta – in improved water supplies it is possible to improve water quality monitoring, to develop 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 km²), with a population of more than 2 million (51% female) in an area of 17,000 km² (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.
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.

\[
\text{Sample size (n)} = \frac{[\text{DEFF} \times N \times (1 - p)]}{\frac{d^2}{Z^2} + \frac{1}{\alpha^2} \left( \frac{N - 1}{1} \right) + \frac{P(1 - P)}{1}}
\]

Equation (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](http://iwaponline.com/wpt/article-pdf/doi/10.2166/wpt.2022.016/1002950/wpt2022016.pdf)

**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; Levy et al. 2014; Kulinkina et al. 2016).

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 $\leq$ 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 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 — e.g., 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).

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

| Detected enterotoxins | PCR test results | Rate in % (n/N) |
|------------------------|------------------|-----------------|
| EAST1                  | Positive         | 33 (82/248)     |
|                        | Negative         | 67 (166/248)    |
| stx1                   | Positive         | 13 (53/248)     |
|                        | 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

| Single or more enterotoxins | Rate in % (n/N) |
|-----------------------------|-----------------|
| Positive                    | 156             |
| Negative                    | 44              |

#### Grown coliform counts

| Total coliforms in CFU/100 mL | Rate in % (n/N) |
|------------------------------|-----------------|
| Zero (0)                     | 26 (65/248)     |
| 1–220 count/s                | 74 (183/248)    |

| Thermotolerant coliforms in CFU/100 mL | Rate in % (n/N) |
|----------------------------------------|-----------------|
| Zero (0)                               | 61 (150/248)    |
| 1–84 count/s                           | 40 (98/248)     |

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

| Water source types | 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 (50/248)        | 100 (248/248) |
| Molecular markers  |                  |              |                          |                        |                    |       |
| EAST1              | % (n/N)          | 42 (45/108)  | 19 (99/53)               | 7 (37/33)              | 3 (13/30)          | 29 (72/248) |
| stx1               | % (n/N)          | 16 (17/108)  | 19 (10/53)               | 3 (1/33)               | 3 (1/30)           | 17 (4/24)  |
| stx2               | % (n/N)          | 11 (12/108)  | 13 (7/53)                | 0 (0/33)               | 3 (1/30)           | 4 (1/24)   |
| Stx                | % (n/N)          | 4 (4/108)    | 0 (0/53)                 | 3 (1/33)               | 0 (0/30)           | 1 (1/24)   |
| LT                 | % (n/N)          | 9 (10/108)   | 6 (3/53)                 | 3 (1/33)               | 0 (0/30)           | 0 (0/24)   |
| Total toxin genes  | % (n/N)          | 81 (88/108)  | 74 (39/53)               | 30 (10/33)             | 20 (6/30)          | 54 (13/24) |
| Total water sampled| % (n/N)          |              |                          |                        |                    | 100 (248/248) |

*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 \( E. \) \( \text{coli} \) 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 \( \text{stx1} \) and \( \text{stx2} \) 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 miss-application of disinfectants in piped systems. Both \( \text{stx1} \) and \( \text{stx2} \) can return to lysogenize \( E. \) \( \text{coli} \) 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, \( \text{stx1} \), \( \text{stx2} \), 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 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). This supports previous results that young peoples susceptibility to \( E. \) \( \text{coli} \) 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).

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

| Improved household water ladder | Molecular markers PCR result – % (n/N)* | 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)  |

*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 enterotoxin detectionism enterotoxin 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 residue 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 \( E. \) \( \text{coli} \) 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,
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. 2012).
This study, Wu et al. (2020) 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; Widiasih et al. 2019). A study in Egypt found a link between E. coli toxin genes and cattle waste (Tahamtan et al. 2010; Widiasih 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).

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

| Methods* | Knowledge | Practice |
|----------|------------|----------|
|          | 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 (158) | 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) |

*The household level water treatment methods included in this study were cloth filters – the pouring of water through clean clothes 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 disinfectant – using solar heat to kill microorganisms.

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

**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

| 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     | 151       | 49       | 37             |       |         |              |         |
| Age      | Very young <5 | 54     | 19       | 35             | 0.46  | 0.794   |              |         |
|          | Child 5–18 | 58     | 24       | 41             |       |         |              |         |
|          | Adult >18 | 156    | 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 | 33   | 22       | 42             | 2.03  | [0.92, 4.49] | 0.078 |         |
| Water ladder | Safely managed | 28  | 11       | 39             | 1.726 |         | 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, 2.55] | 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*** | 185     | 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.
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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The protocol for this study was approved by the College of Natural and Computational Science Institution (CNS-
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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
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**AUTHORS’ CONTRIBUTIONS**
STG led the research from experimental design to drafting the manuscript. AFD and SRG draft and experimental
design of the manuscripts, JJ design and review of the manuscript and, 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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