# Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia

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This is to certify that the thesis prepared by Shibabaw Tadesse, entitled: *Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia* and submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Water and Health with Specialization in Water and Public Health complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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## Abstract

The world's most vulnerable communities frequently drink contaminated water, which is linked to a variety of public health issues. *Escherichia coli* toxins are the most common types of contaminants found in association with disease in *E. coli* bacteria. They are found in nearly all pathogenic *E. coli* bacteria either released from the bacteria or *E. coli* cell or both transmitted via the feco-oral route. The objective was to evaluate the safety of improved water supplies using toxins as a biomarker and correlate with the toxins found in patient stools, and identify gaps between water quality and the global classification of improved water supplies in the South Wollo zone, Ethiopia. This dissertation reports the results of a laboratory-based cross-sectional study conducted in South Wollo, Ethiopia, from January 2019 to June 2020. Two hundred forty-eight samples of household water and patient stool were collected (in a 1:1 match) and toxins were tested using polymerase chain reaction (PCR). The targeted toxins used were (enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), heat-stable (Sta); shiga-like toxin 1 (Stx1), shiga-like toxin 2 (Stx2), and heat-liable (LT)), which cause diarrhea when people drink contaminated water. A survey questionnaire was used to gather information on the types of water sources, as well as the knowledge and practices of the respondents about household water treatment methods. To analyze the data and examine the relationship between the variables, descriptive statistics, Chi-square ($\chi 2$), Fisher's exact test, logistic regression, and Pearson's bivariate correlation coefficients were used. In general, 24% of households had positive results from both water and stool samples, 63% [Confidence Interval (CI): 55- 67%] had positive results from water samples only, and 46% [CI: 37- 49%] had positive results from only stool samples for toxins. Out of the five toxins examined, EAST1 was the one that was found the most

frequently, with 33% in the water and 38% in the stool. Frequently founded contaminants in household water brought from improved water sources were the toxins ESAT1, Stx1, and LT, with Sta the least founded (2%). There were fewer (13%) toxins detected in shallow groundwater sources than in piped water, a statistically significant difference (P=0.031). There was a lower proportion of toxins detected in those who did not know about and used cloth filters than in those who did, and the negative relationship is statistically significant (P=0.017). Approximately one-third of the improved water services sampled, which are considered 'functional' by international standards, do not provide potable water due to fecal and toxin contaminants. There was a significant positive correlation (r = 0.412) between toxins in the stool and toxins in the water ($P < 0.05$). The findings indicate that toxin biomarkers can be used to monitor water safety. Water quality parameters are not currently considered in the classification of basic water services. This suggests that international efforts to address SDG 6 should incorporate water quality and its indicators as a key parameter to better track international progress towards 'clean water and sanitation' efforts. More research is needed to determine the sources of pathogenic microbial markers that cause water-related diseases, such as diarrhea, as well as focus areas for water contamination prevention.

**Keywords:** Biomarker; Correlation; *Escherichia coli*; Ethiopia; Improved water; Stool; Toxins; Water quality.

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## Abbreviations

| | |
|---|---|
| AHRI | Armauer Hanssen Research Institute |
| AOR | Adjusted Odds Ratio |
| bp | Base Pairs |
| 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* |
| EiWR | Ethiopian Institute of Water Resources |
| EPEC | Enteropathogenic *E. coli* |
| ESA | Ethiopian Standard Agency |
| ETEC | Enterotoxigenic *E. coli* |
| ISO | International Organization for Standardization |
| JMP | Joint Monitoring Program |
| LMIC | Low-and Middle-income Countries |
| LT | Heat-liable |
| MAC | MacConkey Agar |
| MF | Membrane Filtration |
| MLSB | Membrane Lauryl Sulphate Broth |
| mPCR | Multiplex Polymerase Chain Reaction |
| MoH | Ministry of Health |
| MUAC | Mid Upper Arm Circumference |
| NTC | No Template Control |
| OR | Odds Ratio |
| PCR | Polymerase Chain Reaction |

| | |
|---|---|
| SDG | Sustainable Development Goal |
| SMDWS | Safely Managed Drinking Water Services |
| Sta | Heat-stable |
| STEC | Shiga toxin-producing *E. coli* |
| Stx1 | Shiga-like toxin one |
| Stx2 | Shiga-like toxin two |
| TC | Total Coliform |
| TTC | Thermo-tolerant Coliform |
| UN | United Nations |
| UNICEF | United Nations International Children's Emergency Fund |
| VTEC | Verocytotoxin *E. coli* |
| WHO | World Health Organization |

## Organization of the dissertation

This Ph.D. dissertation report on 'Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia' was divided into five chapters. Furthermore, the limitations, references, operational definitions of terms, and annexes of this work were included.

**Chapter 1:** This chapter deals with the introduction and background information, rational and significance of the study, research questions, research objectives, and how this dissertation paper is organized.

**Chapter 2:** Focuses on a literature review on improved water supply and the importance of water quality, toxins in improved water and human feces, and mapping of pathogens detected by the PCR-based method in improved water supply in low-middle-income countries (LMICs).

**Chapter 3:** The material and method section of the dissertation paper focuses on the flow diagram of the entire study process, the study area, sample size determination method, water sampling and DNA extraction, stool sampling and DNA extraction, detection of toxins in improved water and stools, survey data collection method, data encoding, and statistical analysis were covered in this chapter.

**Chapter 4:** Summary results and discussions of objective 1: Evaluate the water quality status of improved water supplies using toxins as a biomarker. This section contains the findings and discussions on toxins as biomarkers of water quality.

**Chapter 5:** Summary result and discussion of objective 2: Identify gaps between water quality and the global classification of improved water supplies by evaluating the basic

water quality parameters of 'improved' water supplies. The importance of water quality in classifying improved water services,

**Chapter 6:** Summary results and discussion of objective 3: Investigate the relationship between toxins found in water from improved drinking water sources and toxins found in patient stools using these improved drinking water sources in Ethiopia's South Wollo Zone. Evaluation of toxins in improved water in households and stool of diarrhea patients.

**Chapter 7:** Summary synthesis chapter summarizes the general discussion that generates key knowledge and brief findings obtained from the different chapters of the dissertation mentioned above. Informs its contribution to the science, SDGs, Ethiopia and the community, and to reach those its dissemination strategy.

**Chapter 8:** Conclusions summarizes the dissertation report's major conclusions.

**Chapter 9:** Recommendations, which summarizes the dissertation report's major recommends that they be given due consideration.

# Chapter 1: Introduction

## 1.1. Background

The world's most vulnerable communities frequently drink contaminated water, which is linked to a variety of public health issues. Sustainable Development Goal (SDG) 6 aims to coordinate international efforts toward 'clean water and sanitation' (WHO/UNICEF 2017). However, water contaminated with pathogenic bacteria will not meet the SDG target of clean water in the lives of people around the world. Despite the decade-long construction of improved water supplies in South Wollo, Ethiopia, nearly 266,000 people were infected with diarrhea-causing agents in one year during a time period when a decrease in cases was expected (South Wollo 2017).

Pathogenic *Escherichia coli* are the most common and widespread water contaminants in developing countries. *E. coli* is an important enteric bacterial species found in the intestinal tract of many species and is used as a biomarker of fecal contamination. Enteric (enterovirulent), diarrheagenic or pathogenic *E. coli* are groups of *E. coli* that can cause severe diarrheal diseases in humans mediated by toxins, adhesins, or intimin (US-FDA 2012). The most common causes of diarrhea outbreaks related to human morbidity and mortality are the water transmission of enteric *E. coli* pathogens that carry toxin genes (Croxen et al. 2013). Every year, bacteria that encode shiga-like toxin 1 (Stx1), shiga-like toxin 2 (Stx2), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) account for 75% of infections and 380,000 deaths worldwide, primarily among children (US-FDA 2012).

Six waterborne *E. coli* pathotypes are associated with diarrhea:

- Enterohemorrhagic *E. coli* (EHEC), which mainly produces Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2).
- Enteropathogenic *E. coli* (EPEC), which produces heat-stable enterotoxin 1 (EAST1).
- Enteroaggregative *E. coli* (EAEC), which produces heat-stable enterotoxin 1 (EAST1*)*.
- Enterotoxigenic *E. coli* (ETEC), which produces heat-stable (Sta) and heat-liable (LT) toxins (Hitchins et al. 2001; Weintraub et al. 2005).
- Enteroinvasive *E. coli* (EIEC), which carries the Ial (*ipa*) target gene.
- Diffusely adherent *E. coli* (DAEC), which carries the target toxins 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). In Spain, enteric *E. coli* toxins, such as shiga toxin encoding (Stx) bacteria groups (Stx1 and Stx2), have developed resistance to chlorine water treatment, which can lead to a persistent contamination of improved water (Croxen et al. 2013).

According to international standards “improved water supply” refers to the following categories of water services: (1) safely managed water sources that are accessible on premises, available on demand, and free from contamination; (2) basic water sources that do not meet any of the three criteria listed under safely managed and require a round trip of 30 minutes or less to collect, including queuing, or (3) limited water sources that do

not meet any one of the three criteria under safely managed and require more than 30 minutes to collect (WHO 2017a).

Water from improved sources is generally considered pathogen-free and safe for consumption by users and providers (WHO/UNICEF 2015a). The definition of pathogen-free water supply contradicts with the definition of basic and limited water supply. Improved water supply should imply contamination-free water. As a result, improved must imply pathogen-free, and the definitions are ambiguous. Moreover, in many low-income countries, improved contaminated water continues to cause diarrheal diseases (Soboksa et al. 2020; US-FDA 2012). Global 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; Goma et al. 2019; Harada et al. 2018). The biomarkers of enteric *E. coli* contamination are their toxin genes, and these were identified from patients who drank water contaminated with human and animal waste, which served as a reservoir for these microbes (Ahmed et al. 2020). In Ethiopia, approximately one-third of basic water services classified as 'improved water' do not provide potable water (Gemeda et al. 2021). Furthermore, 23 million (51%) of the population live in rural areas with inadequate water supplies (WHO/UNICEF 2015a).

Following the adoption of water quality goals, targets, and indicators in the era of the Sustainable Development Goals (SDG), 11% (4% rural and 38% urban) of Ethiopians use safely managed water services in the category of improved water (WHO/UNICEF 2017). Poor management of improved water at both the source and the point of use has resulted in the consumption of contaminated water, leading to sporadic and epidemic diarrhea (Rusin et al. 1997; WHO 2011). The level of contamination increases in areas where

open defecation is practiced and animal waste is poorly managed (Vannavong et al. 2018). The results of various global studies on the evaluation of enteric biomarkers using water supply were presented in this paper (Table 1).

**Table 1.** Summary of previous studies on the evaluation of biomarkers in improved and unimproved water

| Biomarkers | Country | Molecular method | Water sources Ladder | Sampling point | Prevalence rate | References |
|---|---|---|---|---|---|---|
| EAST1, Stx1, Stx2, Sta & LT | Zagazig city, Egypt | PCR | Improved | Sources | 50% | Fakhr, A.E., et al 2016 |
| 3 genes including Stx2 & EAST1 | Rural area of Upper Austria | PCR | Improved | Source | 4% | Halabi, M., et al 2008 |
| Stx2 | Central Java Province, Indonesia | PCR | Improved-tap water | Slaughter house-tap | 1 isolate | Widiasih, D.A., et al 2019 |
| 10 toxin genes including Stx1, Stx2, LT, Sta & EAST1 | Southeast Queensland, Australia | PCR | Improved-Rainwater Tanks | Source | 22% | Ahmed, W., et al 2012 |
| 17 genes including EAST1, Stx1, Stx2, Sta & LT | Zliten and Alkhomes, Libya | PCR | Improved-well & reservoirs | Sources | 17% | Ali, M.M., et al 2012 |
| 7 genes including EAST1, Stx1, Stx2 | Gauteng, South Africa | PCR | Unimproved-River | Source | 49% | Abia, A.L.K, et al 2016 |
| 8 genes including EAST1, Stx1, Stx2 & Sta | peri-urban, South Africa | RT-PCR | Unimproved-(wells, boreholes and a river) | HDWs, boreholes and a river | 99% | Roux, W.L., et al 2017 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 12 genes including EAST1, Stx1, Stx2, Sta & LT | Paraná, Southern Brazil | PCR | Unimproved -(Ground water) | Source-Shallow wells & mines | 28% | Pelayo, J.S., et al 2019 |

## 1.2. Statement of the problem

Despite the fact that diarrhea is a public health concern, no research has been done in Ethiopia to evaluate toxins as biomarkers in improved water services. There is no a baseline data and extent to which toxins contribute to diarrhea in Ethiopia, which would help for further researches.

Due to unclarity in defining improved water supplies, water quality targets, indicators are less prioritized by sector actors. Achieving safe drinking water in developing countries and resource-poor environments requires sustained effort. In this contribution, we demonstrate that current international standards for improved water have not been properly specified for water quality. Without a stronger incorporation of water quality parameters, international targets risk focusing in improved basic water services means water free from contamination that merely supply users with non-potable, and contaminated water that would end up with nonfunctional. Water contaminated with thermotolerant coliforms (TTC) will not achieve the SDGs in the lives of people around the world.

There is less uptake of recently developed and well recognized molecular technics for water quality monitoring in developing countries. Due to the high public health burden associated with contaminated water, significant effort has been focused on how to evaluate and maximize water quality testing methods. The use of polymerase chain reaction (PCR) to analyze water samples from cohabiting microbial populations is a

sensitive and rapid strategy (Hugenholtz and Tyson 2008). PCR methods are used to monitor and track pathogen and source-specific markers in water systems and are known as microbial source tracking (MST) (Girones et al. 2010). Previous studies using PCR and MST monitored toxins in infected patients (Seid et al. 2018) or suspected unimproved water sources and found biomarkers in different prevalence rates (Abia et al. 2017; Abia, Ubomba-Jaswa, and Momba 2016; Ali et al. 2012). In Ethiopia, the most common methods used are traditional culture-based methods, which have a limited detection capacity where some diarrheagenic strains of *E. coli* cannot grow in culture (Edge et al. 2007; Egli et al. 2008; Liu et al. 2008). About 93–99.9% of bacteria in drinking water (Hammes et al. 2008), more than 90% of bacteria in wastewater (Manz et al. 1994) and 99–99.9% of soil bacteria have not yet been cultured (Schmeisser, Helen, and Streit R. 2007). This indicates that to better address water quality, it is important to look at the monitoring methods. As a result, the use of PCR techniques is more effective in detecting and characterizing enteric *E. coli* pathogens in improved water supplies.

To date, no studies have been conducted in Ethiopia to detect toxin *E. coli* pathogens in water using PCR techniques. The need to conduct this study in Ethiopia is clearly outlined in the 'significance of the study' section here below. Despite the increased use of improved water supply (South Wollo 2017) people continue to report various types of diarrhea, according to health facilities. Therefore, using the PCR method is a good replacement or supplement for the traditional culture method, as it is precise and time-saving (Fiedoruk et al. 2015; Hugenholtz and Tyson 2008).

## 1.3. Significance of the study

Pathogenic *E. coli* is widely and commonly cause epidemics in developed and developing countries, and they sometimes have fatal consequences. Since many of these pathotypes have low infectious doses like toxins and are spread through water, they pose a serious threat to public health. In other way, the improvement in the level of toxins in water contamination may vary from the perspective of developed and developing countries. Factors such as age, sex, type of water supply, prevalence of toxins, and nutritional status are some that could be treated differently. Therefore, it is necessary to know the background of water contamination by toxins in Ethiopia. outbreaks; unfortunately, this surveillance is often lacking in developing countries.

By the year 2015, the millennium development goal's target of providing access to clean water to half of Ethiopia's population (57%) had been met (WHO/UNICEF, 2015a). On the other hand, the prevalence of diarrhea caused by contaminated water is rising in the nation, indicating that even improved water services are still cause diarrhea. There is a gap between water quality and the global classification of improved water supplies, which could contribute to give less attention for water quality monitoring using reliable methods. According to the goal of SDG 6.1, 'universal access to safe drinking water' water quality parameters are optionally considered, as the current definitions state. Given these facts, Ethiopia does not place a high focus on water quality monitoring.

Therefore, the purpose of this study was to demonstrate by examining toxin biomarkers in household water that was obtained from improved sources. This would offer conclusive proof for the Ministry of Water, Irrigation and Energy, which implements water quality standards, and the Ministry of Health, which regulates them, to pay due attention to routine water quality monitoring and take appropriate action to reduce diarrhea caused by contaminated water. Encourages strengthening national surveillance

programs that keep an eye on and track the toxins released by pathogenic *E. coli*. Because of this, the study has pioneered the identification of biomarkers in water samples and consumers and revealed the disparity between the water quality state and the ostensibly improved water. The research lays the foundation for more studies in this field.

## 1.4. Objectives

### 1.4.1. General Objective

The general objective was to evaluate the safety of improved water supplies using toxins as a biomarker and correlate with the toxins in the patient stools, and identify gaps between water quality and the global classification of improved water supplies in the South Wollo zone, Ethiopia.

### 1.4.2. Specific Objectives

1. Evaluate the water quality status of improved water supplies using toxins as a biomarker.
2. Identify gaps between water quality and the global classification of improved water supplies by evaluating the basic water quality parameters of 'improved' water supplies.
3. Investigate the relationship between toxins found in water from improved drinking water sources and toxins found in patient stools using these improved drinking water sources in Ethiopia's South Wollo Zone and

## 1.5. Research Questions

1. Can toxins be used as a biomarker to evaluate the water quality status of improved water supplies?

2. What are the gaps between water quality and the global classification of improved water supplies by evaluating the basic water quality parameters of 'improved' water supplies?
3. What is the relationship between toxins found in water from improved drinking water sources and toxins found in patient stools using these improved drinking water sources in Ethiopia's South Wollo Zone?

# Chapter 2: Literature review

## 2.1. Improved water supply and importance of water quality

World Health Organization (WHO)/United Nations Children's Fund (UNICEF) joint monitoring programme (JMP) coordinate the Sustainable Development Goals (SDGs) of international efforts to improve the lives of people around the world. SDG 6 broadly outlines 'clean water and sanitation', while target 6.1 specifies 'By 2030, achieve universal and equitable access to safe and affordable drinking water for all' (WHO/UNICEF 2017). Using the case of water in Ethiopia, this document documents the existence of diarrheal bacteria (TTC) in water services currently considered functional by international standards. Although our data are specific to Ethiopia, we find that 34% of improved water services currently classified as functional do not offer 'safe drinking water' as SDG 6.1 states as a global goal (Gemeda et al. 2021). This is supported by other studies conducted in Ethiopia that resulted in the key factors for improved water services functionality being improved water quality by 61% of the respondents (Anthonj et al. 2018).

Furthermore, the study conducted in developing countries recommends harmonizing and standardizing water functionality monitoring; it is necessary to address water quality

(Carter and Ross 2016). In a study conducted in Malawi, the water in the samples (32%) did not meet the Malawi government's drinking water standard (50 CFU/100 ml) for TTC (*Escherichia coli)* and most of the samples (87%) did not meet the WHO's drinking water standard of zero CFU/100 ml) of *E. coli.* Therefore, well-functioning water services tend to have the lowest levels of *E. coli* contamination (Holm et al. 2015). This demonstrates the importance of including a water quality parameter, such as the presence of TTC, to measure basic water functionality. Basic water services are the main source of drinking water for communities around the world; therefore, understanding water quality is an essential component to achieve universal access to safe and clean drinking water (WHO 2017a).

International standards measure and classify water services and water quality in various ways. At the basic level, water services are deemed improved or unimproved, and water services are classified as functional or nonfunctional (Bonsor et al. 2018; WHO 2017a). Unimproved sources include drinking from unprotected dug wells or springs or directly from the source, such as a river, lake, pond, or irrigation canal/channel. This dissertation is focused on improved water services that include: rural piped system (RPS) from a spring, borehole or shallow wells, hand-dug wells with hand pumps (HDW), rainwater harvesting structures (RWH), constructed on-spot springs, reservoirs, river intakes (RI), and slow sand filters (SSF) (South-Wollo-Water-Office 2016). The improved water services are then understood as water services (accounting for both the source and the water delivery systems), classified as safely managed, basic, or limited with set parameters for each (Table 2). Improved water services are classified as functional (fully

operational system at the time of survey) or partial or fully nonfunctional (fails to operate at the time of survey) (Bonsor et al. 2018).

**Table 2. SDG definitions for the classification of improved water services** (WHO 2017a)

| | |
|---|---|
| **Safely managed services** | 1. Source is accessible on premises **and**<br>2. Water is available on demand **and**<br>3. Water is free from contamination |
| **Basic water services** | If the source does not meet any **one** of the three criteria **and** a round trip to collect water takes 30 minutes or less including queuing. |
| **Limited water services** | If the source does not meet any **one** of the three criteria **and** a round trip to collect water exceeds 30 minutes. |

However, as seen in Table 2, water quality (measures that assess contamination) is a mandatory criterion for safely managed services but becomes an *optional criterion* in the definitions of basic and limited water services. In other words, a basic water service can be classified as 'functional' even if the water is not, in practice, 'safe drinking water' the goal of SDG 6.1. The flipside of this is that water services classified as 'nonfunctional' do not incorporate water quality. The parameters of water quality include testing samples in three dimensions: biological (total coliforms (TC) and TTC in colony forming unit (CFU) per 100 ml of water samples), physical (turbidity in nephlometeric turbidity unit (NTU)) and chemical ('pH' and free residual chlorine (FRC) in milligrams (mg) per liter (lit) of water samples). The SDGs classify safe drinking water according to the updated ladders of the World Health Organization (WHO)/United Nations Children's Fund

(UNICEF) joint monitoring programme (JMP) of safe drinking water (WHO/UNICEF 2017). This classification does not, we argue, properly account for 'safe' drinking water in basic water services. The inclusion of a fecal indicator bacteria (FIB) as part of water quality in the commonly accepted definition of basic water (WHO/UNICEF 2017) is a gap that obscures the risk of diarrheal diseases from water services classified as both improved and functional. Given the morbidity and mortality caused by diarrheal disease and the disproportionate burden placed on under-five children and women (Cumming and Cairncross 2016; UNICEF-Ethiopia 2018), a stronger incorporation of water quality parameters is essential to drive investment in water services that produces meaningful improvements in the lives of Ethiopians and citizens of developing countries in general.

Functioning water services are fundamental for social and economic development: improving the quality of health and educational achievement by reducing morbidity and mortality, malnutrition, stunting rates of people in community and improving livelihoods (Cumming and Cairncross 2016; UNICEF-Ethiopia 2018). Global data on water services show that 844 million people still lack basic drinking water. Two hundred sixty-three (263) million people spent more than 30 minutes per round trip collecting water from improved sources due to the lack of drinking water services (WHO/UNICEF 2017). One hundred fifty nine (159) million people, of which 58% are in sub-Saharan Africa, continue to collect drinking water directly from unimproved sources, such as unprotected wells and springs or surface water services (WHO/UNICEF 2017). In fact, enhancing existing basic water services to meet water quality standards is a strategic development area for increased investment.

Despite efforts to increase safe water service with the ultimate result of improving the health and nutritional status of the community (Pahlenberg and Weimann-Koinzack 2015; WHO 2015) in food-insecure rural areas of Ethiopia, with special attention to the regional states of Amhara and Afar, the malnutrition rates in these two regions exceed the national average (40%) (Federal Democratic Republic of Ethiopia 2016). In Ethiopia, lack of access to water supplies causes around half of all health complications from under nutrition (UNICEF-Ethiopia 2018). Since 1990, joint efforts by the Ethiopian government and numerous nongovernmental organizations (NGOs) have expanded water, sanitation, and hygiene services (WASH) through the construction of water infrastructure in Amhara and Afar of Ethiopia (South-Wollo-Water-Office 2016; WHO/UNICEF 2017). Although much attention has been paid to the construction of new water services, there is inadequate or no data on existing water services that, after construction, require ongoing maintenance. Once a water service is constructed and, therefore, 'improved', it can then be considered as safely-managed, basic, or limited. Studies reported the negative impact of water quality in addition to physical drying or low yield, as well as weak operation and maintenance of water, on the functionality of basic water services (Anthonj et al. 2018; Carter and Ross 2016; Mason et al. 2013). In rural Ethiopia, partial functionality and nonfunctionality of basic water services were reported as prevalent challenges (Mason et al. 2013). Ethiopia is not alone in its development struggle to find clean water accessible from improved sources.

In Ethiopia, the proportion of people using a **safely managed** water service(accessible on premises, available when needed, and free from contamination) is only 11% (4% rural and 38% urban) (WHO/UNICEF 2017). These data indicate a significant gap between

urban and rural residents, while demonstrating that even urban living in Ethiopia does not equal access to clean water. To understand this on a national scale, out of the sixteen small to medium towns in Ethiopia, only two towns provide water that meets the Ethiopian government's Growth and Transformation Plan I standard, which requires that the water be free from contamination (Adank et al. 2016). Moreover, where water quality may or may not meet the definition of **basic water services** standards of the Joint Monitoring Program (JMP), in Ethiopia, the national drinking water coverage of basic water services is 39% (30% rural and 77% urban) (WHO/UNICEF 2017). In Ethiopia, 12% of the national population still relies on untreated surface water (WHO/UNICEF 2010), and within the rural population 47.2% use surface water and 28.1% use spring water services, meaning that nearly 75% of the rural and 7% of the urban population uses water from unimproved sources (WHO/UNICEF 2015; DuChanois et al. 2019).

Rural Ethiopian communities have struggled to first gain access to basic water services and then maintain their functionality. In rural Ethiopia in 2013, the Ministry of Water, Irrigation and Electricity inventoried a total of 92,588 water services. As can be seen in the inventory data, the overwhelming majority, 78.4% of rural Ethiopians receive water from basic water services. The types of basic water services include hand dug wells with hand pumps, springs without distribution networks, shallow wells with hand pumps and rope pumps (Butterworth, J., Welle, K., Hailu, T., Boestoen, K. and Schaeffer 2013). As in many other countries, in addition to limited *access* to basic water services, the non-functionality of newly built or existing basic water services is a major problem in Ethiopia (Mason et al. 2013). A study, based on 12 countries on the African and Asian continents, found that 28.7% were nonfunctional or partially functional (Wiles, J. and

Mallonee 2017). In the same study, the nonfunctionality of water systems in sub-Saharan African countries (focused on Ethiopia, Ghana, Kenya, Rwanda, Uganda and Zambia) was 24.8%, followed by 21.3% in Asia (India) and 7.3% in Latin America & the Caribbean (El Salvador, Guatemala, Haiti, Mexico, Nicaragua) (Wiles, J. and Mallonee 2017). According to the report of the water trrigation and electricity ministry inventory report, the national nonfunctionality rate for Ethiopia is estimated to be 26%, with the highest nonfunctionality rate (34%) in the Afar region (Butterworth, J., Welle, K., Hailu, T., Boestoen, K. and Schaeffer 2013). In Ethiopia, the rate of nonfunctionality of water services was found to be 20-30% nationally and up to 50% in rural Ethiopia (DuChanois et al. 2019). Furthermore, in a study in four Ethiopian regional states that are considered to have stronger infrastructure than the remaining five regions, 20% of the water services were found to be nonfunctional (Anthonj et al. 2018).

Our findings suggest that water quality is an important, yet currently under-researched aspect of 'safe drinking water'. By incorporating water quality parameters into basic water services, investments could be better directed to making basic water services potable rather than investing first in infrastructure development. Given the morbidity and mortality caused by diarrheal disease (Majowicz et al. 2014; WHO 2017b), international investments should invest in functionality *and* water quality. Water quality as a metric should remain a consistent priority during the planning, implementation, maintenance, and monitoring and evaluation of water services. The findings of this study may be of use to water scholars, global development organizations, national policymakers, as well as grass-roots implementers.

## 2.2. Toxins in improved water and feces

*E. coli* toxins are an Enterobacteriacea family (Shanker, Vootla, and Pindi 2020) transmitted through the orofecal route (De Graaf et al. 2017) and a vicious cycle of toxins between water sources, stool and the local population (Ahmed et al. 2020; Obi et al. 2004; Pouladfar et al. 2017). Cycling of enteric pathogens between stored water to humans and animals results in a sustained, stable transmission. *E. coli* toxins are a more common public and veterinary health concern that requires continuous monitoring, and molecular analysis is a most effective approach (Szmolka and Nagy 2013). Diarrhea continues to be the most common cause of morbidity and mortality among infants, children under five years of age, girls, boys, women and men in developing countries such as Ethiopia. Diarrhea is the second leading cause of death after pneumonia in children under five years of age and kills around 525,000 children every year (WHO 2017b).

Most of diarrheal disease transmission in Ethiopia is believed to occur due to pollution of the water supplies by human and/or animal feces (Haileamlak 2016). However, very few studies have documented toxins within the improved water supply in households and the stool of patients with diarrhea in South Africa. Furthermore, no such studies have been conducted in Ethiopia so far (Abong'o and Momba 2008; Omar and Barnard 2014). Few initiatives have been taken to study toxins in Ethiopia, either in water or stool samples where the samples were not statistically representative. Among these are stool samples from patients with human immunodeficiency virus (HIV) in Gondar, Ethiopia, and tap water used in slaughter operations in the Export Abattoir in Modjo, Ethiopia (Mersha et al. 2010; Seid et al. 2018). In these two studies, toxins with a detection rate of 2.3% (n =

100) in the stool (Seid et al. 2018) and the *E. coli* shiga toxin (Stx1 or Stx2) at a rate of 4.3% (n = 23) were found in water samples (Mersha et al. 2010).

The capacity of a given *E. coli* strain to cause disease is determined by specific virulence factors that include adhesion to other cells, penetration of invasions, contact with toxins or absorption of the toxic effect and capsule cover (Kuhnert, Boerlin, and Frey 2000). In this study, toxins were chosen as the most obvious and prominent virulence factors found in all pathogenic *E. coli* strains. Among the once targeted toxins were enteroaggregative *E. coli* heat stable enteric toxin 1 (EAST1), shiga toxin 1 and 2 (Stx1 and Stx2), heat stable toxin a (Sta) and heat labile (LT) toxins (Boerlin et al. 1999; Masters et al. 2011).

EAST1 is a small protein toxin that was first found in the stool of a child who had diarrhea in Chile (Ménard and Dubreuil 2002). Since EAST1 is not specific to a pathotype, it can be found in a variety of clinically significant enteric *E. coli* and is more common than previously known (Savarino et al. 1996). Despite being separate enterotoxins, EAST1 and the Sta enterotoxins have some physical and mechanical similarities. Sta is an enterotoxin that is heat-stable and known to cause secretory diarrhea (Lawal, Parreira, and Goodridge 2022; Ménard and Dubreuil 2002; Veilleux et al. 2008). A human pathogenic strain of *E. coli* that produces Shiga toxin producing *E. coli* (STEC) is a human pathogen with two of its key virulence factors being the toxins, Stx1 and Stx2 (Bai et al. 2019; Beutin and Martin 2012).

STEC strains that cause severe diarrhea are endemic to Central Africa and have spread to Europe and Asia (Beutin and Martin 2012). One of the most potent bacterial toxins known as shiga toxin (Stx1 and Stx2) causes bloody diarrhea, hemolytic uremic

syndrome, kidney failure, and death in humans (Crespo-Medina et al. 2020). The EAST1 and Stx2 toxins of the *E. coli* toxins were found in drinking water from improved sources and stools of patients in Finland and the USA associated with gastroenteritis and diarrhea outbreaks using multiplex PCR (mPCR) (Cooley et al. 2013; Crespo-Medina et al. 2020; Lienemann et al. 2011). The detection of enteric *E. coli* toxins in both the water supply and the stool indicates that the outbreak of diarrhea was due to contamination of the water by human stool. However, this conclusion was based on the analysis of stool samples taken from five patients and one water sample from the water distribution system alone (Lienemann et al. 2011). Therefore, it would be better to see the associations of enteric *E. coli* toxins in the water and stools with representative samples and a statistically significant sample size.

However, various studies are conducted on the association between enteric bacterial pathogens in water at sources rather than in household drinking water and stool using culture-based methods. The causative agent of an average of up to 40% of diarrhea cases cannot be identified by the culture method (Ugboko et al. 2020). This approach created a limited ability to identify pathogens (Ashbolt 2015; Seid et al. 2018). On the other hand, studies conducted using PCR techniques are conducted largely in developed countries or a few middle- and low-income countries in detecting pathogens only in stool samples (Sjoling Asa, Sadiphipoorjahromi Leila, Daniel Novak 2015) or water samples (Mattioli et al. 2014; Shanker et al. 2020; Titilawo, Obi, and Okoh 2015). There were also time, money, and infrastructure barriers to investigate toxins in stool and water samples, and statistically significant associations were found (Omar and Barnard 2014; Vendruscolo et al. 2017). There are studies that recommend the use of a combination of traditional

culture and molecular methods (Cooley et al. 2013; Tornieporth et al. 1995; Youssef et al. 2000). Therefore, this study used the molecular technique; Multiplex polymerase chain reaction (mPCR) recommended as a first step in microbial source tracking studies in combination with culture methods to allow a reliable and maximum identification of toxins in both improved water and stool (Baker-Austin et al. 2009; Savichtcheva, Okayama, and Okabe 2007). Furthermore, most of the studies used water samples from sources that overlooked the possibility of water contamination during distribution to the point of use. Managing water treatment at water sources may not be enough to access and consume safe water. Treatment of water must be aimed at the whole distribution system, from households. The report of a meta-analysis conducted in developing countries shows a non-compliance rate of 26% of the water quality standard between water samples taken from households and 'improved' water sources such as piped water, water from boreholes and protected shallow wells (Shields et al. 2015).

## 2.3. Mapping pathogens detected by PCR-based method in improved water supply in LMICs

The genetic diversity of human enteric bacterial (Kumar, Tripathi, and Garg 2013; Potgieter et al. 2018), viral (Haramoto et al. 2018; Sadik et al. 2017; Spilki et al. 2017; Titilawo et al. 2015) and protozoal (Bonilla et al. 2015) genomes in water is increasing and becoming more complicated as a result of contamination of improved water at the sources, collection and home storage, creating increased public health threats in low and middle-income countries (LMICs). Detecting microbial pathogens in water supplies in a comprehensive and accurate way is beneficial to ensuring the safety of water in LMICs where water contamination is a major concern (Alhamlan, Al-Qahtani1, and Al-Ahdal

2015). Traditional techniques of using pure cultures grown in laboratories to characterize microbial water quality have limitations due to false positive results (Brenner et al. 1996; Chao, Chao, and Chao 2004; Fricker and Eldred 2009; Harwood et al. 2002; Schets et al. 2001) and inability to identify non-culturable microbes, since only <1% of natural microorganisms are cultureable (Hugenholtz and Tyson 2008; Staley and Konopka 1985). The causing agent of up to 40% of diarrhea cases cannot be identified using the culture method (Ugboko et al. 2020). Water-related pathogens were known to cause persistent or acute diarrhea in humans, dehydration-related diarrhea in infants and children, traveler's diarrhea, watery diarrhea lasting up to a week, and bloody diarrhea or dysentery (South Wollo 2017). Very little attention has been paid to the non-culturable but harmful microorganisms (Pommepuy et al. 1996; Roszak and Colwell 1987) such as enteric toxin *E. coli* (Pommepuy et al. 1996) and *Legionella pneumophila* (Ducret, Chabalier, and Dukan 2014). These are causative agents for diarrhea and pneumonia, which are particularly common in LMICs. A method to characterize microbial quality that provides a relatively unbiased picture on the type, quantity, distribution, and functionality of pathogenic microorganisms in the water supply within the context of LMICs is essential. This can help to monitor progress towards achieving the UN Sustainable Development Goal (SDG6) target 6.1, such as safely managed drinking water services (SMDWS) (WHO 2017a).

The different types of waterborne pathogen detected using PCR methods, as well as their magnitude in terms of rates or concentrations in various developed countries, have been outlined. In the United States of America (USA), *E. coli* O157: H7 was found in drinking water at a concentration of 30 - 40 cells per 100 ml) (Sen et al. 2011). In another study

conducted in Florida, United States, *Cryptosporidium* rates were 45%, poliovirus rates were 55%, and *Giardia* rates were 41% (Bonilla et al. 2015). *E. coli* cells were detected at a mean concentration of 310 cells per 100 ml isolated from rainwater tanks in Australia (Ahmed et al. 2012).

DNA denaturation, primer annealing, and DNA synthesis are the three measure steps in the DNA amplification steps (Figure 1).

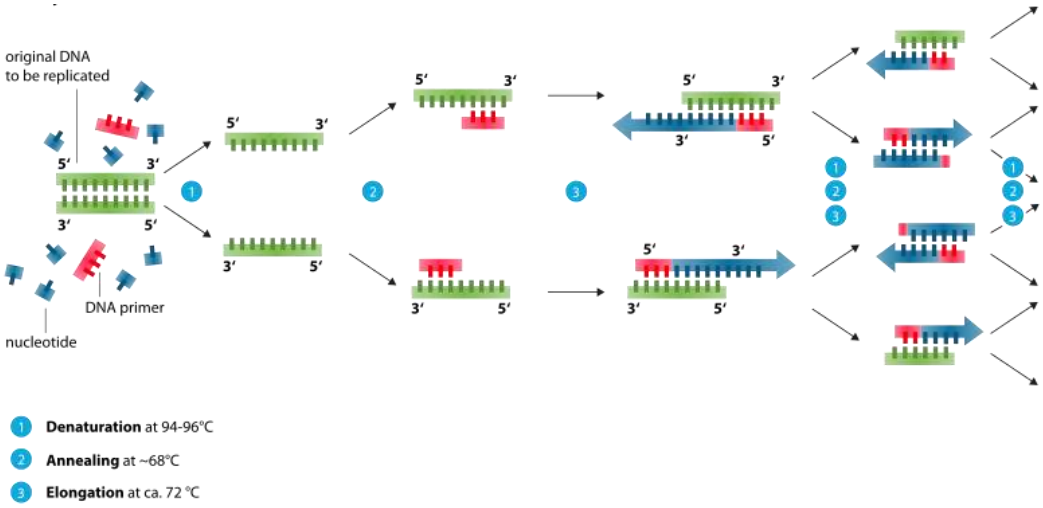


**Figure 1.** Polymerase Chain Reaction of original DNA replication using three steps.
**Source:** (Loman et al. 2012)

The methods are powerful molecular techniques that can be used for a range of applications, from detecting up to quantification of bacterial, viral, and protozoal pathogens in small quantities (1-2 microliters) of water samples (Domingues and Silva 2014). Furthermore, PCR, compared to conventional methods, increases the overall detection frequency by 4% of bacterial enteropathogens (Fiedoruk et al. 2015), measures specific gene expressions more rapidly (Sen et al. 2011), and detects enteric viruses and enterococci, which can appear in the form of viable but non-culturable pathogens

(VBNC) quantitatively and rapidly in water samples (He and Jiang 2005). Moreover, some PCR techniques can distinguish viable cells from dead cells (Domingues and Silva 2014).

Due to advances in pathogenic microbial testing methods, a better understanding of newly emerging waterborne diarrheal pathogens is occurring. For example, reports showed an increasing number of different microbial genotypes that can cause diseases (Standridge 2008). The water quality results obtained through PCR methods would provide a better picture of the provision of sensitive and rapid results (Servais et al. 2002) and can be applied as a microbial source tracking tool (MST) to generate more credible information, which in turn contributes to the realization of the SDG target for water supply in LMICs. Despite its importance, the use of PCR in identification and quantification of water pathogens has not been uptake by the LMICs. However, the disadvantages of PCR-based water quality methods for environmental water samples, which may not all be the case with samples from improved sources, are: (1) the need to concentrate the microorganisms due to the dilution effect (low concentration of microorganisms per volume) or require a culture step to increase the number of microorganisms in the sample. (2) Pollutants can accumulate along with the target microorganisms for environmental water, which may not be the case for improved water samples. (3) Membrane clogging during filtration of environmental water samples, which is not the case with water samples from improved water sources. (4) Loss of target cells and/or nucleic acids during the various water sample preparation steps (concentration, extraction, purification), which can result in inefficient concentration of the target microorganisms to be detected and consequent false negative results. (5) Long and

complex procedures; and (6) inhibitors are difficult to remove and are not completely eliminated (some have the same solubility properties as nucleic acids) (Domingues and Silva 2014). Those disadvantages have to be taken care during applying PCR techniques to meet the validity of the results.

The molecular-PCR consists of a range of methods known as traditional PCR, quantitative polymerase chain reaction (Q-PCR), real-time polymerase chain reaction (RT-PCR), real-time reverse transcription polymerase chain reaction (RT-qPCR), multiplex PCR, competitive PCR, and SmaRT-PCR (Hugenholtz and Tyson 2008). In traditional PCR, the detection of the DNA is carried out at the end-point of the reaction, and in the case of RT-PCR the detection was carried out while the reaction is occurring. Q-PCR is a strongly recommended PCR method for quantification of the molecular index of viral contamination in water samples (Nestor Albinana-Gimeneza, c, Marize P. Miagostovichb, Byron Calguaa and Lleonard Matiac 2009). Multiplex PCR allows simultaneous detection of different strain targets within the same species, which gives its advantage over the other type of PCRs and demonstrated superior detection ability when tested with laboratory collected strains (Molina et al. 2011). On the other hand, real-time reverse transcriptase PCR (RT-qPCR) uses RNA templates in the molecular process and commonly uses fluorescent dyes for the detection of quantitative estimation of the amplified segment in water samples (Girones et al. 2010). SmaRT-PCR has the advantages of not requiring additional equipment and is easier to automate the procedures and the calculation of molecules (Szibor and Morawietz 2001).

Based on the length of bases and the sequence type, PCR amplifications can be grouped into three different categories. Standard PCR that involves amplification of a single DNA

sequence that is less than 5 kb in length. Useful for cycle sequencing, cloning, and mutation detection. Long PCR that is used for amplification of a single sequence that is longer than 5 kb and up to 40 kb in length used for long-range sequencing of complete genes. Multiple PCR used for amplification of multiple sequences that are less than 5 kb in length. Used for forensic studies, pathogen identification, linkage analysis, template quantification, genetic disease diagnosis, and population genetics (Grunenwald 2003).

Advances in *E. coli* testing methods, new understanding of newly emerging waterborne diarrheal pathogens, and growing international acceptance of *E. coli* as a viable indicator of fecal contamination are now growing (Standridge 2008). The identified methods can be applied to improved drinking water sources that, by nature of their construction, adequately protect the source from outside contamination and, particularly, fecal matter (WHO/UNICEF 2015b). The usual recommended volumes of drinking water samples are 100 ml. However, PCR-based techniques generally use small volumes of samples, usually 1 or 2 μL (Domingues and Silva 2014). After the water samples are collected, the sample preparation procedures for PCR-based analysis comprises five steps; concentration of the target organism by filtration, centrifugation, extraction of the nucleic acids by heat shock, chemical, mechanical and/or enzymatic lysis, purification from chemical precipitation, solvent extraction, magnetic separation to eliminate possible inhibitors, amplification and sequencing. Purifying ethanol and isopropanol is difficult in the water sample and can function as inhibitors. Therefore, to avoid this interference, the use of designed polymerases such as KAPA2G and Solys biodyne DNA polymerase is recommended. Amplification is done by thermal cycling by repeated heating and cooling

to exponentially copy a segment of DNA in thousands to millions (Domingues and Silva 2014).

PCR is subject to the confounding problem of contamination. Cross-contamination of samples is of concern in any discipline. For this, an appropriate control has to be used. A separate ‘no DNA’ control should be set for each master mix or each individual reaction. Positive control reaction with a template known to contain the sequence and internal control amplify a target known to be consistently present in the test DNA (Stirling 2003). Furthermore, one of the drawbacks of the PCR method is that the detection of genomes does not provide information about the infectivity of the pathogen detected or the level of risk to the population (Girones et al. 2010). This drawback of the PCR technique can be addressed by associating the genomes detected with the type of diarrheal disease using diagnosed diarrheal cases.

## 2.4. Conceptual framework

The conceptual framework below clearly shows the process how to associate the detected pathogens with diarrheal cases (Figure 3). The association of diarrheal disease with water can be concluded by two major evidences; pathological and epidemiological evidence. A conceptual framework considers the steps to test the statistical association between enteric *E. coli* toxins identified in the improved water and stool of the diarrheal patient is indicated. The application of singleplex and multiplex PCR in the epidemiological evidence. The framework indicates the exclusion criteria used in this study. The exclusion criteria were those factors that could contribute as a means of transmitting toxins to patients other than the improved contaminated household water they use regularly. This can be concluded by two major evidences; pathological and

epidemiological evidences. These evidences can be verified by agent identification in the water sample and stool samples from human cases and a strong statistical association (Stanwell-Smith 1994).

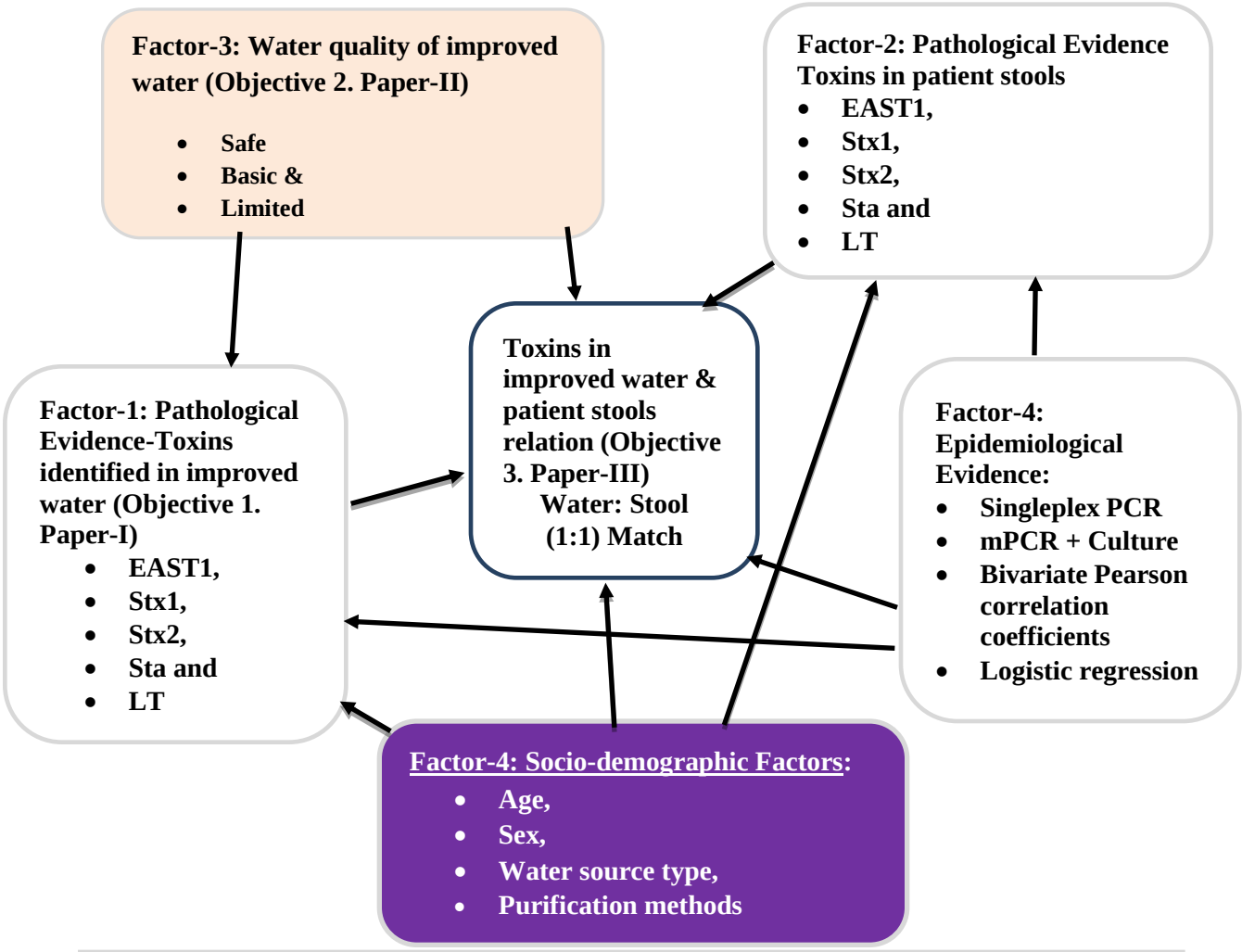


**Figure 2.** Adopted conceptual framework to examine evidence for association of *E. coli* toxins in water and stools in South Wollo, Ethiopia (Stanwell-Smith 1994).

## Chapter 3: Methods and materials

The research follows the steps outlined in the diagram below, in general. This diagram is used to identify toxins in improved household water samples and patient stool (Figure 3). The study was conducted from June 2019 to February 2020.

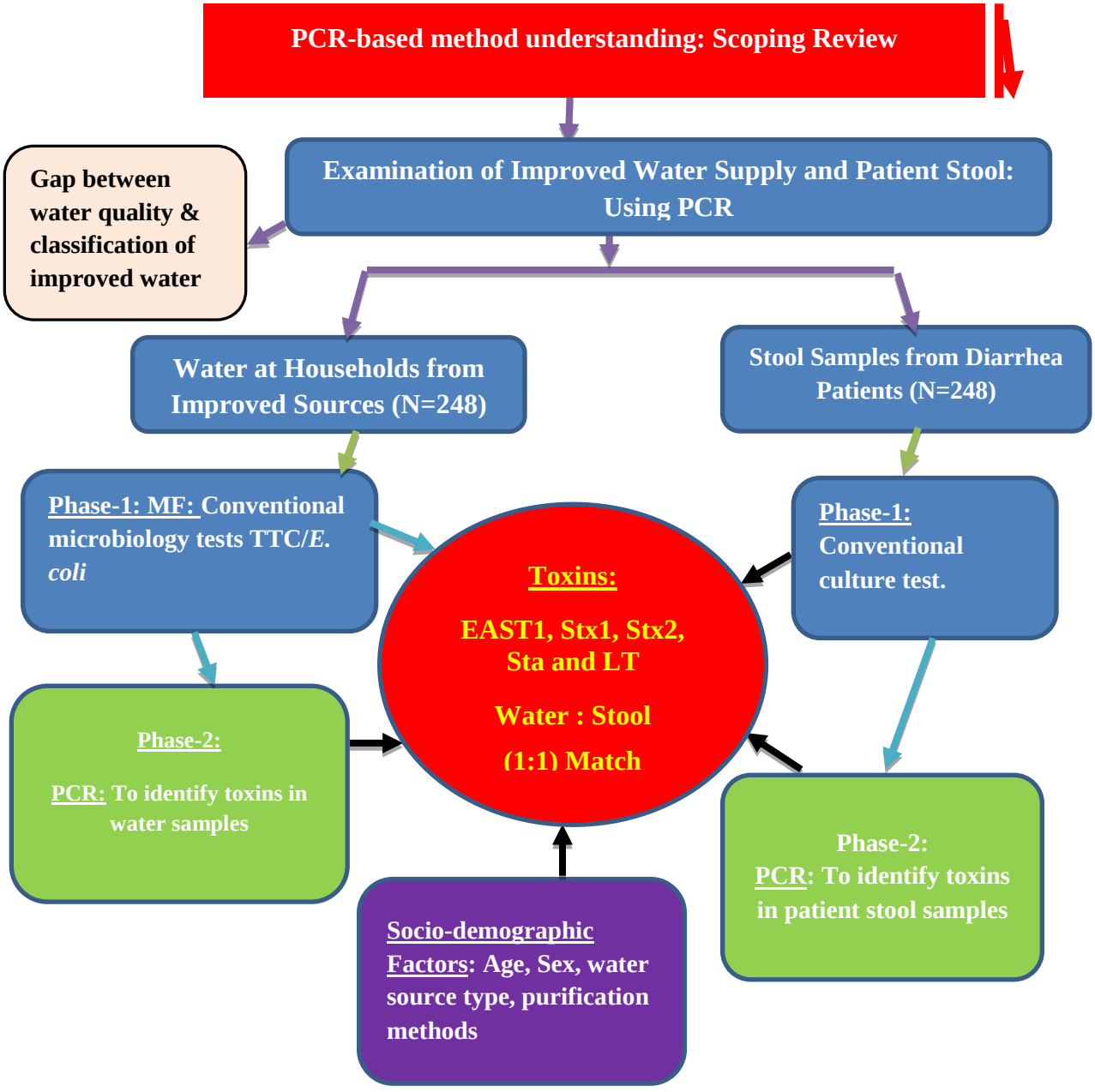


**Figure 3.** Flow diagram of the dissertation work

## 3.1. Study area

The study was carried out in South Wollo, Ethiopia. South Wollo is one of the 10 Zones found in the Amhara Regional State with the capital city Diesse. This Zone has a total population of about 2.5 million. South Wollo is bordered on the north by North Wollo, on the northwest by South Gondar, on the west by East Gojam, on the south by North Shewa and on the east by Afar Region. The South Wollo Zone has an average annual maximum temperature of 22.2 °C (72.0 °F) and an average annual minimum temperature of 2.6 °C (36.7 °F). Mount Tabor in Amhara Sayint, which rises 4247 meters above sea level (MASL), is its highest peak. In 2017, the Zonal authorities announced that approximately 540 safe water units were constructed during this budget year at a cost of more than 23 million birr, while other 878 units were repaired. This has improved the access to improved water of 51% to 61% of the Zone's inhabitants (ENA 2017). The population density is associated with the 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 and ICF 2016; Fakhr, Gohar, and Atta 2016). Governments and nongovernmental organizations in the area were actively intervening, including constructing new and improved water supplies. The health facilities and woredas (districts) targeted in this study are shown in Figure 4. A woreda is an administrative division of the Ethiopian local government.

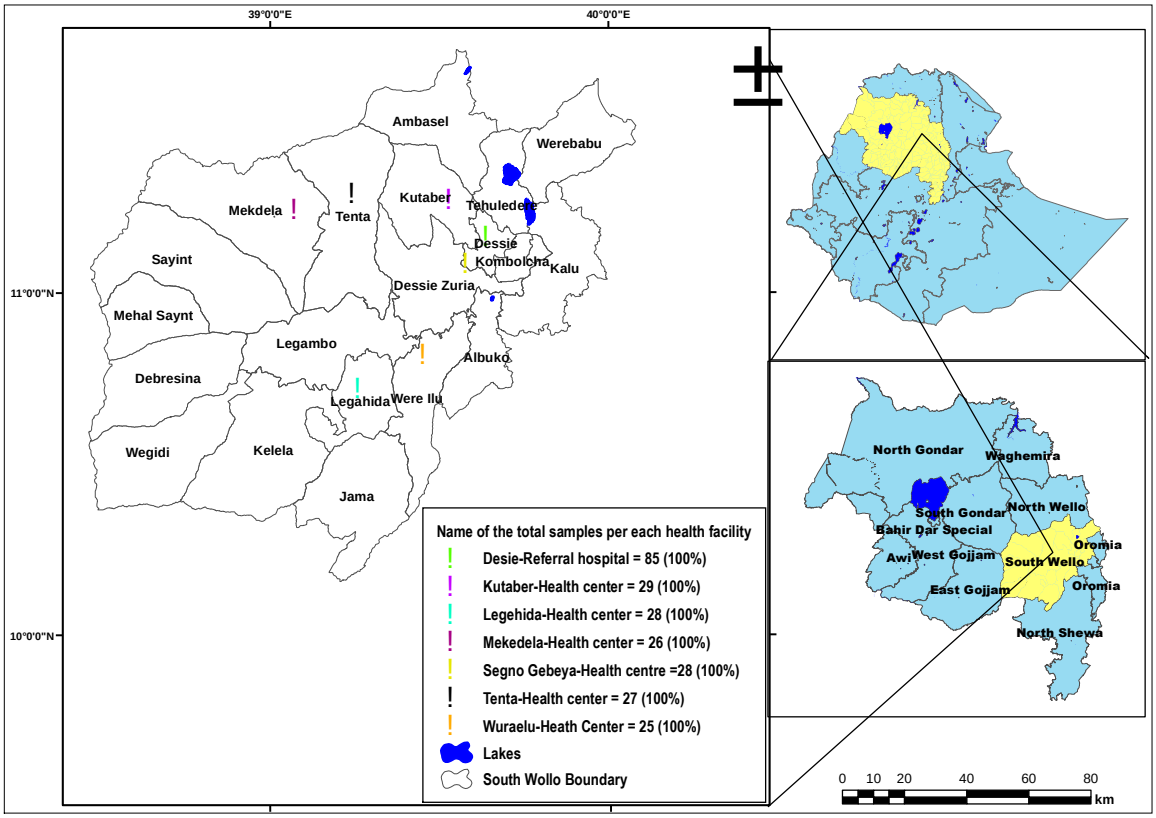


**Figure 4.** Administrative map of the study area

## 3.2. Sample size determination

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

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

where; design effect (DEFF) is 1.5, population size (**N)** 2,518,862, prevalence rate of diarrhea in the area (**p)** 11%, confidence limits (**d)** ($p$<0.05) with 95% confidence interval (CI) and $Z^2_{1-\propto/2}$ = Confidence interval and $\propto$ = estimated standard deviation. **(n)** = 225 and considering a 10% nonresponse 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 and ICF 2016; South Wollo 2017). The sampling began with the random selection of districts (n = 6 of 20 listed), which is approximately 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 due to its important position in the health system. Six of the eight health centers in the selected districts were randomly selected. The size of the water sample was determined based on the standard number of people served by the 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 referral hospital, were included in the study if they met the inclusion criteria. Six health centers' patients represent 66% of the respondents, while 34% came from the referral hospital. Diarrhea patients were subsequently interviewed at the healthcare facility. Additional interviews were conducted in households and water samples were collected for analysis.

### 3.3. Water sample and *E. coli* DNA extraction

QIAamp® DNA Mini Kit (Qiagen, Germany) of Quick-Start Protocol April 2018 was applied for the DNA extraction in water samples (Hinlo et al. 2017). DNA concentration and quality was measured using a Nanodrop and > 50 (ng/uL was used as a cut-off point (Thermo Scientific, USA). The water in each household was collected aseptically using gloves and sterile 500 mL polyethylene bottles within 12 hours of the patient's visit of

healthcare facility. Polyethylene bottles are chemically resistant containers, lightweight, prevent breakage, and make laboratory work easier. The samples were transported in a cold box to the regional branch of the Ethiopia Public Health Institute (EPHI) laboratory for analysis within four hours of collection. The prevalence of total coliforms (TC) and thermotolerant coliforms (TTC) was determined by membrane filtration (MF) (Cobetter MicroDisc gamma sterile membrane filter, China) and incubated at 37 ºC and 44 ºC, respectively (WHO 2011). A volume of 100 mL water was filtered through a 0.45 µM pore size and a diameter of 47 mm sterile membrane filter and cultured in membrane lauryl sulphate broth (MLSB) (AVONCHEM-ACM-1820-O, Avonchem Ltd, Waterloo, St. West, Macclesfield) or MacConkey agar (MAC) (ThermoFisher Scientific ™ Oxoid™, UK). Colony-forming units (CFU) per 100 mL of water were determined for each sample. The estimated total number of coliform organisms and the thermotolerant coliform organisms were determined presumptively by counting yellow colonies from membranes after they had been incubated at 37 °C and 44 °C, respectively (Figure 5).

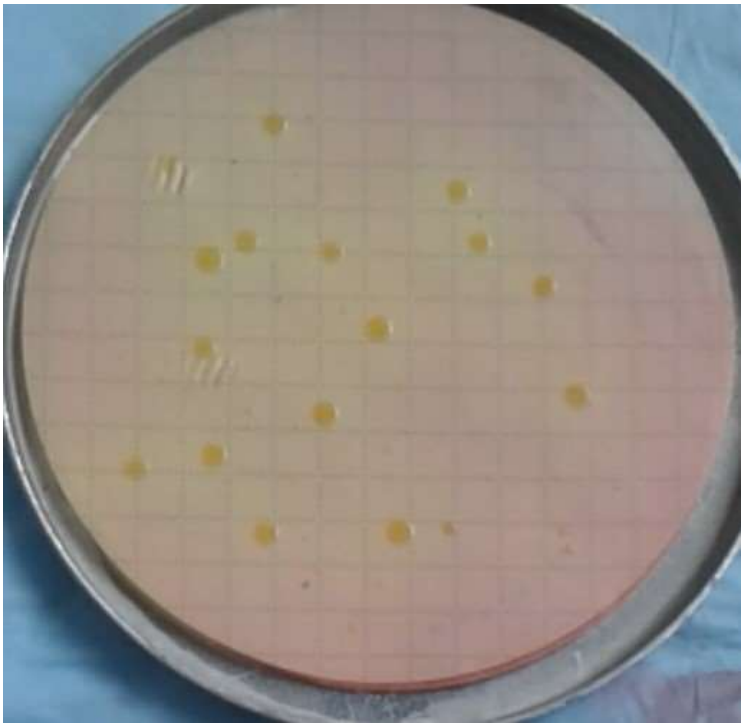

**Figure 5.** Colonies of grown fecal coliforms on filter paper using membrane filter/culture technique at 44 $^{0}$C.

Full-size filter papers with bacterial colonies were transferred to 2 mL cryo-tubes and transported to the AHRI, Addis Ababa, where they were stored at -20 $^{0}$C. The filter paper was aseptically cut into small pieces and placed into a 1.5 mL micro-centrifuge tubes for lysis and isolation as per the manufacturer's instructions. Total DNA was extracted from 183 cultured water samples.

In addition, water samples were tested for selected chemical parameters; pH, turbidity and residual chlorine using Spectrophotometer-Photometer 7100 of Palintest with preprogramed test calibrations, absorbance (A) and transmittance (T) of accuracy + or – 1%T. The Spectrophotometer was adjusted with automatic wave length selected from minimum 450 nm and maximum of 650 nm and water test tables. Phenol red, turbidity

tube and N,N´-diethyl-p-phenylenediamine (DPD No-1) tablets, and double distilled water as blanks were used to test pH (in number), turbidity (in nephelometric turbidity unit (NTU)) and residual chlorine (mg/lit $Cl_2$) respectively and the results were recorded. The selected chemical tests were conducted at the sampling location, either in the home for samples taken at a distance, or in a laboratory for samples taken at a closer proximity.

### 3.4. Stool sampling and DNA extraction

Fresh 25 g stool samples and swabs of 248 diarrhea patients were collected at seven selected health facilities using covered sterile collection bottles. Each sample was labeled and 2 mL of saline was added before being delivered to the regional public health laboratory in Dessie (approximately in 2 hours) using a cold box. The stool samples were cultured on MacConkey (MAC) agar medium at 36 °C for 24 hours following standard procedures targeting *E. coli* (ThermoFisher Scientific Inc., Oxoid Limited). Pink color colonies isolated to be used for further identification using selected biochemical tests. Oxidase test using Oxidaze detection strips of code-microbact@TM-MB0266A (Figure 6).

**Figure 6.** Biochemical test using Oxidase test by Oxidaze detection strips by indicating a deep blue color in the strip after sample smeared.

Sulphide reduction, indole production and motility (S.I.M.), and gas production were among the biochemical tests used. The test used S.I.M. medium-IVD-Ref-610181 after culturing the inoculates on Trypticasein Soy Agar (TSA) of Conda, category number of 1068.00 of Farm Europea of 40 g per one liter at 37 $^{0}$C for 24 hours. By mixing three drops of Kovac's Indole Reagent, indole was produced (Cat. no. 49431AR) (Figure 7).

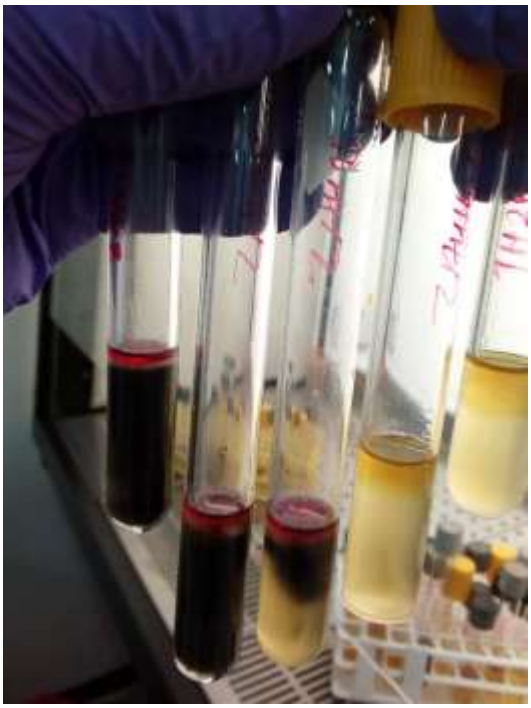

**Figure 7.** Biochemical test result using sulphide reduction, indole production and motility (S.I.M.) and gas production. The production of indole with top rings in all the five test tubes, the sulphide reduction indicated by the black precipitate from three left test tubes, motility by forming central lines in test tubes and gas production by observing bubbles and turbid nature in the two right test tubes.

A total of 390 presumptive *E. coli* isolates from the 248 samples were transported to the Armaur Hansen Research Institute (AHRI) in 2 mL brain heart infusion (BHI) in cryotubes. DNA was extracted from the 390 isolates using the boiling method (Mandour, Hinawi, and Xiulan 2016).

The following figures are shown during DNA extractions both in water and stool samples (Figure 8).

**Figure 8.** gDNA extraction from improved water and patient stool samples at AHRI lab.

The concentration of extracted bacterial genomic DNA (gDNA) in terms of mass unit; nanogram per microliter (ng/µl) and the purity ratio of A260/280 and A260/230 were measured using Thermo Scientific NanoDrop One$^{C}$ Spectrophotometer with absorbance measurement normalized to equivalent path length of 10mm and detection limit of 0.2 – 27,500 ng/µl. The amount of dsDNA used was 1µl of aliquots. The blank solution used was molecular grade water, which is the same as the solution used for DNA extraction to maintain the same PH and similar ionic strength. The total number of stool isolates measured using the NanoDrop instrument is 263. The widely accepted value of double stranded (dsDNA) concentration is 50 ng/uL and the purity ratio ranges from 1.5 to 2.2. Accordingly, most of the results were within acceptable values for both the concentration and purity ratio, as shown in the graph below (Figure 9).

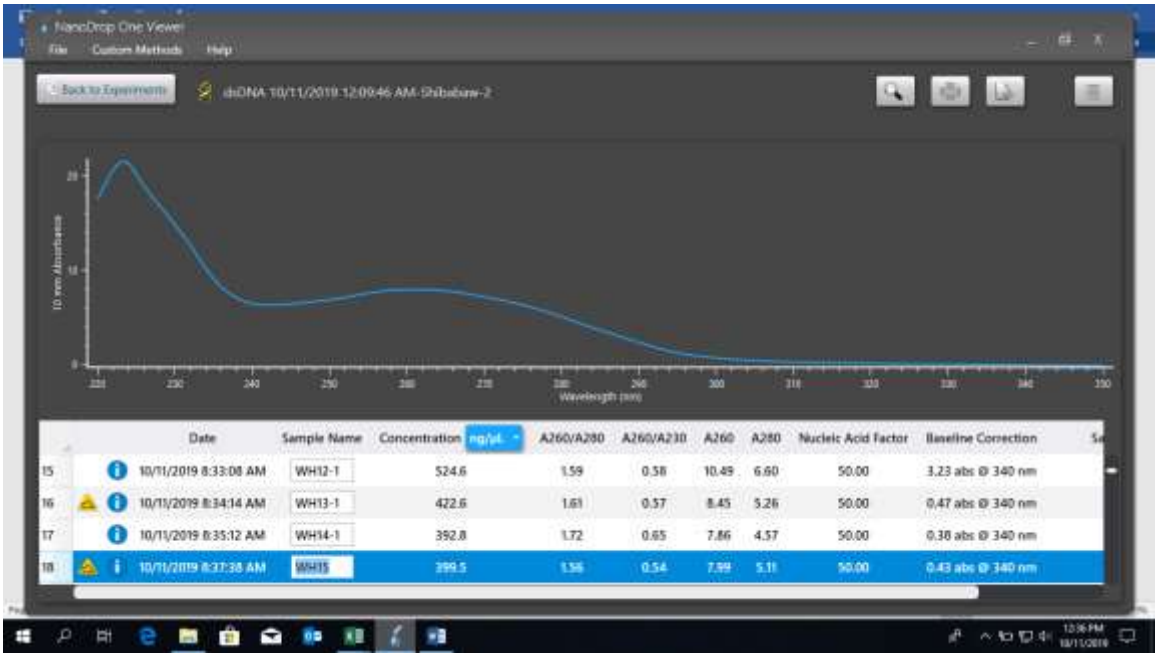


**Figure 9**. Sample graphs of the result of the DNA concentration and purity ratio

## 3.5. Detection of toxins in improved water and stools

### 3.5.1 Water

For PCR-based detection, five genes encoding enteric toxins were chosen using the primers listed in Table 3. The KAPA2G hotstart mastermix (Kapa Biosystems, Wilmington, Massachusetts) was used for PCR, with cycling conditions of initial denaturation at 95 $^{o}$C for five minutes followed by 40 cycles of denaturation at 95 $^{o}$C for 15 seconds, annealing temperature at 60 $^{o}$C for 15 seconds, extension at 72 $^{o}$C for 15 seconds and final extension at 72 $^{o}$C for one minute. Laboratory *E. coli* toxins named enteroaggregative heat-stable enterotoxin 1 (EAST1), shiga-like toxin 1 (Stx1), shiga-like toxin 2 (Stx2), heat-stable (Sta) and heat-liable (LT) were used for positive control and molecular grade water as no template controls (NTC). The presence or absence of toxin genes was determined using 2 % agarose gel and electrophoresis of PCR products.

### 3.5.2. Stool

Five *E. coli* toxins were selected for five-plex PCR-based detection using the primers listed in Table 3. Multiplex polymerase chain reaction (mPCR) was conducted using Solis BioDyne mastermix (European Union (EU) registered trademark of Solis BioDyne OÜ, Tartu, Estonia). The reaction components were 2 µl of 5x hot firepol multiplex mix, 1 µl of forward primer pool, 1 µl of the reverse primer pool, 1 µl of molecular grade water, and 5 – 10 ng of template DNA in a total of 10 µl reaction. Each primer was at 0.2 µM final concentration in the final reaction mix. The PCR cycling conditions were initial denaturation at 95 $^0$C for 12 min followed by 30 cycles of denaturation at 95 $^0$C for 30 sec, annealing temperature at 61 $^0$C for 30 sec, extension at 72 $^0$C for 2 min and final extension at 72$^0$C for 10 min.

**Table 3** Primers used to evaluate *E. coli* toxins in improved water and stool samples.

| **Primer Names** | **Primer sequences (5' - 3')*** | **Amplicon sizes** | **Pathotypes** | **References** |
|---|---|---|---|---|
| EAST1-F | CCATCAACACAGTATATCCGA (21) | 111 | EAEC, EPEC | (Choi, Kwon, and Chae 2001) |
| EAST1-R | GGTCGCGAGTGACGGCTTTGT (21) | 111 | | |
| Sta-F | TCTTTCCCCTCTTTTAGTCAG (21) | 166 | ETEC | (Moseley et al. 1982) |
| Sta-R | ACAGGCAGGATTACAACAAAG (21) | 166 | | |
| Stx1-F | CGCTGAATGTCATTCGCTCTG (21) | 302 | EHEC/STEC/VTEC | (Mora et al. 2007; Rey et al. 2003) |
| Stx1-R | CGTGGTATAGCTACTGTCACC (21) | 302 | | |
| Stx2-F | GGCACTGTCTGAAACTGCTCC (21) | 255 | EHEC/STEC/VTEC | (Paton and Paton 1998) |
| Stx2-R | TCGCCAGTTATCTGACATTCTG (22) | 255 | | |
| LT-F | ATTTACGGCGTTACTATCCTC (21) | 280 | ETEC | (Choi et al. 2001) |
| LT-R | TTTTGGTCTCGGTCAGATATG (21) | 280 | | |

* Nucleotide sequences of the primers that had been used to amplify enteric toxins (EAST1, Sta; Stx1, Stx2, and LT using genomic DNA extracted from improved water as a template in South Wollo, Ethiopia. The name of the enteric toxin gene target is given on the left, followed by the primer name and the primer sequence (5'-3').

Laboratory *E. coli* strains named EAST1, Stx1, Stx2, Sta, and LT were used for positive control and molecular grade water as no template controls (NTC). The presence or absence of toxins was determined by electrophoresis of agarose gel of a single-plex (Figure 10) and the 5-plex polymerase chain reaction (PCR) products in 3% agarose gel (Figure 11).

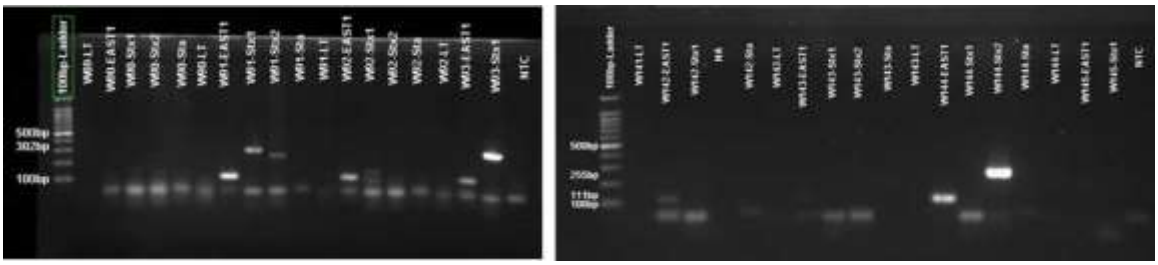

**Figure 10.** Agarose gel read in a Bio-Rad using a 100 bp ladder and a singleplex PCR.

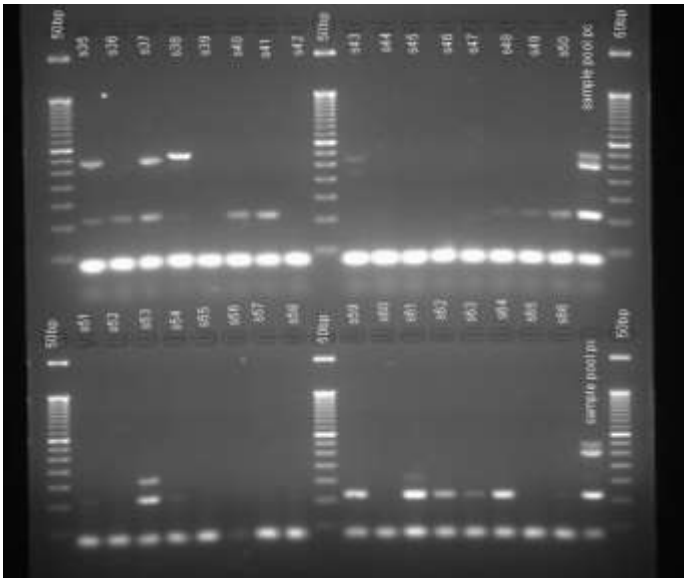

**Figure 11.** Agarose gel read in a Bio-Rad using a 50 bp ladder and multiplex PCR

EAST1- enteroaggregative *Escherichia coli* heat-stable enterotoxin 1, Stx1- shiga-like toxin 1, Stx2 - shiga-like toxin 2, LT - heat-liable toxin and Sta - heat-stable toxin)

detected and examples of typical colony morphology grown in improved water. It depicts three steps: The membrane filtration (MF) technique, culture at 37 and 44 degrees centigrade using MacConkey (MAC) agar & Membrane lauryl sulphate broth (MLSB) and PCR with gDNA extracted from improved water samples (Figure 12).

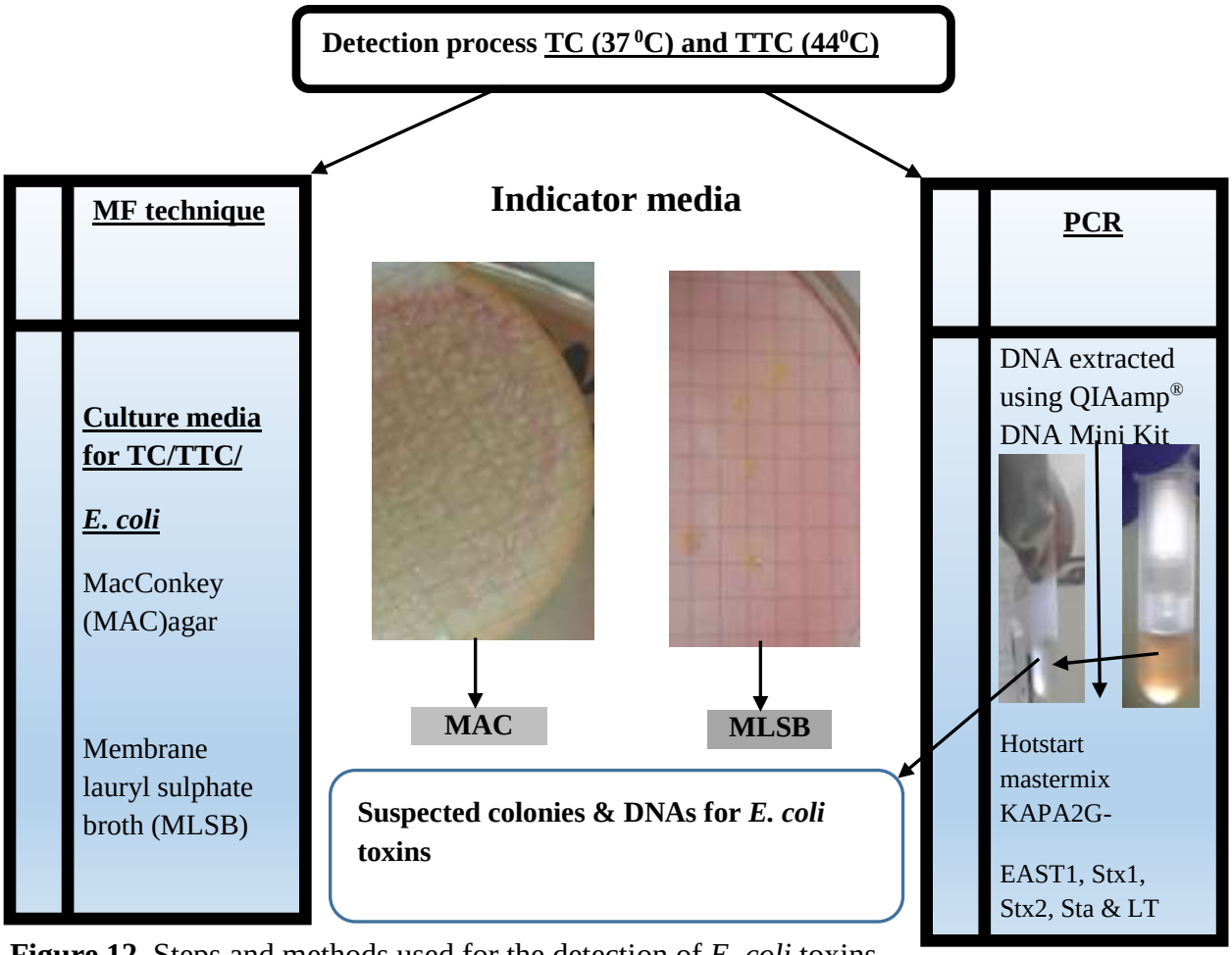


**Figure 12.** Steps and methods used for the detection of *E. coli* toxins.

## 3.6. Water supply and survey data collection

The availability of water, type of water source, the handling of water, and the presence of human and/or animal waste are all factors that influence the contamination of water by toxins 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 symptoms of diarrhea in the health facilities sampled. The interviews were conducted at home using KOBO Toolbox and KOBO Collect, on iPads and smartphones. Data collectors received one day of training on an adapted questionnaire used by trained healthcare professionals in healthcare facilities, as well as ethical research issues, and a 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 in the dry season. The risk of water contamination increases due to runoff that could transport bacteria into the water (Kulinkina et al. 2016; Sidhu et al. 2013).

### 3.7. Data encoding and statistical analysis

Data were encoded using KOBO Toolbox and KOBO Collect on the iPad and smart phones. Data from the 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 toxin 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 knowledge and practice of the respondents 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 toxins (EAST1, Stx1, Stx2, Sta and LT) detected (0 = absent, 1 = present).

Descriptive statistics, Chi-square ($\chi 2$), Fisher's exact test, logistic regression, and bivariate Pearson's correlation coefficients were used to analyze the data and examine the relationship between variables. Variables with *p*-values $\leq 0.25$ in the Chi-square and Fisher's exact test analyses were considered candidates for multivariable logistic regression analysis. In the binary logistic regression model, variables with $p \leq 0.05$ were considered significantly associated with the outcome variable – the detection of at least one toxin in a water sample. The statistical significance of the variables for the correlations was calculated based on Pearson's correlation rank (r). A p-value $< 0.05$ was considered statistically significant. A 95% confidence interval was used to calculate the adjusted odds ratios (AOR) to assess the level of significance of the associations between variables.

### 3.8. Ethics approval and consent to participate

The protocol for this study was approved by the College of Natural and Computational Science Institution Review Board (CNS-IRB), Addis Ababa University (CNSDO/729/10/2018) dated July 24, 2018. The data obtained for this research studies were approved by the Ethiopian Institute of Water Resources, Addis Ababa University and by local kebele leaders of the study target areas. Participants provided informed consent verbally as most participants were illiterates. In order to protect the dignity, rights and welfare of the study participants, confidentiality of information was respected and standards and operational guidance for ethics review of health-related research with human participants of World Health Organization (WHO) followed up. Patients who visited health facilities with complaints of diarrhea received treatment there. The complete water quality report was shared with the EPHI Dessie branch and Zonal Water

Department to help treat the sources and encourage people to practice home water treatment techniques including chlorination.

# Chapter 4: Results and discussion - Summary result of objective 1: Evaluate the water quality status of improved water supplies using toxins as a biomarker

## 4.1. Water treatment – public knowledge and practice

The interviews were used to assess the knowledge of the respondents about water treatment for drinking. Of 90% (222) of the respondents reported that they were aware of the use of boiling for water treatment. In addition, 56% (138) knew about settling, 46% (114) knew about chlorine disinfection, and 41% (102) knew about cloth filters. However, the respondents were unaware of solar water disinfection techniques (Table 4).

As to the frequency table, there was a significant difference between the number of participants, 46% (114), who knew about the use of chlorine for disinfection and those, 16% (40)**,** who practiced it (Table 4). This might be as a result of the lack of chlorine in nearby markets or the participants' negative perception toward chlorine use for water treatment. For instance, some people could think that chlorine will change the taste and odor of water (Mitro et al. 2019). Some 44% (108) of the respondents used cloth filters for drinking water treatment, while 42% (105) used settling, 25% (61) boiling and 16% (40) chlorine. However, none of the respondents used solar disinfection (Table 4). A study in Nigeria reported that the most common 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 at home.

**Table 4** Knowledge and practice of the respondent about the methods of household water treatment.

| 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 (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

***** The household-level water treatment methods included in this study were cloth filters – pouring water through clean clothes into a clean container, settling – allowing water to settle before use, boiling – heating water to 100 $^{0}$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.

## 4.2. Toxins as biomarkers of water quality

Of the 248 samples cultured, 74% (183) tested positive for TC bacteria and 40% (98) for TTC. Some 59% (147) samples were analyzed for toxins and 156 toxin biomarkers of *E. coli* were detected. The prevalence of one or more biomarkers in improved water samples was 39% (96) (Table 4). 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 avian, such as birds, while the water sources included in this study could be exposed to contamination from humans and any type of animals. On the other hand, the result of this study was slightly lower than that of Egypt (50%; n = 300 from potable water from 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 explain the slightly increased prevalence of toxins. The findings of previous studies show that seasonal variation could affect the prevalence of toxins by an increase of 10 to 14% between the dry and wet seasons (Bhavnani et al. 2014; Sidhu et al. 2013).

In this study, 17% (41) of the water samples revealed multiple *E. coli* toxins, 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 risk of infection, depending on the type, amount, and viability of the bacteria that carry them (US-FDA 2012; WHO 2011). The detection of large amounts of toxins from *E. coli* 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, was detected in 33% (82) of the samples (Table 5).

**Table 5.** PCR and membrane filtration technique test results of toxins and coliform counts.

| Detected toxins and Coliforms | PCR test results | Rate in % (n/N) |
|---|---|---|
| EAST1 | Detected | 33 (82/248) |
| | Not | 67 (166/248) |
| Stx1 | Detected | 13 (33/248) |
| | Not | 87 (215/248) |
| Stx2 | Detected | 9 (21/248) |
| | Not | 91 (227/248) |

| Detected toxins and Coliforms | PCR test results | Rate in % (n/N) |
|---|---|---|
| LT | Detected | 6 (14/248) |
| | Not | 94 (234/248) |
| Sta | Detected | 2 (6/248) |
| | Not | 98 (242/248) |
| **Total enterotoxin markers** | **Detected** | **156** |
| Single or more enterotoxins | Detected | 39 (96/248) |
| | Not | 61 (152/248) |
| Two or more enterotoxins | Detected | 17 (41/248) |
| | Not | 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** |

EAST1 occurs in many bacterial strains or pathotypes of *E. coli* (EAEC and EPEC), which could explain its high prevalence (Sidhu et al. 2013). Early treatment of hosts is thought to decrease or prevent the occurrence of toxins in the host. The high prevalence of EAST1 could indicate continuous contamination by feces of untreated hosts (adults, children and/or animals), since a greater number of copies or cells of gene ($1 \times 10^6$) is required to cause diarrhea than that of other toxins. For example, Stx1 and Stx2 – which require only 10 to 500 copies/cells to induce this phenotype, thus reducing the chance of treating 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. *E. coli* toxins were detected in 74% (39) public stands, of which 36% contained EAST1, 19% Stx1, 13% Stx2, and 6% LT. The highest detections of EAST1 and LT 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 equipped with hand pumps (Table 6).

**Table 6.** Toxins detected by water source types in the 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 (30/248)** | **10 (24/248)** | **100 (248/248)** |
| **Biomarkers of toxins** | **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 toxins in water samples** | | **% (n/N)** | **81(88/108)** | **74 (39/53)** | **30 (10/33)** | **20 (6/30)** | **54 (13/24)** | **63 (156/248)** |

This contradicts the findings of a previous study in developing countries that reported that 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 (Ahmed et al. 2020; Allué-Guardia, Martínez-Castillo, and Muniesa 2014). In this study, only 11% (28) of the 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 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 toxins were detected in basic household piped water supplies – 68% (106/156). The detection of toxins with the category of the water ladder; water samples in the "limited" category reported 23% (36) and the safely managed category of the water ladder reported 9% (14) contamination with toxins (Table 7). 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 the water is from improved sources with good quality construction, there is a strong possibility of toxin contamination before consumption during collection, storage and / or use. This could arise from poor maintenance of the system or household water handling, including storage (Shaheed et al. 2014). Inadequate household water treatment practices were reported. For example, only 16% of the respondents reported that they applied chlorine in their drinking water. The presence of

STEC in 22% of the 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 defection (approximately 21% of the respondents reported having no access to latrines).

**Table 7** Test results of biomarker of toxins by household water ladder

| **Improved household water ladder** | **Biomarkers PCR result -% (n/N*)** | | | | | |
|---|---|---|---|---|---|---|
| | **EAST1** | **Stx1** | **Stx2** | **Sta** | **LT** | |
| | **Detected** | **Detected** | **Detected** | **Detected** | **Detected** | **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)** |

* Results of the toxin PCR tests in improved household water according to the drinking water ladder. (NOTE where n is the detection frequency per the water ladder and N is the total number of toxins detected of the types in question.

## 4.3. Sociodemographic and risk factors for contamination

In this study, 117 (47%) of the respondents were female (Table 8). Of the diarrheal cases, 66% (163) came 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 of toxins – 41% (24) was reported in young people (5 to 18 years) (Table 8). This supports previous results that the susceptibility of young people to *E. coli* toxins is higher than that of adults in settings where hygiene is poor, as natural immunity increases with

age. It could also be due to the diversity of individual awareness and cleanliness and the behavior of children (Qadri et al. 2005).

**Table 8** Responses of participants 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* | No | 106 | 43 |
| | Yes | 142 | 57 |
| Human waste observed* | No | 134 | 54 |
| | Yes | 114 | 46 |

*Animal and human waste observed near households or water sources.

A linearity test was predicted for the detection of toxins by the age of the respondents in improved water, and the linearity plot with continuous variable age and residue errors is presented in. As can be seen, the observations follow the horizontal line flow and, as the age of the respondent increases, the detection of the toxin gene increases or vice versa (Figure 14).

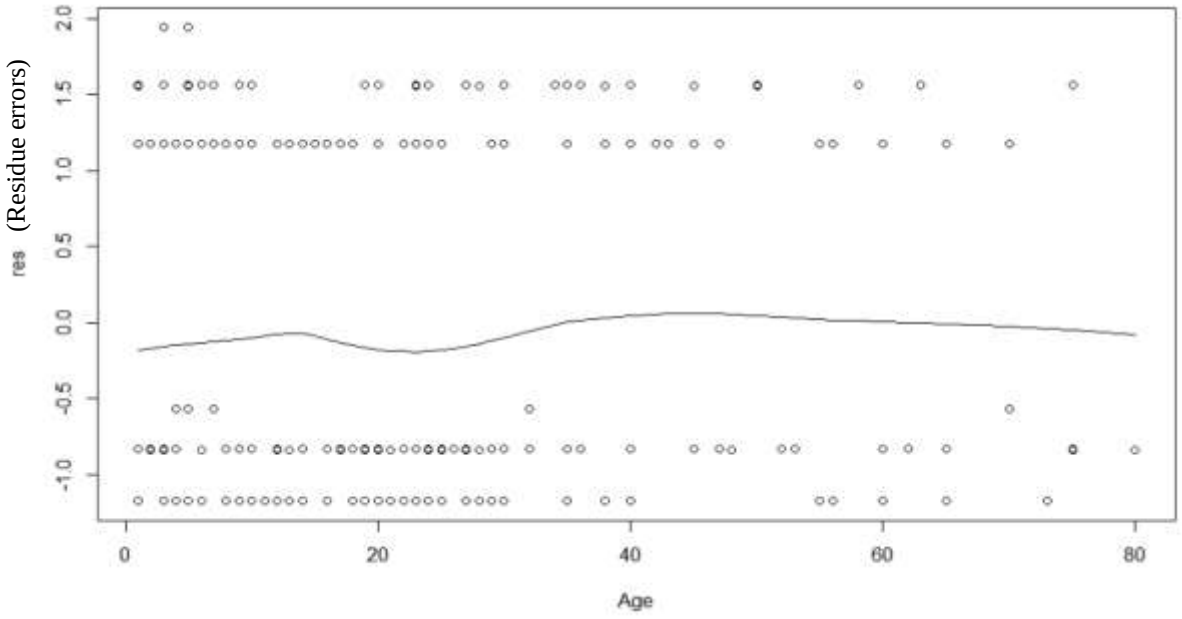


**Figure 13.** Linearity test of age and detection of toxin genes in improved water samples.

### 4.4. Association between toxins and risk factors of contamination

The univariable Chi-square ($\chi 2$) and Fisher's exact tests showed that the types of water source (P=0.001), using a cloth filter for water treatment (P=0.004), animal waste (P=0.044) and human waste (P=0.006) observed near homes and/or water sources, and TC (P=0.001) were statistically significantly associated with the detection of at least one water toxin. Water source type, use of a cloth filter, and TC were also statistically significant variables with the detection of at least one toxin using multivariable logistic regression. The results also showed that the use of a shallow well reduced the chances of

finding toxins in the water by 13%, compared to the use of piped systems connected to yards (P = 0.031).

A lower proportion of toxins was detected in relation to those who did not know and used cloth filters than those who knew about 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 and practice cloth filter treatment. Incorrect use of the cloth filter 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 toxins (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 detected TC 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 but not to *E. coli* or TTC, suggesting that TC are related to pathogens (Wu et al. 2011). This is similar to the concept of *E. coli* genomes in whole, pan and core genomes; of 15,741 gene families, only six *E. coli* strains are human and animal feces-related pathogens, which means the majority of *E. coli* strains are non-pathogens (Lukjancenko, Wassenaar, and David 2010). TTC counts were not significantly associated (P = 0.185) with the presence of one or more biomarkers of toxins, although toxins were detected in a high proportion of the

samples. This supports a previous study showing that $10^7$ number per gram of feces of TTC are *E. coli*, but mostly nonpathogenic (WHO, 2011) and TTC are not associated with diarrhea (Gruber et al. 2014). Furthermore, there was no association between TTC, *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). As a result, the presence of markers like TTC and *E. coli* does not necessarily indicate a connection to pathogens and only small groups are pathogens (US-FDA, 2012).

In this study, human and animal waste observed near homes and improved water sources were not statistically associated with toxin detection in water samples, which may be due to greater influence by other variables (Table 9). While 142 respondents – more than half – reported animal waste and 114 (46%) human waste were observed within 10 meters of houses or water sources (Table 7). This result contradicts those of studies that confirm that human and animal waste are equally important contaminants that affect water quality (Ercumen et al. 2017; Goma et al. 2019; Harada et al. 2018). A study in Egypt found a link between *E. coli* toxin genes and cattle waste (Ahmed et al. 2020; Goma et al. 2019; Tahamtan, Hayati, and Namavari 2010). This variation may arise from the storage of animal waste, which gradually reduces the detection of bacterial toxin genes in rural and peri-urban areas where animal waste is a common fertilizer and household energy source (Avery, Killham, and Jones 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 data on water quality when the data and samples were

collected. Therefore, the free residual chlorine results were used to determine whether the water sources were managed safely as recommended by WHO/UNICEF (WHO/UNICEF 2017). Due to this, the number of groups in the category of 'safely managed' may not be accurate.

**Table 9.** Distribution and probability of occurrence of 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 between factors (Chi-square test)** | | | | | | | **Probability of occurrence of toxins between 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 | | | | |
| | Child 5-18 | 58 | 24 | 41 | 0.46 | 0.794 | | |
| | Adult >18 | 136 | 53 | 39 | | | | |
| Water source | PS-Yard | 108 | 55 | 51 | | | Ref. | |
| | Dug well | 24 | 8 | 33 | | | 2.50 [0.92, 6.76] | 0.071 |
| | Shallow well | 30 | 4 | 13 | 19.66 | 0.001* | 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 | 0.422 | | |
| | Basic | 149 | 62 | 42 | | | | |
| | 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, | 0.712 |

| Distribution of occurrence of one or more toxin/s between factors (Chi-square test) | | | | | | | Probability of occurrence of toxins between risk factors (logistic regression) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2.35] | |
| | 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 | |
| | 1 – 84*** | 98 | 43 | 44 | | | 0.62 [0.32, 1.20] | 0.163 |

*$p$-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. The type of water source and the total coliform counts were positively associated with at least one detected water toxin. Knowledge of cloth filters was negatively associated with at least one detected water toxins.

***Results of total coliform and thermotolerant coliforms in colony-forming unit (CFU) count per 100 ml of water sample.

# Chapter 5: Summary result of objective 2: Identify gaps between water quality and the global classification of improved water supplies by evaluating the basic water quality parameters of 'improved' water supplies.

## 5.1. The importance of water quality in classifying basic water services: The case of Ethiopia, SDG6.1, and safe drinking water

Currently, water quality is not considered a parameter under the classification of 'functional improved basic water'. Water quality is a neglected topic in global debates, and quality is not given the due attention as it should when it comes to basic water services (Satterthwaite 2016; WHO/UNICEF 2017). Preserving the quality of basic water services, to which most people in developing countries have access, is important for better use of drinking-water supply and decreased morbidity. The water samples for lab analysis were taken from the sources of functional basic water services, and for the nonfunctional 24 (21.62%) of the water services, the samples for lab analysis were taken from the sources, storages, or pipelines. In laboratory analysis, measured colony forming counts per 100 ml of water samples for **bacteriological tests** of total coliforms (TC) were detected in 46 (41.44%) taken from basic water services. Of which 40 (36.03%) of basic water services are functional and the remaining 6 (5.41%) were not functional.

The other test conducted from the priority bacteriological tests is the thermotolerant coliforms (TTC). The presence of TTC serves as an indicator of water contamination by human or animal fecal matter. TTC was detected in 44 basic water services (39.64%) of which 38 (34.23%) are operationally 'functional' and 6 (5.41%) are not functional (Table

10). Therefore, TC for about 41% and TTC for nearly 40% of water services were above the acceptable standard values, which is 0 CFU/100 ml.

**Table 10.** Water quality parameters versus services functionality in the Amhara and Afar regions.

| | | **Water services** | | |
|---|---|---|---|---|
| **Parameters** | **Classifications** | **Functional** | **Non-functional** | **Total n (%)** |
| **Total Coliform** | Meets standard of 0 CFU/100ml | 47 (42.34) | 18 (16.22) | **65 (58.56)** |
| | Above standard of 0 CFU/100ml | 40 (36.03) | 6 (5.41) | **46 (41.44)** |
| **Thermotolerant Coliform** | Meets standard of 0 CFU/100ml | 49 (44.14) | 18 (16.22) | **67 (60.36)** |
| | Above standard of 0 CFU/100ml | 38 (34.23) | 6 (5.41) | **44 (39.64)** |
| **'pH'** | Below the standard – 6.5 | 1 (0.90) | 1 (0.90) | **2 (1.80)** |
| | Meets standard of – 6.5-8.5* | 47 (42.34) | 17 (15.32) | **64 (57.66)** |
| | Above the standard – 8.5 | 39 (35.13) | 6 (5.41) | **45 (40.54)** |
| **Turbidity** | Meets standards of less than 5 NTU | 83 (74.78) | 24 (21.62) | **107 (96.40)** |
| | Above standard greater than 5 NTU | 4 (3.60) | 0 (0.0) | **4 (3.60)** |
| **Residual Chlorine** | Below the standard – 0.2 mg/lit | 86 (77.48) | 23 (20.72) | **109 (98.20)** |
| | Meets standard – 0.2-0.5 mg/lit* | 1 (0.90) | 0 (0.0) | **1 (0.90)** |
| | Above the standard – 0.5 mg/lit | 0 (0.0) | 1 (0.90) | **1 (0.90)** |

* WHO drinking water quality guideline recommended standard.

Although the international water standard for thermotolerant coliform (TTC) levels is 0 CFU/100ml, in our sample of 111 water services, the maximum TTC counts were 71

CFU/100 ml and the mean was 4 CFU/100 ml. Thermotolerant coliform counts were above the acceptable standard values for nearly 40% (n = 111) of basic water services. TTC was detected in 44 (39.64%) (n = 111) basic water services. Of these, 38 (34.23%) were operationally functional, while 6 (5.41%) were not functional. Approximately one third of the basic water services sampled, deemed 'functional' by international standards, do not provide *potable* water due to the levels of thermotolerant coliform (TTC).

## Chapter 6: Summary result of objective 3: Evaluation of toxins in improved water in households and in the stool of patients with diarrhea.

Human feces are a potential risk contaminant for improved water supply in water sources or in household water storage, where pathogenic bacteria such as enteric toxins that cause diarrhea and gastroenteritis thrive (Bonkoungou et al. 2021). Most of this transmission is believed to occur in Ethiopia as a result of feces polluting water supplies along their routes up to home consumption (Melake et al. 2013; Seid et al. 2018). In this study, the stool of each patient was matched with their household water samples in a 1:1 match. Overall, of the 248 households, 24% (59) have positive results in both water and stool samples, 63% [CI: 55- 67%] have positive results in water samples only, and 46% [CI: 37- 49%] were found to be positive for toxins in stool samples only. The EAST1 toxin was detected in most stool samples (38% (n = 95), in water samples 33% (n = 82) and in both 21% (n = 52). The LT toxin was detected in a small proportion (1.21%) in both water and stool samples (Table 11). EAST1 was found to be the enterotoxin most commonly detected in both stool and water samples. This high rate of occurrence could be due to the fact that the toxin encoding EAST1, unlike other toxins, originates in three pathotypes namely; enteroaggregative *E. coli* (EAEC), enterohemoregic *E. coli* (EHEC), and enteropathogenic *E. coli* (EPEC) (Savarino et al. 1996). As a result of its dominant presence in the stool and/or the fact that it is more easily detected, this result suggests that EAST1 in the stool of the diarrhea patient is a significant contaminant of stored household drinking water from improved water sources. The detection rate of toxins in stool samples from people who used groundwater sources (protected dug wells with pumps and protected shallow wells with pumps) was much higher than the detection rate

of people who used piped distribution (yard connection and public stand) and on spot springs. This variation could be due to socioeconomic status factors for these households, the fact that groundwater sources with hand pumps are commonly located far from houses, requiring long travel to fetch, and might have been exposed to contamination during transportation, and the exposure of water collection and storage vessels to contamination.

A significant association was found between toxins in the stool of diarrheal patients and in their drinking water (P= 0.000) taken from improved water storage at home. This finding is similar to a study in Lagos, Nigeria, which showed the relationship between *E. coli* toxins (Stx1, Stx2, and LT) in water samples (n = 117) from different sources and stool samples (n = 91) in the same localities (Igbokwe et al. 2015). Furthermore, in Gwangju Metropolitan City, South Korea, an association of enteric *E. coli* toxins in improved groundwater water sources with patients' stools was reported (Park et al. 2018). This similarity in results suggests that toxins in human feces and stored household drinking water have a strong relationship.

**Table 11.** Association of toxins detected by mPCR in stool and water samples.

| **Type of Toxins** | **Detected samples in stool (n) (%)** | **Detected samples in water (n) (%)** | **Detected samples in water & stool (n) (%)** | **Toxins combinations in stool & water (n) (%)** | | **X2** | ***P*-value** |
|---|---|---|---|---|---|---|---|
| EAST1 | 95 (38) | 82 (33) | 52 (21) | One | 13 (25%) | 32.67 | 0.000* |
| Stx1 | 5 (2) | 33(13) | 4 (2) | Two | 20 (38%) | 19.67 | 0.001* |
| Stx2 | 0 (0) | 21 (9) | 0 (0) | Three | 13 (25%) | - | - |
| Sta | 5 (2) | 6 (2) | 0 (0) | Four | 5 (10%) | 0.1265 | 1.000 |
| LT | 10 (4) | 14 (6) | 3 (1) | Five | 1 (2%) | 116.039 | 0.001* |
| **Total** | **115 (46%)** | **156 (63%)** | **59 (24%), N** = 248** | **N*** = 52 samples** | | | |

* The association is significant at the 0.05 level.

** N is equal to the total water and stool samples, which is 248 each.

*** N is equal to the combination of water and stool samples that are positive for toxins, which is 52.

Toxins in stool and water samples in South Wollo, Ethiopia. The result shows the total detected rate for each toxin and the total sum. EAST1 - enteroaggregative *E. coli* heat-stable enteric toxin 1, Stx1 & Stx2 - shiga producing toxin 1 and 2, Sta - heat-stable toxin and LT - heat-labile toxins.

In stool samples, 4% were positive for LT, 2% for Stx1, and 2% for Sta toxins. On the other hand, 13% of the water samples were positive for Stx1, 9% Stx2, 6% LT, and 2% Sta. The prevalence rate of toxins in children under five years of age (female 37% and male 63%) was found to be 45% (24) in their stool and 43% (23) in the water they drink. The detection rates in both the stools and the water were closer to each other. The age-wise proportion analysis showed that 54% of the toxins were detected in stool aged 5 to 18 years with a sex composition of (female 42% and male 58%) and 75% toxins in the water samples, indicating a higher prevalence of toxins in water than in the stool (Table 12).

The prevalence of toxins was found to be 45% in children under age of five from stool samples, which is nearly similar to the prevalence of 43% in the water they drink. This may be the case in addition to the use of stored household drinking water contaminated by poor hygiene practices in children. Similar results were reported in a study conducted in Europe, Sweden, using the multiplex PCR (mPCR) technique in stool (Sjoling Asa, Sadiphipoorjahromi Leila, Daniel Novak 2015). Furthermore, in a study of residential water and stool samples from healthy volunteers in a defined geographic area of Germany and India, no intestinal *E. coli* pathogens were found in the water isolates, and less than 5% of the stool samples contained pathogenic *E. coli* toxins (Mühldorfer et al. 1996; Singh et al. 2019). These non-detection and lower rate of detection of toxins in Germany could be attributed to stool samples taken from healthy volunteers and sample size difference (131 stool samples and 95 water samples).

In South Africa studies, none of the Stx1 and Stx2 in water samples (n = 40 and n=228) and 1 (0.05%) and 15 (5.8%) in stool samples (n = 20 and n=252) were detected using

PCR (Ateba and Mbewe 2011; Obi et al. 2004). The absence of the Stx2 gene in stool samples in this study could probably be due to adapted immunity to Stx2 toxin in the study area or a difference in the Stx2 toxin that harbors not susceptible *E.coli* and resistance to antibiotics, unlike the study reported by Iranian and Egyptian studies (Ahmed et al. 2020; Pouladfar et al. 2017). This finding was supported by an antimicrobial resistance (AMR) test performed on bacterial isolates in a few selected stool samples using four types of antibiotic discs that indicated bacterial susceptibility and resistance to antibiotics (Annex 5.1). The other reason could be other sources of water contamination, which were confirmed in different studies, concluding that a quarter of African countries reported isolation of shiga toxin-producing *E. coli,* either from animal waste or raw and/or cooked but contaminated food by cross-contamination (transfer of bacteria from those items into the water) (Fagan et al. 1999; Lupindu 2018).

**Table 12.** *E. coli* toxins detected in stools and water samples by age category

| | | | Toxins in stool | | | | | | | | | | | | Toxins in water | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age*** | **Age sum** | | **EAST1** | | **Stx1** | | **Stx2** | | **Sta** | | **LT** | | **Total** | | **EAST1** | | **Stx1** | | **Stx2** | | **Sta** | | **LT** | | **Total** | |
| **category** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** | **n** | **%** |
| < 5 years | 54 | 27.8 | 21 | 38.9 | 1 | 1.9 | 0 | 0 | 1 | 1.9 | 1 | 1.9 | 24 | 44.6 | 17 | 31.5 | 3 | 5.6 | 2 | 3.7 | 0 | 0.0 | 1 | 1.9 | **23** | **42.7** |
| Young 5 - 18 years | 59 | 30.4 | 26 | 44.1 | 3 | 5.1 | 0 | 0 | 1 | 1.7 | 2 | 3.4 | 32 | 54.3 | 21 | 35.6 | 8 | 13.6 | 4 | 6.8 | 3 | 5.1 | 8 | 13.6 | **44** | **74.7** |
| Adult > 18 years | 135 | 69.6 | 48 | 35.6 | 1 | 0.7 | 0 | 0 | 3 | 2.2 | 7 | 5.2 | 59 | 43.7 | 44 | 32.6 | 22 | 16.3 | 15 | 11.1 | 3 | 2.2 | 5 | 3.7 | **89** | **65.9** |
| **Total** | **194/248** | **100** | **95** | **38** | **5** | **2** | **0** | **0** | **5** | **2** | **10** | **4** | **115/248** | **46** | **82** | **33** | **33** | **13** | **21** | **9** | **6** | **2** | **14** | **6** | **156/248** | **63** |

* The age category is under five years of age, adolescents and adults who were patients with diarrhea visited health facilities in South Wollo, Ethiopia.

The combination of toxins detected both in stool and in water samples was calculated using detected toxins. A single toxin was detected in 20 samples, double toxins in 13 samples, triple toxins in 13 samples, four toxins in five samples, and five toxins in one sample. Of 52 samples found toxins in both stool and water, 2%, 10%, 25%, and 25% were positive for five, four, three and two toxins, respectively. The remaining 38% were tested positive for a single toxin per sample (Table 11).Three or more toxins were detected simultaneously in 37% of the (positive) samples in our study, while in a study conducted in Libya, 50% of the toxins were found to be three or more (Ali et al. 2012). Variations in the level of detection of toxin *E. coli* could be explained by differences in population density and socioeconomic characteristics such as occupation and household income (Healy-Profitós et al. 2016).

The type of water sources and toxins detected in these water samples and stools were analyzed. Of 248 stool and water samples, at least one or more toxins were detected in 41% (102) and 39% (96) of stool and water samples, respectively. 22% (55) of the piped water sources connected to households and 21% (51) of the stool samples collected from those who use these piped systems were found to be positive for toxins (Table 13).

**Table 13.** *E. coli* toxins detected in stool and water samples in South Wollo, Ethiopia, by water sources.

| **Water source types*** | **Water sampled** | | **S-toxins** | | **W-toxins** | |
|---|---|---|---|---|---|---|
| | **n** | **%** | **n** | **%** | n | % |
| PS- connected to yard/household | 108 | 44 | 51 | 21 | 55 | 22 |
| PS-connected to public stand | 53 | 21 | 20 | 8 | 22 | 9 |
| Protected on SS | 33 | 13 | 7 | 3 | 7 | 3 |
| Protected SW-HP | 30 | 12 | 12 | 5 | 4 | 2 |
| Protected DW- HP | 24 | 10 | 12 | 5 | 8 | 3 |
| **Total** | **248** | **100** | **102** | **41** | **96** | **39** |

* PS-piped system, SS-spot spring, SW-HP-shallow well fitted with hand pump, DW-HP-dug well fitted with hand pump. S-stool and W-water. n-the number of water sampled, S-toxins-toxins detected in stools of the water users and W-toxins are toxins detected in the water samples.

Detection of Stx1 and Stx2 in stool and improved water samples were highly varied compared to EAST1, Sta, and LT. The total number of toxins detected in the stool 46% (115) was less than the toxins detected in the water 63% (156) as shown in Table 11.

According to the Chi-square test of the distribution of toxins in water and stool among different factors, a statistically significant difference (P=0.001) was observed between the five water sources included in this study. The prevalence of toxin in household connections was higher than that obtained from yard connections (51%) and piped systems connected to public stands (42%) compared to the prevalence in other types of water sources. Additionally, the highest number of toxins (50%) was detected in stool samples from those using protected hand dug wells. 47% of toxins were detected in stool

samples from diarrhea cases using yard connections. However, there was no statistically significant variation (P>0.05) in toxin detection between stool toxins with respect to the type of water source (Table 14). Diarrheal toxins in the stool were statistically associated (P=0.0001) with toxins found in the water. The target toxins (ESAT1, Stx1, and LT) found in the stool were also found in high proportions in the water. Only the Stx2 virulence gene was detected in water at a lower rate of 9% (21) (Table 11).

In this study, as indicated in Table 11, the total amount of detectable toxins in water was higher than in the stool (Table 11). The highest detection rate in water in the current study differed from studies in Germany and South Africa that found the highest detection rate in stools (Mühldorfer et al. 1996; Obi et al. 2004). This could be attributed to the fact that the water could be exposed to additional contaminants originating from other sources than directly from the stool.

**Table 14.** Distribution of the water and stool toxins across factors (sex, age and water source type)

| | | Water toxin | | | | | Stool toxin | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Category** | **Tested (n)** | **Detected** | **Prevalence (%)** | **$X^2$** | ***p*-value** | **Tested (n)** | **Detected** | **Prevalence (%)** | **$X^2$** | ***p*-value** |
| Sex | Female | 117 | 47 | 40.17 | 0.19 | 0.655 | 117 | 52 | 44.44 | 1.00 | 0.316 |
| | Male | 131 | 49 | 37.40 | | | 131 | 50 | 38.17 | | |
| Age | Adult | 136 | 53 | 38.97 | 0.46 | 0.794 | 136 | 53 | 38.97 | 0.96 | 0.61 |
| | Medium | 58 | 24 | 41.38 | | | 58 | 27 | 46.55 | | |
| | Small | 54 | 19 | 35.19 | | | 54 | 22 | 40.74 | | |
| Water source | Protected dug wells | 24 | 8 | 33.33 | 19.66 | 0.001** | 24 | 12 | 50.00 | 8.11 | 0.088 |
| | Protected shallow wells | 30 | 4 | 13.33 | | | 30 | 12 | 40.00 | | |
| | Protected on spot spring | 33 | 7 | 21.21 | | | 33 | 7 | 21.21 | | |
| | PS-yard | 108 | 55 | 50.93 | | | 108 | 51 | 47.22 | | |
| | PS-public stand | 53 | 22 | 41.51 | | | 53 | 20 | 37.74 | | |

** The association is significant at the 0.01 level.

Logistic regression analysis showed a statistically significant association between toxin in stool and water (P= 0.000). The presence of EAST1 toxin in stools increased the odds of EAST1 occurrence in water (OR=4.96, 95% CI, 2.81–8.74; P=0.000). The odds of Stx1 in water increased significantly with the odds of Stx1 in the stool (OR=29.52, 95% CI, 3.18–273.24; P=0.003). The presence of LT toxin in the stools increased the odds of occurrence of LT in the water (OR=8.84, 95% CI, 2.01–38.21; P=0.004) (Table 15).The findings of our study contradict those of a study conducted in Tanzania, which found that the presence of *E. coli* toxins in water samples was associated with a significant decrease in the risk of childhood diarrhea (OR = 0.51; 95% CI: 0.27–0.93) using mPCR (Mattioli et al. 2014). The presence of EAST1 and Stx1 in the stool increased the probability of the occurrence of EAST1 and Stx1 in the water (OR=4.96) and (OR=29.52), respectively, as indicated in (Table 15). This implies that EAST1 and Stx1 toxins are the main contaminants, originating in the stool. This variation in results could have occurred due to differences in water management and hygiene behaviors in households. Another reason could be due to the development of adaptive immunity by children to *E. coli* toxins (Seid et al. 2018).

**Table 15.** Odds of the occurrence of water toxins in stool samples.

| **Toxins** | **Category** | **OR [95% CI]** | ***P*-value** |
|---|---|---|---|
| EAST1 | No | Ref. | |
| | Yes | 4.96 [2.81, 8.74] | 0.000** |
| Stx1 | No | Ref. | |
| | Yes | 29.52[3.18, 273.24] | 0.003** |
| LT | No | Ref. | |
| | Yes | 8.84 [2.01, 38.21] | 0.004** |
| Water sources | On Spot spring | Ref. | |
| | On spot shallow well | 0.31 [0.08, 1.18] | 0.088 |
| | Protected dug well | 0.53 [0.16, 1.77] | 0.308 |
| | PS-Yard | 2.07 [0.82, 5.25] | 0.123 |

| | | |
|---|---|---|
| RPS-Public | 1.41 [0.52, 3.89] | 0.496 |

** The association is significant at the 0.01 level.

The correlation coefficient analysis between toxins detected in stool and water samples is presented in Table 16. Pearson's correlation coefficients were calculated to determine the possible associations between the detection of toxins in stool and water samples. The correlation coefficient analysis result showed a positive correlation between overall toxins detections (one or more) in the stools and in the water. A significantly positive correlation, at $P< 0.05$ were found between toxins (one or more) in the stool and toxins (one or more) in water ($r = 0.412$). For example, EAST1 in the stool to general toxins (one or more) in water ($r = 0.378$), EAST1 in the stool to EAST1 in water ($r = 0.363$) were significantly related (Table 16).

A significantly positive correlation was found between toxins (one or more) in the stool of diarrheal patients and toxins (one or more) in their drinking water ($r = 0.412$) at $P < 0.05$ could indicate the higher probability of diarrheal disease occurring as a result of contamination of the water with toxins sourced from human feces (Mattioli et al. 2014; Savichtcheva et al. 2007). However, *stx2*, which is a significant contributor to improved water contamination, was not detected in stool samples.

**Table 16.** Correlation coefficients between toxins detected in stool and improved water samples.

| Parameters | S_toxin | S_EAST1 | S_Stx1 | S_Stx2 | S_Sta | S_LT | Age | W_toxin | W_EAST1 | W_Stx1 | W_Stx2 | W_Sta | W_LT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **S_toxin** | 1.00 | | | | | | | | | | | | |
| **S_EAST1** | **0.943**** | 1.00 | | | | | | | | | | | |
| **S_Stx1** | 0.172 | 0.005 | 1.00 | | | | | | | | | | |
| **S_Stx2** | .[b] | .[b] | .[b] | .[b] | | | | | | | | | |
| **S_Sta** | 0.172 | 0.182 | -0.021 | .[b] | 1.00 | | | | | | | | |
| **S_LT** | 0.245 | 0.091 | 0.116 | .[b] | 0.116 | 1.00 | | | | | | | |
| **Age** | 0.022 | -0.016 | -0.066 | .[b] | -0.011 | 0.060 | 1.00 | | | | | | |
| **W_toxin** | **0.412*** | **0.378*** | 0.180 | .[b] | 0.063 | 0.090 | 0.022 | 1.00 | | | | | |
| **W_EAST1** | 0.353 | **0.363*** | 0.082 | .[b] | 0.082 | 0.030 | 0.012 | **0.884**** | 1.00 | | | | |
| **W_Stx1** | 0.227 | 0.180 | 0.282 | .[b] | 0.113 | 0.101 | 0.082 | **0.493**** | 0.305 | 1.00 | | | |
| **W_Stx2** | 0.217 | 0.207 | 0.059 | .[b] | 0.266 | 0.085 | 0.137* | **0.383*** | 0.217 | **0.393*** | 1.00 | | |
| **W_Sta** | 0.135 | 0.092 | 0.164 | .[b] | -0.023 | 0.101 | -0.049 | 0.198 | 0.057 | 0.093 | 0.046 | 1.00 | |
| **W_LT** | 0.222 | 0.167 | 0.338 | .[b] | 0.089 | 0.216 | -0.051 | 0.308 | 0.125 | 0.264 | **0.365*** | 0.303 | 1.00 |

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

b. Cannot be computed because Stx2 is not detected in the stools.

## Chapter 7: Synthesis

This Ph.D. dissertation report on 'Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia' has answered four questions. The first work targeted the evaluation of toxins as biomarkers of water quality. Safety of improved water supplies using toxins as a biomarker is evaluated. Water samples from improved water sources at the households were tested for toxins using polymerase chain reaction (PCR). The toxins targeted in this study fall under four diarrhea pathotypes out of six diarrheagenic *E. coli* pathotypes. Namely; enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and enterotoxigenic *E. coli* (ETEC) (Hitchins et al. 2001; Trung et al. 2005; US-FDA 2012). 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 toxins. The type of water source, cloth filter water purification and TC were significantly associated with at least one toxin detected. In this particular work it was concluded that toxin biomarkers can be used to monitor water safety. The detail can be referred in a published article on a journal called ***Water and Practical Technology***, which is the International Water Associations (IWA) publishing titled 'Enterotoxins as a biomarker of water quality'.

The second objective of this work targeted the identification of gaps between water quality and the global classification of improved water supplies by evaluating the basic water quality parameters of 'improved' water supplies. Approximately one third of the basic water services sampled were deemed "functional" and "safe" by international standards, do not provide potable water due to thermotolerant coliform (TTC) levels. The detail about this work was elaborated in an article published in a journal ***PLoS One*** titled

‘The importance of water quality in classifying basic water services: The case of Ethiopia, SDG6.1, and safe drinking water’. World Health Organization (WHO)/United Nations Children’s Fund (UNICEF) joint monitoring programme (JMP) coordinate international efforts toward improving the lives of people around the globe using Sustainable Development Goals (SDGs). SDG 6 broadly outlines “clean water and sanitation” while target 6.1 specifies: “By 2030, achieve universal and equitable access to safe and affordable drinking water for all” (WHO/UNICEF 2017). Yet achieving safe drinking water in developing countries and resource-poor environments requires sustained effort. In contribution, we demonstrate that current international standards around improved water have not properly specified standards around water quality. The SDGs classify improved household drinking water based upon the updated World Health Organization (WHO)/United Nations Children’s Fund (UNICEF) joint monitoring programme (JMP) ladders of drinking water services (WHO/UNICEF 2017). This classification does not, we argue, properly account for “safe” drinking water in improved water services. The inclusion of a fecal indicator bacteria (FIB) as part of water quality in the commonly-accepted definition of basic water (WHO/UNICEF 2017) is a gap that obscures the risk of diarrheal diseases from water services classified as both improved and functional.

The third objective evaluated the relationship between enteric *E. coli* toxins/enterotoxins detected in **‘improved’ drinking water** sources and toxins found in **stool samples from individuals with diarrhea** that consumed the water. *E. coli* isolates were analysed for 5 major toxins (enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1), heat-stable (Sta); shiga-like toxin 1 (Stx1), shiga-like toxin 2 (Stx2), and heat-stable (LT)) using a multiplex polymerase chain

reaction (mPCR). A positive correlation and a significant association was observed between toxins in drinking water from improved sources and those in patient stools. Enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) was most frequently detected in both drinking water from improved sources and in patient stools. ESAT1, Stx1, and LT toxins were the most pertinent contaminants found in household drinking water from improved sources. See the detail in a manuscript accepted and under revision for the second time in a journal Environmental Challenge titled 'Diarrheagenic toxins in stool correlate to drinking water from improved water sources in Ethiopia'.

The final objective focused on mapping of pathogens detected by the PCR-based method in improved water supply in low-middle-income countries (LMICs). We designed this work to address in scoping review using existing literatures. Thus, this work is divided into two phases; first publishing a scoping review protocol and secondly, working the scoping review. Exploring water quality outcomes generated through PCR-based methods is important to better understand the status and monitor progress towards internationally set goals for LMICs. This scoping review aims to map the existing evidence on the magnitude and characteristics of diarrheagenic pathogens as detected by PCR-based methods in improved water sources within the context of LMICs. The review protocol titled 'PCR-based detection of pathogens in improved water sources: a scoping review protocol of the evidence in low- and middle-income countries' was published on British Medical Journal (BMJ) (see the attached published article Annex-4). The next way forward in this work would be finalizing the publications of those two manuscripts under pipelines.

## Chapter 8: Conclusions

One or more *E. coli* toxins were detected in 39% of samples taken from improved water supplies at the household level. The toxin encoding EAST1 was the most detected, while Sta was the least prevalent. The type of water source, the use of a cloth filter for water treatment, and TC were significantly associated with the detection of at least one toxin biomarker in improved water. As a higher number of toxins were detected in the stored drinking water of the household fetched from the surface than from groundwater sources, it is obvious that improved water from surface sources are more prone to contamination.

A significant positive correlation was observed between toxins in drinking water and those found in stools of individual households. There is evidence that drinking water contributes to diarrhea cases and human feces contribute to drinking water contamination (EAST1, Stx1 and LT toxins). The absence of Stx2 and Sta in human feces suggests that other factors may also play a role in diarrhea such as viral infections or other toxins. Even though water supplies are categorized as “improved” they may still be contaminated during transport and storage. *E. coli* toxins have been found to be effective biomarkers for detecting possible drinking water contamination in households with diarrhea.

Our findings demonstrate that water quality parameters are not currently considered when classifying basic water services.

## Chapter 9: Recommendations

Biomarkers for the targeted *E. coli* toxins can be used for regular water safety monitoring and development of water safety strategies. In addition, *E. coli* toxins may act as reliable biomarkers for spotting potential water contamination, which may help to prevent, treat and control water related diarrheal illnesses. Long-term planning for the protection and/or treatment of stored household drinking water to ensure toxins free water at the point of use are required.

Working toward a safely managed household water category that includes a water quality parameter is critical for ensuring the safety of household water. More attention should be paid to water quality monitoring and treatment measures for water fetched and transported from surface water sources (piped system connected in the yard, public stand, and on spot springs) than groundwater sources (protected dug wells with pumps and protected shallow wells with pumps).

It is suggested that international efforts to address SDG 6 should incorporate water quality as a key parameter to better track international progress towards 'clean water and sanitation' efforts. We discuss two potential pathways for the inclusion of water quality parameters in international definitions: (1) to include water quality criteria within 'functional' and 'nonfunctional' definitions or (2) to add a ladder rung titled 'safe basic water services' to the international drinking water ladder, or revise the definition of improved water supply as following; i) Safely managed: accessible on premises, available when needed and free from fecal and priority chemical contamination (keeping the existing definition as it is), ii) Basic: free from fecal and priority chemical contamination, round trip $< 30$ minutes, and iii) Limited: free from fecal and priority chemical contamination, round trip $> 30$ minutes.

| | Functional Basic Water Service | Non-functional Basic Water Service |
|---|---|---|
| **SDG Definition** | If the water from improved source does not meet any **one** of the three criteria (source is accessible on premises, water is available on demand, water is free from contamination and a round trip to collect water takes 30 minutes or less including queuing.<br><br>*Water quality optional* | If the water service from improved source fails to fully operate or only partially operates at time of survey.<br><br>*No water quality priority parameter considered* |
| **Suggested Revision** | If the water from improved source is free from contamination of priority parameters (TC and Toxins/*E. coli*), **and** it is not on premise **or** available on demand, **and** a round trip to collect water takes 30 minutes or less including queuing.<br><br>*Priority water quality parameter mandatory* | If water service is fails to fully operate or only partially operates at time of survey**or** is not free from priority contaminants (TC and Toxins/*E. coli*).<br><br>*Priority water quality parameter mandatory* |

The global sector actors leading the Joint Monitoring Programme (JMP) must consider the revision suggested in number 3 above and nations has to push and implement the revision. UNICEF has taken the recommended revision of the definitions as a result of proper dissemination of the findings, which would further be a contribution to influencing the global community.

Our findings from Ethiopia suggest that additional research should be carried out in development contexts to assess whether 'functional' basic water services provide safe drinking water to users. More studies are recommended to explore the sources of pathogenic microbial markers that cause water-related diseases such as diarrhea and help identify the focus areas of the prevention of water contamination, as well as to investigate the influence of demographic and socioeconomic factors on water quality via microbial

risk assessments. Moreover, future studies on toxins should incorporate contaminant factors that consider human feces, animal waste, food, and soil.

Display the application of PCR-based methods in the water sector for improved water quality monitoring by sharing findings using the Scoping Review (see the published protocol for the Scoping Review).

We developed article dissemination strategy and distributed the articles published and those in the pipelines. The distribution reached a total of 128 target sector actors from Policymakers in government, Ministry of Water, Irrigation and Energy (MoWIE) & Ministry of Health (MoH), Bilateral & Multilateral donors- DFID, ADB, UNICEF, WB, EU. Non-governmental organizations (NGOs)- CO-WASH, WaterAid Ethiopia (WAE), SNV, PLAN International, Save the Children (SCI), World Vision International (WVI). Universities and research institutes of scholars and water management bodies. Further, the dissemination strategy included, 275 and 171 friends in the social media such as Facebook and LinkedIn respectively (see the detail in Annex 8. Article dissemination strategy).

Due to the strong dissemination effort, the publications results were broad casted in an International Well known New Paper called *The New York Times*. This is believed to influences the global community interested in the subject matter for up taking the findings and addressing.

This research is relevant for bilateral, multilateral donors, policy makers and researchers because it helps to understand the context of drinking water quality in LMICs such as Ethiopia and recommends that water quality be included in the drinking water basic ladder or improved definition. It adds value to the development and revision of organizational water quality strategies and fills gaps in the definition of improved water supply classification. It lays the groundwork for future additional research to assess whether or not “functional” basic water services provide safe drinking water to users and consider monitoring of toxins using PCR. It improves the situation of

water quality on the ground and places an emphasis on monitoring and reporting water quality in a consistent manner in order to meet the "clean water for all" initiative. This means that Ethiopian statistics around access to water and water services do not properly capture the safety of water in our rural, peri-urban and urban areas. If we collect this data, we could more effectively and efficiently target water services for the communities. We demonstrated how poor water quality leads to increased diarrheal disease, which negatively impacts Ethiopian life and decreases our productivity as a nation.

## Limitations

The limitation of this study was that, despite the exclusion criteria applied to eliminate the possibility of other causes of water contamination such as malnutrition, fetching water from unprotected sources, cross-contamination and animal waste to the detection of toxins could not be entirely avoided, as it is uncontrollable due to its complex transmission and contamination routes (Navab-Daneshmand et al. 2018). The major issue is that water analyzed in the household is not the one consumed by the patients when they became ill. Microbiological contamination can vary over time.

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## Operational Definitions

**Diarrhea** is defined as the passage of three or more loose or liquid stools per day or more frequent passage than is normal for the individual. It does not include frequent passing of stools by breastfed babies. In addition, the clinical definition at one of the referral hospitals that found in the study area as cascaded by the Ministry of Health (MoH) in Ethiopia is; diarrhea is watery stools that manifests in less than 14 days and savior dehydration is called watery diarrhea with disease code of 105. A diarrhea type called, dysentery is with the manifestation in greater than 14 days and persistent (with bloody) diarrhea that is caused from unknown sources with disease code of 106 and diarrhea type of acute or persistent diarrhea, which is with non-bloody type with disease code of 104 (WHO 2017b).

**Improved drinking water** 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).There are three categories in the WHO/UNICEF Joint Monitoring Program for Water Supply and Sanitation (JMP) ladder for household improved 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).

**Membrane filter technique (MF):** International Organization for Standardization (ISO) standard title (water quality): ISO: 9308-1:2000 or Ethiopian Standard (ES) ES ISO 9308-1 Detection and enumeration of *Escherichia coli* and coliform bacteria. A technique that consists of filtering a water sample on a sterile filter with different pore sizes mostly recommended a 0.45 μm pore size using pressure that retains bacteria. Then incubating this filter on a selective medium and enumerating typical colonies on the filter by expressing in colony forming unit (CFU/100ml) (Grabow and du Preez 1979; Rice et al. 1987; WHO 2011).

**Polymerase Chain Reaction (PCR) method:** ISO 22118:2010 to the detection of pathogens in environmental samples. A simple chain reaction or technique in molecular genetics that permits the synthesis and analysis of short lengths of single-stranded sequence DNA (oligonucleotides)or RNA in samples containing only minute quantities of DNA or RNA by amplifying or reproducing selected sections of DNA or RNA for analysis (Joshi and Deshpande 2011). Previously, amplification of DNA involved cloning the segments of interest into vectors for expression in bacteria, and took weeks. Now, with PCR done in microfuge tubes, it takes only a few hours. The most frequently used nucleic-acid-based methods for coliform detection in drinking water are the PCR method (Hugenholtz and Tyson 2008).

**Safely Managed Drinking Water** is drinking water from an improved water source which is located on premises, available when needed and free of fecal and priority chemical contamination(WHO/UNICEF 2017).

**Water quality** as defined by WHO/UNICEF is an improvement and protection of the microbiological or chemical, such as fluoride and physical quality of drinking-water through water treatment and safe storage or by improving existing water sources to protect them from outside contamination at household or water source level (WHO/UNICEF 2015b).

**Water quality as defined in this research** is intervention to improve the primary microbiological parameter in terms of detection, enumeration and characterization of total coliform (TC), thermo-tolerant coliform (TTC) and toxins in comparison to the standard of WHO and Ethiopia Standards Agency (ESA).

## Biography/Profile

My name is Shibabaw Tadesse Gemeda. I am a PhD candidate in Water and Health with a specialization in Water and Public Health at Addis Ababa University, Ethiopian Institute of Water Resources, Addis Ababa Ethiopia. My dissertation research focuses on the 'Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia. Methods used were mixed and laboratory based cross-sectional 1:1 match studies using water samples from households that use water from improved sources and patient stools from a household member visit a health care facility due to diarrhea using digital polymerase chain reaction (PCR) techniques. I received my MSc in Water and Environmental Management from Loughborough University, WEDC, in the United Kingdom. I completed my BSc in Environmental Health Science at Jimma University, Jimma, Ethiopia. I am passionate about understanding the role of water and sanitation in Ethiopia development to improve people life/health in accessing clean water services. I held various positions with European Union-funded multi-sectoral project being a Key Expert for AMREF/CCM, WaterAid Ethiopia (WAE), Save the Children International (SCI) in WASH, Health and Nutrition, Food Security and Livelihood Projects and in governmental organizations. My future career objective is to be a competent professional in Africa's water, sanitation, and hygiene (WASH) sector, as well as an excellent researcher in the WASH and Health sectors, in order to contribute to the achievement of the UN Sustainable Development Goals' country commitments.

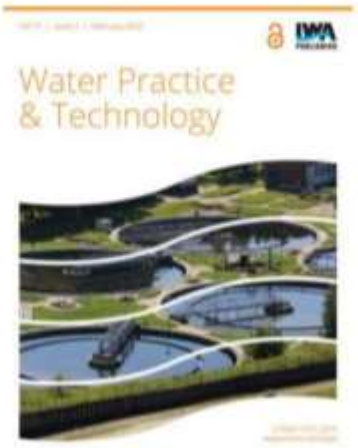
# Annexes

## Annex-1. Published on reputable journal- titled ‘Enterotoxins as a biomarker of water quality’

Link: http://iwaponline.com/wpt/article-pdf/17/2/623/1012151/wpt0170623.pdf

DOI: https://doi.org/10.2166/wpt.2022.016





Water Practice & Technology



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# Enterotoxins as a molecular marker of water quality

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## ABSTRACT

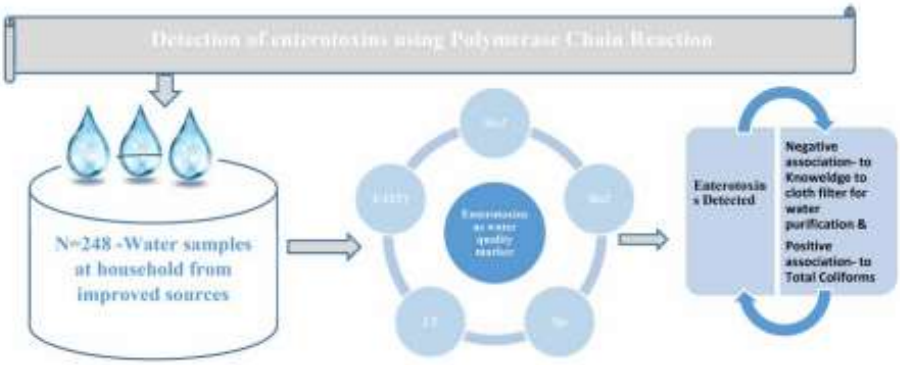
Safety of improved water supplies using enterotoxins as a molecular marker is evaluated. Water samples were collected from 248 households and tested for enterotoxins using polymerase chain reaction (PCR). The relationships between the presence of at least one enterotoxin and independent variables were investigated using Chi-square ($\chi^2$), 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 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.

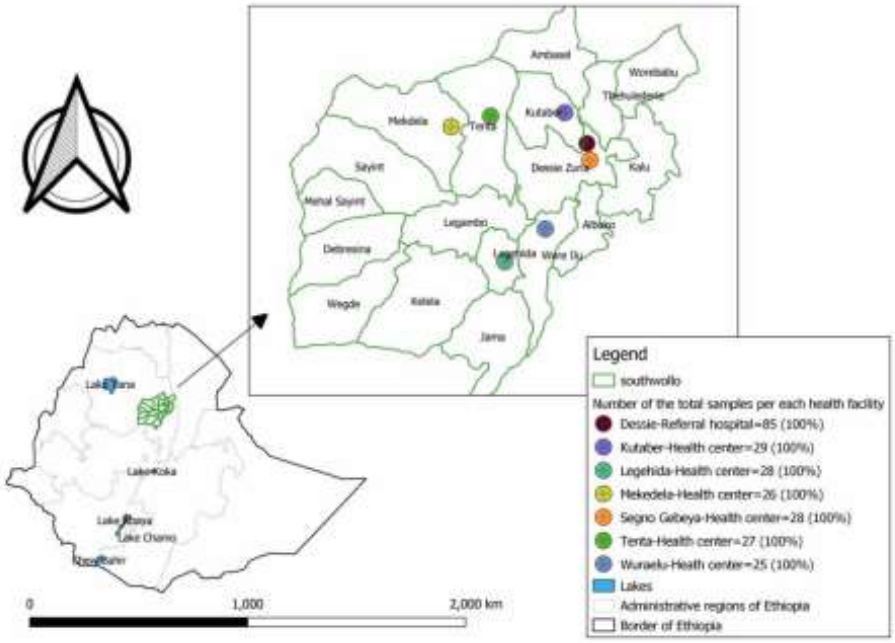



**Figure 1** | Map of Ethiopia and South Wollo (developed from Ethiopian Administrative shape files 2013 using Quantum Geographic Information System (QGIS) Software version 3.2.1, 2020).

### 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.

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

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.

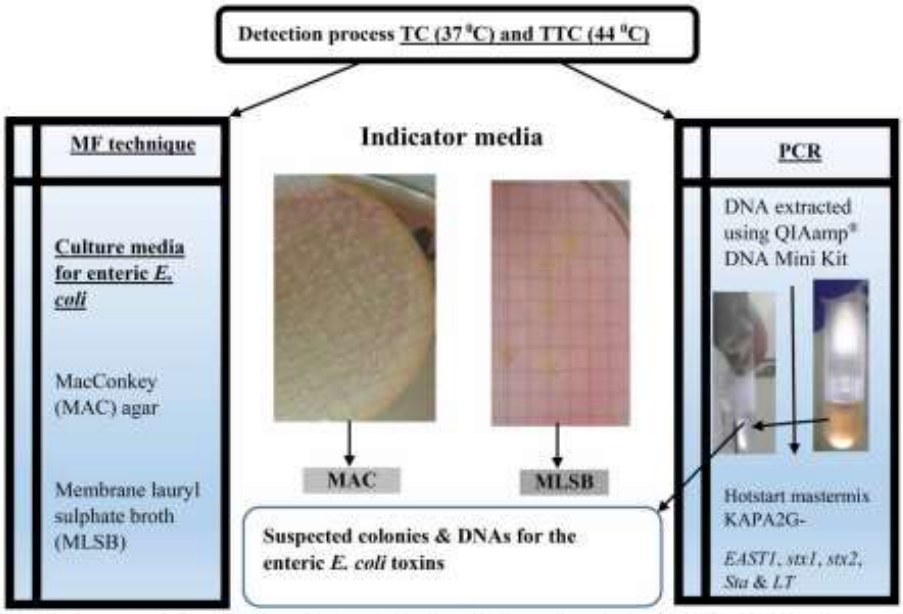



**Figure 2** | Three steps in *E. coli* enterotoxin detection. Membrane filtration, culture at 37 and 44 °C using MacConkey agar and membrane lauryl sulphate broth, and PCR with gDNA extracted from samples.

### 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 $\leq 0.25$ 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 $p<0.05$ 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 ($1\times10^6$) 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.

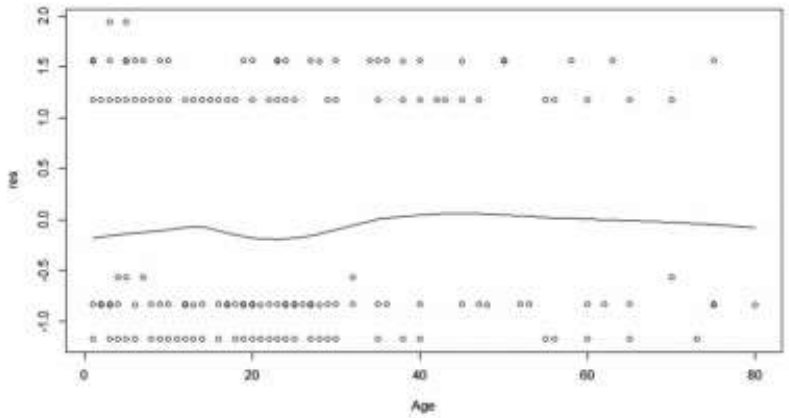



**Figure 3** | Linearity test of age and toxin gene detection in the samples.

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 ($\chi^2$) 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

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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 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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**Annex-2. Published on the press-titled 'Millions More People Got Access to Water. Can They Drink It?'**

Link: https://www.nytimes.com/2021/12/02/world/clean-water-to-drink.html


The New York Times

Millions More People Got Access to Water. Can They Drink It?

The U.N. pledged to halve the proportion of the world without access to clean drinking water by 2015.

The New York Times | https://www.nytimes.com/2021/12/02/world/clean-water-to-drink.html

HINDSIGHT

# Millions More People Got Access to Water. Can They Drink It?

The U.N. pledged to halve the proportion of the world without access to clean drinking water by 2015.

By Lucy Tompkins
Dec. 2, 2021

**Hindsight** is a series from the Headway team looking back at predictions and promises from the past.

For a long, long time, subsistence farmers in Ethiopia's northern highlands have risked illness, and even death, in the essential act of drinking water. Gathering water from the surface of contaminated ponds or springs, villagers coped with cholera outbreaks and children dying of diarrhea before even reaching their fifth birthday. Until recently, this was their only option.

But in 2015, after a concerted effort by the government and aid organizations to dig wells and install communal taps, the World Health Organization recognized Ethiopia for its remarkable progress. In 1990, it reported, just 14 percent of the country's population had access to clean and safe water; by 2015, 57 percent did.

People come to a stream to fetch water from a stream in northern Ethiopia in 2006. Lynsey Addario for The New York Times

The push for access to clean water in Ethiopia came in part out of a goal set by United Nations member countries in 2000. Those countries vowed to cut in half the proportion of the world without access to safe drinking water by 2015, compared with 1990 levels, and on paper, progress looked spectacular. In 2012, Ban Ki-moon, then the U.N. secretary general, announced that the safe water goal had been met ahead of schedule.

And yet villagers in Ethiopia and elsewhere continued to get sick.

Shibabaw Tadesse, a researcher at the Ethiopian Institute of Water Resources at Addis Ababa University, set out to discover why. In 2019, he and a group of researchers identified 248 households that met two criteria: They got water from a so-called improved source, like a pipe or covered well, and someone in the household had been treated for diarrhea. The researchers collected their water samples and brought them back to the lab.

Researcher Shibabaw Tadesse tested water samples from 248 households where people got sick even when their water came from an improved source.
Shibabaw Tadese

The results? In two-thirds of the households, the water was contaminated with E. coli or other diarrhea-causing toxins, making it unsafe to drink. For all the progress Ethiopia has made, far fewer people were actually drinking clean water than the W.H.O. reported.

## What went wrong?

The decades after the U.N. summit coincided with a period of rapid growth in countries like China, India and Brazil, which helped to spur infrastructure improvements. Most of the money for these efforts came from the countries themselves, though aid organizations also helped, said Rick Johnston, a technical officer at the World Health Organization's Joint Monitoring Program for Water Supply, Sanitation and Hygiene.

But when it came to verifying the pledge, there was a problem. The only way to know whether water coming out of pipes or wells is safe to drink is to test it, and at the time, few countries had the means to do that at scale. Instead, W.H.O. researchers relied on the one thing they could measure: the number of people getting water from improved sources.

Pipes, however, can deliver dirty water. In Bangladesh, for instance, piped water in urban areas is more likely to be contaminated than water from a primitive well or borehole in the countryside, according to a 2021 report by W.H.O./UNICEF researchers. If you drill a hole in the ground in Bangladesh, sand and gravel act as natural filters, said Mr. Johnston, one of the report's authors. Piped systems in urban areas, on the other hand, deliver water sporadically, creating low pressure that allows contaminated water to seep in through cracks and misaligned joints.

When a team of researchers led by the University of Bristol and University of North Carolina at Chapel Hill measured water quality in five countries in 2008, they found that many supposedly safe sources were actually contaminated. The U.N. had said that some 800 million people still did not have access to clean drinking water in 2010, but the researchers estimated that the figure was really more like two billion.

Ethiopia built a large number improved water sources, like wells, between 2000 and 2015. Eduardo Soteras/Agence France-Presse — Getty Images

Unimproved water sources like open ponds are more likely to be contaminated, Mr. Johnston said. "But even among improved sources, we've found examples of them being contaminated — even heavily contaminated."

## So has the world achieved the U.N.'s goal or not?

We don't actually know. The pledge was based on improving levels from 1990, and there isn't robust data on safe water going back that far. But significant progress has been made. For one thing, deaths from diarrheal disease linked to contaminated water have fallen significantly, to about half a million a year — down from more than two million in 2000, even as the global population has grown.

Researchers are also working with better information. Technological advances since 2015 have made it easier to test water quality, so now the U.N. not only can track how water is delivered, but also know whether it's safe to drink. Researchers are also asking more questions: Is the water available at home, or are people, women and girls in particular, spending an hour a day collecting it? Is it available when needed, or only sporadically? Is it affordable? The result is a new metric called "safely managed drinking water sources," which means water that is clean and available when it's needed on site. About 74 percent of the global population now meets this standard, the U.N. recently reported.

The most recent set of U.N. goals aim for universal access to safe water by 2030. It is an ambitious bar that is extremely unlikely to be met. This time, at least, there will be a better idea of where the world stands.

**Action:** Press release provided for a known International Company called 'The New York Times' with a headline 'Millions More People Got Access to Water. Can They Drink It?'

**Annex-3. Published on reputable journal- titled 'The importance of water quality in classifying basic water services: The case of Ethiopia, SDG6.1, and safe drinking water'**

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PLOS ONE

RESEARCH ARTICLE

# The importance of water quality in classifying basic water services: The case of Ethiopia, SDG6.1, and safe drinking water

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## Abstract

### Introduction

Sustainable Development Goal (SDG) 6 aims to coordinate international efforts toward "clean water and sanitation." However, water contaminated with pathogenic bacteria or thermotolerant coliforms (TTC) will not achieve the SDG target of clean water in the lives of people around the world. The aim of this study is to assess the water quality parameters of basic water services in Amhara and Afar regions of Ethiopia as well as the role and importance of local managerial committees in ensuring basic water functionality.

### Methods

This mixed methods research, conducted in January-June 2019, sampled 22 districts from food-insecure areas in the Amhara and Afar regions of Ethiopia. From the 22 districts, which represent nearly one third of all districts in each region, 111 water services classified as "basic" were randomly selected. For each selected water service, research included: water quality sample testing, visual observation of water services, interviews and focus group discussions with the associated water managerial committee members. Descriptive statistics frequency, percent, mean, median, standard deviations, normal tables, cross-tables and graphs are used to present the data.

### Results

Although the international water standard for thermotolerant coliform (TTC) levels is 0 CFU/100ml, in our sample of 111 water services, the maximum TTC counts were 71 CFU/100 ml and the mean was 4 CFU/100 ml. Thermotolerant coliform counts were above the permissible standard values for nearly 40% (n = 111) of the basic water services. TTC was detected

services sampled, deemed "functional" by international standards, do not provide *potable* water due to thermotolerant coliform (TTC) levels.

## Conclusion

Our findings from the Amhara and Afar regions of Ethiopia demonstrate that water quality parameters are not currently considered in classifying basic water services. This suggests that international efforts to address SDG 6 should incorporate water quality as a key parameter to better track international progress toward "clean water and sanitation" efforts. We discuss two potential pathways for stronger inclusion of water quality parameters in international definitions: (1) to mandate water quality within "functional" and "non-functional" definitions or (2) to add a ladder rung titled "safe basic water services" to the international drinking water ladder. Our findings from Ethiopia suggest that additional research should be undertaken in development contexts to assess whether or not "functional" basic water services provide safe drinking water to users.

## Introduction

The Sustainable Development Goals (SDGs) coordinate international efforts toward improving the lives of people around the globe. SDG 6 broadly outlines "clean water and sanitation" while target 6.1 specifies: "By 2030, achieve universal and equitable access to safe and affordable drinking water for all" [1]. Yet achieving safe drinking water in developing countries and resource-poor environments requires sustained effort. In contribution, we demonstrate that current international standards around improved water have not properly specified standards around water quality. Without a stronger incorporation of water quality parameters, international targets risk focusing in improved basic water services means water free from contamination that merely supply users with non-potable, and contaminated water that would end up with non-functional. Water contaminated with thermotolerant coliforms (TTC) will not achieve the SDGs in the lives of people around the world. Using the case of water in Ethiopia, this paper documents the existence of diarrheal bacteria (TTC) in water services currently deemed functional by international standards. We then utilize qualitative methods to contextualize water service management by local citizen committees, called WASHCos (Water, Sanitation, and Hygiene Committees). While our data is specific to Ethiopia, we find 34% of basic water services currently classified as functional, do not offer "safe drinking water" as SDG 6.1 states as a global goal. This is supported by other study conducted in Ethiopia that resulted the key factors for improved water services functionality were improving water quality by 61% of the respondents [2]. Further, study conducted in developing countries recommends to harmonize and standardize water functionality monitoring, it is must to address water quality [3]. In a study conducted in Malawi, the water in samples (32%) conformed not met to the drinking water standard for the Malawi Government (50 CFU/100 ml) for TTC (*Escherichia coli)* and most samples (87%) did not meet the WHO drinking water standard of zero CFU/100 ml) of *E. coli*. Thus, the water services that are working well tend to have the lowest *E. coli* contamination levels [4]. This demonstrates the importance of including a water quality parameter, such as the presence of TTC, to measures basic water functionality. Basic water services are the main source of drinking water for communities around the globe, therefore understanding water quality is an essential component to achieving universal access to safe and clean drinking water [5].

International standards measure and classify water services and water quality in various ways. At the highest level basic, water services are deemed improved or unimproved and water services are classified as functional or non-functional [5, 6]. The unimproved sources include drinking from unprotected dug wells or springs or directly from the source, such as a river, lake, pond or irrigation canal/channel. This article is focused on improved water services which include: rural piped system (RPS) from a spring, borehole or shallow wells, hand-dug wells with hand pumps (HDW), rainwater harvesting (RWH) structures, constructed on-spot springs, reservoirs, river intakes (RI), and slow sand filters (SSF) [7]. Improved water services are then understood as water services (accounting for both the source and water delivery systems), classified as safely managed, basic, or limited with set parameters for each (Table 1). Improved water services are classified as functional (fully operational system at time of survey) or partial or fully non-functional (fails to operate at time of survey) [6].

However, as is seen in Table 1, water quality (measures that assess contamination) is a mandatory criterion for safely managed services yet becomes an *optional criterion* in the basic and limited water services definitions. In other words, a basic water service may be classified as "functional" even if the water is not, in practice, "safe drinking water"—the goal of SDG 6.1. The flipside of this is that water services classified as "non-functional" do not incorporate water quality. Water quality parameters include testing samples across three dimensions: biological (total coliforms (TC) and TTC in colony forming unit (CFU) per 100 ml of water samples), physical (turbidity in nephlometeric turbidity unit (NTU)) and chemical ('pH' and free residual chlorine (FRC) in milligrams (mg) per liter (lit) of water samples). The SDGs classify safe drinking water based upon the updated World Health Organization (WHO)/United Nations Children's Fund (UNICEF) joint monitoring programme (JMP) ladders of safe drinking water [1]. This classification does not, we argue, properly account for "safe" drinking water in basic water services. The inclusion of a fecal indicator bacteria (FIB) as part of water quality in the commonly-accepted definition of basic water [1] is a gap that obscures the risk of diarrheal diseases from water services classified as both improved and functional. Given the morbidity and mortality caused by diarrheal disease and the disproportionate burden placed onto under-five children and women [8, 9], a stronger incorporate of water quality parameters is essential to driving investment in water services that produces meaningful enhancements in the lives of Ethiopians and developing country citizens more broadly.

Functioning water services are fundamental to social and economic development: improving the quality of health and educational achievement by reducing the morbidity and mortality, malnutrition, stunting rates of people in community and improving livelihoods [8, 9]. Global data on water services show that 844 million people still lack basic drinking water. Two hundred sixty-three (263) million people spent over 30 minutes per round trip to collect water from an improved sources due to scarce drinking water services [1]. One hundred fifty nine (159) million people, of which 58% are in sub-Saharan Africa, continue to fetch drinking water directly from unimproved sources such as unprotected wells and springs or surface

**Table 1. SDG definitions for the classification of improved water services.**

| | |
|---|---|
| **Safely managed services** | 1. Source is accessible on premises **and** |
| | 2. Water is available on demand **and** |
| | 3. Water is free from contamination |
| **Basic water services** | If source does not meet any **one** of the three criteria **and** a round trip to collect water takes 30 minutes or less including queuing |
| **Limited water services** | If source does not meet any **one** of the three criteria **and** a round trip to collect water exceeds 30 minutes. |

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water services [1]. Indeed, enhancing the existing basic water services to meet water quality standards is a strategic development area for increased investment.

Despite efforts to increase the safe service of water with an ultimate outcome of improving the health and nutritional status of the community [10, 11] in food insecure rural areas of Ethiopia, with special attention to Amhara and Afar regional states, malnutrition rates in these two regions exceed the national average (40%) [12]. In Ethiopia, lack of access to water supply causes around half of all health complications from undernutrition [8]. Since 1990, joint efforts by the Ethiopian government and numerous non-governmental organizations (NGOs) have expanded services to water, sanitation and hygiene (WASH) through the construction of water infrastructure in Amhara and Afar of Ethiopia [1, 7]. While much focus has been given for the construction of new water services, there is inadequate or no data on existing water services that, after construction, require on-going maintenance. Once a water service is constructed and therefore "improved," it may then be considered as safely-managed, basic, or limited. The negative impact of water quality apart from physical drying or low yielding as well as weak operation and maintenance of water on the functionality of basic water services were reported in studies [2, 3, 13]. In the case of rural Ethiopia, partial functionality and non-functionality of basic water services were reported as prevalent challenges [13]. Ethiopia is not alone in its developmental struggle for clean water accessible from improved sources.

In Ethiopia, the proportion of people using a **safely managed** water service (accessible on premises, available when needed, and free from contamination) is only 11% (4% rural and 38% urban) [1]. This data indicates a significant gap between urban and rural areas residents, while demonstrating that even urban living in Ethiopia does not equate access to clean water. To understand this at a nation-wide scale, out of the sixteen small-to-medium towns in Ethiopia, only 2 towns provide water that meets the Ethiopian government's Growth and Transformation Plan I standard [14] which requires water be free from contamination. Moreover, **basic water services** meaning water from an improved source does not meet any one of accessible on premises, available when needed and free from contamination, but a round trip to collect water takes 30 minutes or less including queuing is the next category in the water ladder. Therefore, where water quality may or may not meet the joint monitoring program (JMP) definition of basic water services standards, in Ethiopia, the national drinking water coverage of basic water services is 39% (30% rural and 77% urban) [1]. In Ethiopia, 12% of the national population still relies on untreated surface water [15], and within the rural population 47.2% use surface water and 28.1% use spring water services, meaning that nearly 75% of the rural population uses water from unimproved sources [16].

Rural Ethiopian communities have struggled to first gain access to basic water services and then maintain their functionality. In rural Ethiopia in 2013, a total of 92,588 water services were inventoried by Ministry of Water, Irrigation and Electricity of Ethiopia. As can be seen in the inventory data, the overwhelming majority, 78.4%of rural Ethiopians get water from basic water services. The types of the basic water services include: hand dug wells with hand pumps, springs without distribution networks, shallow wells with hand pumps and rope pumps [17]. As in many other countries, in addition to limited *access* to basic water services, non-functionality of newly built or existing basic water services is a major problem in Ethiopia [13]. One study, based on 12 countries across African and Asian continents, found that 28.7% were non-functional or partially functional [18]. In the same study, non-functionality of water systems in Sub-Saharan African countries (focused on Ethiopia, Ghana, Kenya, Rwanda, Uganda, and Zambia), were 24.8%, followed by 21.3% in Asia (India) and 7.3% in Latin America & the Caribbean (El Salvador, Guatemala, Haiti, Mexico, Nicaragua) [18]. According to Ministry of Water Irrigation and Electricity inventory report, the national non-functionality rate for Ethiopia is estimated to be 26% with the highest rate of non-functionality (34%) in the Afar region

[17]. In Ethiopia, the non-functionality rate of water services was found to be 20–30% nationally and up to 50% for rural Ethiopia [16]. Additionally, in a study across four Ethiopian regional states that are considered to have stronger infrastructure than the remaining five regions, 20% of water services were found to be non-functional [2].

Studies find different reasons for the non-functionality of water services in Ethiopia. One study [19] attributed that 50.8% (n = 102) of users fail to pay water service fees, which allow for consistent operation and minor maintenance as needed. Another study documented that 53% of water services lack a budget source, 40% had no trained caretakers, and only 7% have limited access to spare parts [20]. Ethiopia utilizes similar water management structures as seen elsewhere, such as Ghana and Uganda [21, 22]: basic water services in rural areas are managed by volunteer citizen Water, Sanitation and Hygiene Committees, popularly called "WASHCos." Each WASHCo consists of 7 or more individuals, with government mandates to include 4 women and 3 men from the community. WASHCo members are trained across a variety of dimensions by both governmental and non-governmental actors [23]. In general, their training aligns with international standards, which typically recognize 5 dimensions that contribute to strong water service management by local citizens. These 5 dimensions include: technical (e.g. water technicians, spare parts), institutional (e.g. meeting frequencies, capacities of members, promotion to potential service users), social (e.g. gender composition, working relationships), environmental (e.g. drought, flooding) and financial (e.g. ability to collect water fees, cost of operation and maintenance). Poor performance across these 5 dimensions is thought to contribute negatively to water service functionality. Despite the limitations of WASHCos, the presence of a WASHCo improves the ongoing functionality and water services, and WASHCos perform better than non-community managed types such as local government and private entities [23]. Lastly, community perceptions of water quality enhance use of basic water services, which further helps drive functionality through perceiving water quality [24], timely paying of fees, and speeding up repair times [2].

Consequently, the aim of this assessment was to assess the status of basic water services functionality with respect to water quality and WASHCo management in Amhara and Afar regions of Ethiopia. The study takes into account water quality, as measured by the presence of fecal indicator bacteria (FIB) as one factor of functionality in addition to water service managerial factors such as technical, institutional, social, environmental and financial aspects. The study also identifies potential gaps to be addressed for improvement. Our data demonstrate that too many factors have been confounded into water services that are classified as "basic." In contribution we suggest that the JMP ladder either incorporate water quality into the criteria for basic water services or add an additional rung to track and develop more nuanced targeting around basic water services that provide "safe drinking water." The study provides evidence in support of the Ethiopian government's commitment to reducing the non-functionality rate (NFR) to 7% as stipulated in the One WASH National Program II (OWNP II), which complements and contributes to the Growth and Transformation Plan II (GTP II) [25].

Our findings suggest that water quality is an important, yet currently under-researched aspect of "safe drinking water." By incorporating water quality parameters into basic water services, investment could be better driven to making basic water services potable rather than investing first in infrastructure development. Given the morbidity and mortality caused by diarrheal disease [26, 27], international investments should invest in functionality *and* water quality. Water quality as a metric should remain a consistent priority during the planning, implementation, maintenance, and monitoring and evaluation of water services. The findings of this study may be of use to water scholars, global development organizations, national policymakers, as well as grass-roots level implementers.

## Methods and analysis

In order to understand the functionality of basic water services, this mixed methods study collected data from 22 food-insecure rural districts, known locally as woredas, selected from two regions—Amhara and Afar. In the Ethiopian context, the country is divided into regions, then zones, then woredas, and, finally the smallest unit named kebele. In the selected woredas/districts, malnutrition rates exceed the national average of 40% [12, 28, 29]. The 111 basic water services sampled were then tested for the required priority water quality parameters such as biological: total coliforms (TC) and thermotolerant coliforms (TTC), physical: turbidity and chemical: 'pH' and free residual chlorine (FRC). Data collection was conducted from January to June 2019: the driest peak season in the area. This season was selected for the reason that the factors related to environment and climate change would be greatest. *E. coli* is higher in the rainy season than the dry season in Ethiopia [30]. Therefore, by collecting data during the peak dry season, we expect our results to provide the most conservative estimations regarding the role of water quality in water service non-functionality.

### Sample size

Amhara and Afar regions were purposively selected due to their high levels of malnutrition and food-insecurity, and then within each region three zones were randomly selected. Within the selected zones, using government lists of food insecure woredas/districts, a total of 22 woredas/districts were selected (Fig 1 and Table 2).

Following the selection of targeted woredas/districts, a total of 111 (79 in Amhara and 32 in Afar) basic water services and respective WASHCos were selected randomly from the water services inventory lists of the Zone Water Offices. This represents approximately 13.1% of the total number of basic water services of the water services found in the 22 woredas/districts and their respective WASHCos (Table 2).

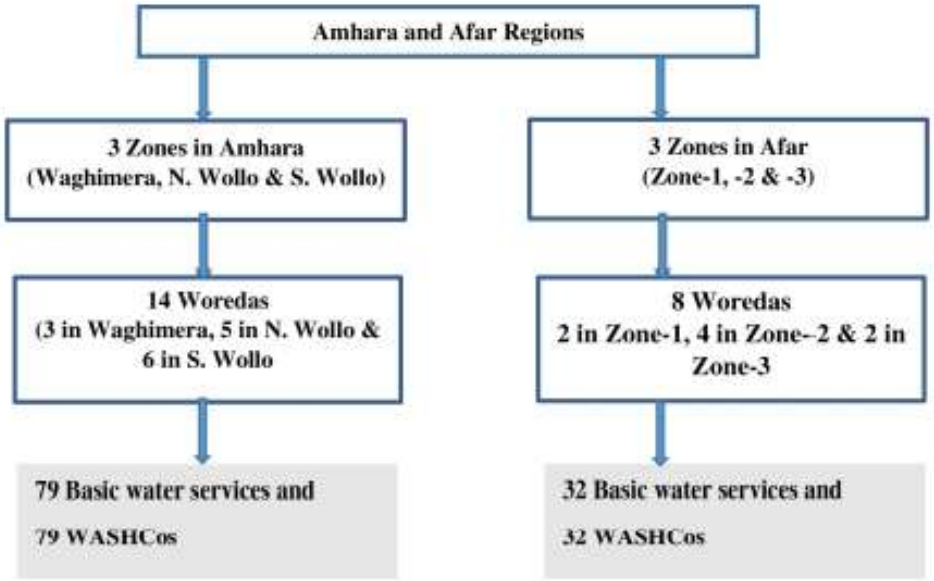


**Fig 1. Sampling structure of selected basic water services and associated WASHCos.**

https://doi.org/10.1371/journal.pone.0248944.g001

Table 2. Summary of sample in comparison to total basic water services population in study area.

| Region | Zone | Total basic water services in Zone n = | Total basic water services in selected Woredas n = (*n = selected Woredas*) | Sampled basic water services (n =) | Sample as a percent of Woreda total |
|---|---|---|---|---|---|
| Amhara | Waghimera | 120 | 82 (3) | 14 | 17.07 |
| | North Wollo | 920 | 327 (5) | 33 | 10.09 |
| | South Wollo | 963 | 346 (6) | 32 | 9.25 |
| | **Sub-total** | **2003** | **755 (*14*)** | **79** | **10.46** |
| Afar | Zone-1 | 37 | 22 (*2*) | 3 | 13.64 |
| | Zone-2 | 46 | 31 (*4*) | 19 | 61.29 |
| | Zone-3 | 54 | 39 (*2*) | 10 | 25.64 |
| | **Sub-total** | **137** | **92 (*8*)** | **32** | **34.78** |
| | **TOTAL** | **2140** | **847 (*22*)** | **111** | **13.11** |

https://doi.org/10.1371/journal.pone.0248944.t002

## Methods

This mixed methods research focused on basic water services as the unit of analysis. Assessing each water service identified by the sampling technique described above, we then created an in-depth understanding of each water service using mixed methods. This study includes five data sources taken from each sampled water service and corresponding WASHCo: geocoded location data for the water service, water samples taken, visual and physical inspection of water service with an observation checklist, interviews of 2–3 WASHCo members using a structured questionnaire, and focus group discussions (FGDs) with the remaining 4–5 WASHCo members using a checklist (S1 File). In addition to the above primary data collection, the research team analyzed individual WASHCo records such as meeting minutes, bank or microfinance books, water fee collection receipts and water users lists that were kept about their basic water services.

First, geocoded data to geo-locate the water services were collected using instruments GPS-garmin-62 with an accuracy to three meters (3m) precision. Second, water samples of 300 ml were taken from all sites for lab analysis, irrespective of the functionality of the basic water services (either from the sources or reservoirs or containers or public water points/tap stands) Water quality was assessed from water samples across biological, physical, and chemical parameter properties that include the following tests: **bacteriological**: total coliforms (TC) and thermotolerant coliform (TTC) using membrane filter (MF) technique of International Standard Organization (ISO): 9308–1:2014 detection and enumeration of TC and TTC bacteria [31–33] were tested. Membrane Lauryl Sulphate Broth (MLSB)-AVONCHEM-ACM-1820-O media was used for the bacteriological- TC and TTC detection. Total Coliforms were detected by culturing at 37 $^{0}$C for 24 hours. Thermotolerant Coliforms were detected by culturing at 44 $^{0}$C for 24 hours. The growths of yellow colonies in each plate on the filter papers were directly counted in each quadrant. The results in numbers and in CFU/100 ml registered in a laboratory. Physical, turbidity (NTU), and chemical, 'pH' and free residual chlorine (FRC) in mg/lit, were tested using photometer 7100 of Palintest (manufactured by Wagtech in United Kingdom) with preprogrammed test calibrations. Water quality test protocol is found in this link: https://dx.doi.org/10.17504/protocols.io.bpc7mizn. Third, direct visual observation of water services was conducted. This included: functionality factors in terms of technical aspects; service type, technology type, the status of water services, training, experience of operation and management (O&M), access to spare parts, water treatment/chlorination collected.

Qualitative methods were used to collect data from WASHCo members. The WASHCo members were randomly assigned to either interviews or FGDs, however women were

purposively sampled for inclusion to ensure the presence of one woman in both the interview and FGD. The questions in the interview and FGD questionnaire are nearly similar (S1 File). In this manner, data was verified across observations, interviews, and FGDs. This triangulation is important to improve data quality since many of the WASHCo members have low literacy and numeracy and may struggle to recall particular details about the water service. Both interviews and focus group discussions were given by research assistants hired for each local area. Interviews and focus group discussions were conducted by research assistants who hold a bachelor's degree and have a background in water and sanitation.

### Data collection and analysis

The study data was gathered using the partially modified and piloted questionnaire from the standard water functionality survey questionnaire, FGD checklist and lab analysis by obtaining informed verbal consent from each of the participants of WASHCo members.

Depending on the nature of variables, an analysis tool was developed using IBM SPSS statistics software version-20. Before the analysis, data were cleaned and validated. Descriptive statistics of frequency, percent, mean, standard deviations, normal tables, cross-tabulations and graphs were used. A one tailed *t*-test at 95% CI was used to evaluate whether the mean for microbial parameters (TC and TTC counts), physical parameter (turbidity) and chemical parameters ('pH' and residual chlorine) in the water were significantly different from the WHO and Ethiopian quality standards.

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. Permission was obtained from the community members orally after explaining the study objectives and how they were selected for this study because of lack of reading and writing by included cases. Confidentiality of information was respected.

## Results and discussions

### Location and type of water services

A total of 111 (Afar: n = 32, 28.8% and Amhara: n = 79, 71.2%) basic water services and their respective WASHCos found in six zonal administrations that cover 22 woredas/districts were included in this study. In Amhara, the water services included were located in North Wollo 33 (10.09%), South Wollo 32 (9.25%) and Waghimera 14 (17.07%) of the total basic water services found in the 14 woredas of Amhara. Likewise, the Afar samples were 19 constituting 61.29% in Zone-2, followed by 10 (25.64%) in Zone-3 and 3 (13.64%) in Zone-1 of the total basic water services found in 8 woredas of Afar see (Table 2) above.

The location map of the 111 basic water services included in this study was geo-referenced. Global positioning system (GPS) reading; X (longitude), Y (latitude)-coordinates and Z (altitude) data were taken. However, some information was difficult to include in the data from Afar (shown in Fig 2). The water points were located at a minimum altitude of negative 91 meters below sea level (BSL) in Afar with arid climatic conditions and a maximum altitude of 3,402 meters above sea level (ASL) in Amhara characterized by cold fertile climatic conditions. The mean altitude reading of the locations of the basic water services was 2,076.62 meters above sea level and standard deviation of 953.338 meters (Fig 2). The type of the basic water services in the Amhara highlands might vary from that of the lowlands in Afar. This would have brought different factors that contribute to the functionality of water services and the water contamination itself as functionality factor. In a review of case study conducted in Malaysia using 68 studies, it was confirmed that factors such as soil erosion, landslides and agricultural activities associated with land use change have significantly influenced the water

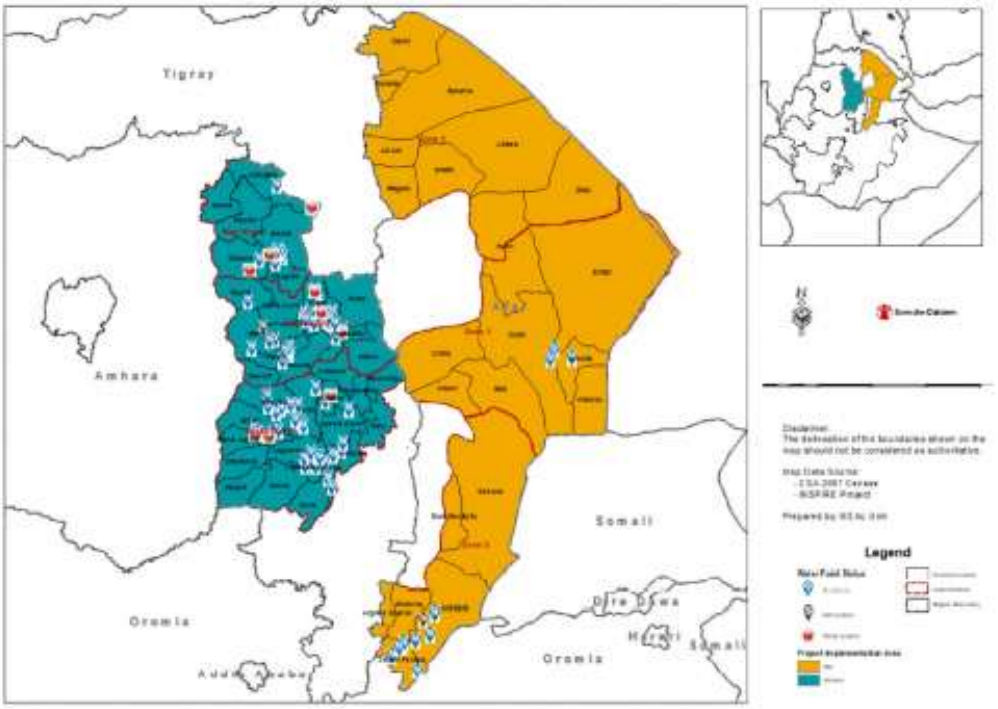


**Fig 2. Location map of the study sites, Amhara and Afar, Ethiopia (Save the Children GIS center is used to develop this map).**

https://doi.org/10.1371/journal.pone.0248944.g002

quality in the highland areas [34] similar to the Amhara region. This might not be the case for Afar region, with lowland areas where the life of the community was dependent on livestock in pastoralist area.

## Water services: Technical findings for functionality

The basic water service technology types included in this study were rural piped system (RPS) using 42 spring sources (37.8%), 25 hand-dug wells with hand pumps (HDW) (22.5%), 22 RPS from a motorized borehole or shallow wells sources (19.8%), 9 rainwater harvesting (RWH) structures (8.1%) and others such as on-spot springs, reservoirs, river intake (RI) and slow sand filters (SSF) jointly account for 13 basic water services (11.8%). It was found out that treatment of water is largely done by WASHCos (n = 43, 38.7%) and Woreda Water Offices (WWO) (n = 38, 34.2%) while very few sources (n = 2, 0.9%) were reported to be treated by Zonal Water Offices (ZWO) and others such as tap attendant (one source by each). The time interval of water chlorination is often quarterly (n = 46, 41.5%), several others yearly (n = 21, 18.9%), and every six months (n = 13, 11.7%) and the remaining few (n = 3, 2.7%) monthly. Unfortunately, as to interviewed WASHCos, a quarter of the water services (n = 28, 25.2%) sampled in this study were disinfected totally by any responsible body including the WASH-Cos who are largely responsible for the disinfection of the water services (Table 3).

**Table 3. Results of the technical variables.**

| Variables | | Size | Percent |
|---|---|---|---|
| Water service types | On spot springs | 6 | 5.4 |
| | RPS-from gravity springs | 42 | 37.8 |
| | Motorized shallow or bore wells | 22 | 19.8 |
| | Hand-dug wells | 25 | 22.5 |
| | Rainwater harvestings | 9 | 8.1 |
| | Water reservoirs | 4 | 3.6 |
| | River intake | 1 | 0.9 |
| | Slow sand filtrations | 2 | 1.8 |
| Status of water services | Functional | 87 | 78.4 |
| | Not functional | 24 | 21.6 |
| Responsible body to treat water | WASHCos | 43 | 38.7 |
| | Woreda Water Office | 38 | 34.2 |
| | Zone Water Office | 1 | 0.9 |
| | Other-tap attendant | 1 | 0.9 |
| Time interval to treat water by WASHCos/others | Monthly | 3 | 2.7 |
| | Quarterly | 46 | 41.5 |
| | Every six months | 13 | 11.7 |
| | Yearly | 21 | 18.9 |
| | Not disinfected | 28 | 25.2 |

https://doi.org/10.1371/journal.pone.0248944.t003

From our sample of 111 water services, 24 sources (21.6%) were categorized as non-functional from technical and management aspect by interviews and FGDs with WASHCos, and water services visual observation. The reasons for non-functionality of basic water services listed in decreasing order of importance, excluding water quality, were poor management (n = 19, 17.1%), unavailability of spare parts (n = 17, 15.3%), lack of maintenance tools (n = 12, 10.8%) and environmental reasons (n = 10, 9.0%). Indeed, lack of skilled technicians and shortage of money were also barriers to functionality (Table 4).

## Water services: Water quality

At present, water quality is not a parameter considered under the classification of "functional improved basic water." Water quality is a neglected topic in global debates and quality is not given due attention as it should be [1, 35] as far as basic water services is concerned. Preserving the quality of basic water services, which the majority of the people in developing countries have access to, is important for better drinking-water supply use and decreasing morbidity. Water quality lab analysis were conducted for all basic water services using lab protocol (S2 File). Water samples for lab analysis were taken from the sources for functional basic water

**Table 4. Reasons attributed to the non-functional water services other than water quality.**

| Reasons for non-functionality | Response frequency (%) | |
|---|---|---|
| | Yes | No |
| Poor management | 19 (17.1) | 5 (4.5) |
| Unavailability of spare parts | 17 (15.3) | 7 (6.3) |
| Lack of tools | 12 (10.8) | 12 (10.8 |
| Environmental reasons | 10 (9.0) | 14 (12.6) |
| Shortage of money | 7 (6.3) | 17 (15.3) |
| Lack of skilled technicians | 6 (5.4) | 18 (16.2) |

https://doi.org/10.1371/journal.pone.0248944.t004

**Table 5. Water quality parameters versus services functionality in Ambara and Afar regions.**

| Parameters | Classifications | Water services | | |
|---|---|---|---|---|
| | | Functional | Non-functional | Total n (%) |
| **Total Coliform** | Meets standard of 0 CFU/100mL | 47 (42.34) | 18 (16.22) | **65 (58.56)** |
| | Above standard of 0 CFU/100mL | 40 (36.03) | 6 (5.41) | **46 (41.44)** |
| **Thermotolerant Coliform** | Meets standard of 0 CFU/100mL | 49 (44.14) | 18 (16.22) | **67 (60.36)** |
| | Above standard of 0 CFU/100mL | 38 (34.23) | 6 (5.41) | **44 (39.64)** |
| **'pH'** | Below the standard– 6.5 | 1 (0.90) | 1 (0.90) | **2 (1.80)** |
| | Meets standard of– 6.5–8.5* | 47 (42.34) | 17 (15.32) | **64 (57.66)** |
| | Above the standard– 8.5 | 39 (35.13) | 6 (5.41) | **45 (40.54)** |
| **Turbidity** | Meets standards of less than 5 NTU | 83 (74.78) | 24 (21.62) | **107 (96.40)** |
| | Above standard greater than 5 NTU | 4 (3.60) | 0 (0.0) | **4 (3.60)** |
| **Residual Chlorine** | Below the standard– 0.2 mg/lit | 86 (77.48) | 23 (20.72) | **109 (98.20)** |
| | Meets standard– 0.2–0.5 mg/lit* | 1 (0.90) | 0 (0.0) | **1 (0.90)** |
| | Above the standard– 0.5 mg/lit | 0 (0.0) | 1 (0.90) | **1 (0.90)** |

* WHO drinking water quality guideline recommended standard.

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services and for the non-functional 24 (21.62%) of the water services, the samples for lab analysis were taken from either at the sources or storages or in the pipelines. In the lab analysis, measured in colony forming counts per 100 milliliters (ml) of water samples for **bacteriological tests** of total coliforms (TC) were detected from 46 (41.44%) taken from basic water services. Of which 40 (36.03%) of the basic water services are functional and the remaining 6 (5.41%) were not functional.

The other test conducted from the priority bacteriological tests is the thermotolerant coliforms (TTC). The presence of TTC serves as an indicator of water contamination by human or animal fecal matters. TTC was detected in 44 basic water services of (39.64%) of which 38 (34.23%) are operationally "functional" and 6 (5.41%) are not functional (Table 5). Therefore, TC for about 41% and TTC for nearly 40% of the water services were above the permissible standard values, which is 0 CFU/100 ml.

To compare the dispersion of the result of the thermotolerant coliform counts detected in functional and non-functional basic water services we used box plot. Thus, comparing the thermotolerant coliform in functional and non-functional basic water services, there is greater variability in detected thermotolerant coliform counts in functional basic water services than non-functional basic water services as shown in the box plot (Fig 3). In other words, the data set is highly dispersed in functional basic water services than non-functional basic water services.

The maximum total coliform (TC) counts was 111 CFU/100 ml and the mean was 11 CFU/100 ml. The maximum TTC counts were 71 CFU/100 ml and the mean was 4 CFU/100 ml. As to priority chemical tests, the 'pH' of 47 basic water services (41.34%) were either below or above the allowable limits which is in the range of 6.5–7.5. In addition, FRC result shows that 109 basic water services (98.20%) were below the allowable ranges– 0.2–0.5 mg/lit. The mean of free residual chlorines and 'pH' were 0.04 mg/lit and 7.4 respectively. The mean 'pH' result was within the range of permissible values of the standard, while the mean of FRC is below the permissible values of the standard-0.2–0.5 mg/lit in basic water services. The low level of the FRC could result in reduced killing of pathogenic microorganisms. This creates a higher risk of contamination of the water services by human and or animal wastes. This high-level contamination was confirmed by the detection of the TC and TTC in the water samples brought from different water services. 107 of the basic water services (96.40%) showed turbidity values

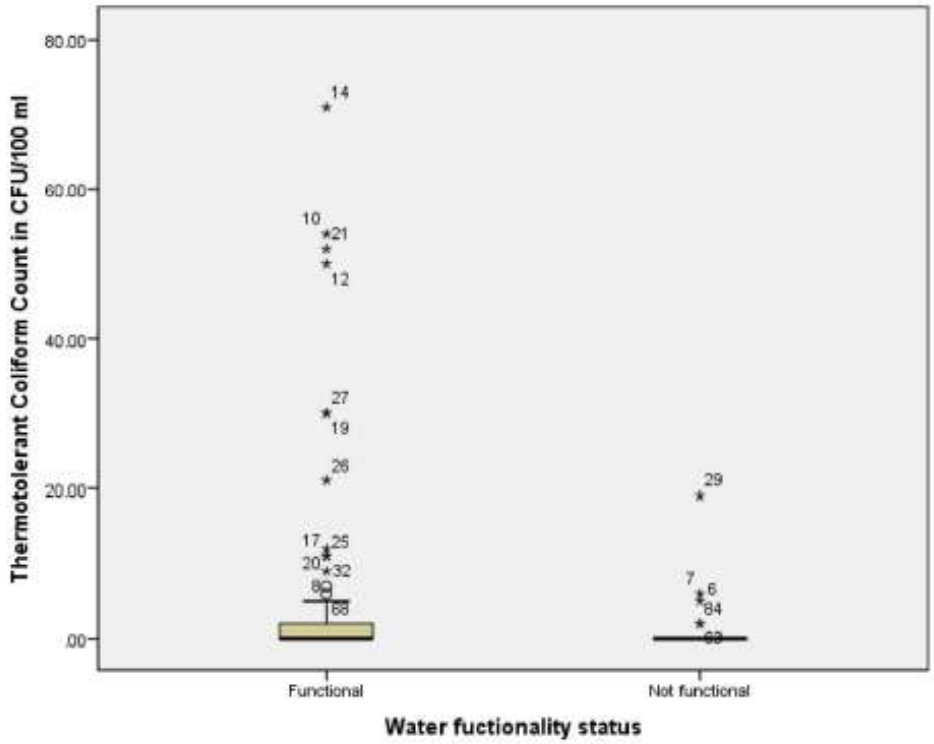


**Fig 3. Water functionality status compared to the detection of TTC in CFU/100 ml.**

https://doi.org/10.1371/journal.pone.0248944.g003

within the acceptable value of the drinking water quality guideline of World Health Organization (WHO) (less than 5 NTU) (Table 6). Therefore, the lab test result of the five (TC, TTC, 'pH', turbidity and residual chlorine) primary water quality parameters minimum, mean, maximum detection level was computed with the WHO and Ethiopian standard (Table 6).

**Table 6. Summary of water quality results of basic water services sample (n = 111) in comparison to WHO and Ethiopian standards.**

| | | Water Quality Parameters | | | | |
|---|---|---|---|---|---|---|
| | | TC Count CFU/100 ml | TTC Count CFU/100 ml | 'pH' | Turbidity (NTU) | Residual chlorine (mg/lit) |
| WHO & Ethiopian Standards* | | 0 | 0 | 6.5–8.5 | < 5 | 0.2–0.5 |
| Sample | Minimum | 0 | 0 | 6 | 0 | 0 |
| | Median | 0 | 0 | 7.48 | 0.75 | 0 |
| | Mean | 11 | 4 | 7.48 | 1.36 | 0.05 |
| | Maximum | 111 | 71 | 8.81 | 31 | 5.3 |
| | Std. Deviation | 23.14 | 11.60 | 0.42 | 3.33 | 0.50 |

*Ethiopian standard authority developed similar standard to the WHO.

https://doi.org/10.1371/journal.pone.0248944.t006

By considering the TTC of which the significant public health concern in causing diarrheal diseases and morbidity specially in children under-five in developing countries like Ethiopia, this study points out the contribution of water quality for non-functionality. Accordingly, an additional 38 functional water services (34.23%) were re-categorized as non-functional because of water quality and 6 (5.41%) of already non-functional as a result of technical and management factors were also unfit for consumption because of water quality, which ended up to double non-functional.

The overall non-functionality rate of basic water services 44 (39.6%) was higher than the findings of other studies. For example, in studies of a twelve-countries in Sub-Saharan African, Latin America & Caribbean and Asian, including Ethiopia (with sample size (n = 120 for Ethiopia) in 2014, the non-functionality rate was 28.7% [18] with nearly similar socio-economic representation. In Ethiopia, 35% of rural water supply systems [2] with almost similar socio-economic condition of our study area and with lesser sample size (n = 74) than our study, the water services were not working at the time of the survey. In other study in Ethiopia, 18% of the hand pump borehole water services [36] that consider water sample size of (n = 200) were not working at the time of the survey. These differences could be due to the consideration of water quality parameter as part of functionality for water services in this study, which was not the case in the previous studies.

Report showed that 20.6% of water services were not working at the time of the survey in Grater Afram Plains region of Ghana [37] with different size of water service sample size (n = 1509) and different socio-economic situation with our study. This variation in results come up because of the percentage variations in water contamination. In fact, the difference in sample size and socio-economic consideration between the study areas might have contributed. On the other hand, the result 44 (39.6%) of non-functionality of our study was lower than the non-functionality rate of other study in Ethiopia that shows 44.4% for shallow and boreholes [19]. This difference in non-functionality was attributed to differences in water service technology types (the present study uses variety of water service technologies and the later study considers only shallow or borehole water services) within similar socio-economic conditions and nearly the same sample size (n = 102) as that of our study. This shows that if water quality indicators are included in the later study, the non-functionality rate would be much higher than our finding.

In summary, the reasons contributed for non-functionality of water services based on the laboratory analysis of water quality was **water quality-thermotolerant Coliforms** count 38 (34.23%) in operationally "functional" basic water services. Poor management 19 (17.1%), absence of skilled technicians 16 (14.4%), lack of spare parts 17 (15.3%) are chronologically identified reasons for non-functionality based on the interview and FGDs results. The findings of our study are consistent with other study [20] which showed that high non-functionality of water services was due to lack of spare parts and long distance to procure spare parts [38]. Lack of maintenance and operation tools 12 (10.8%), environmental factors 10 (9.0%) and shortage of money 7 (6.3%) were also found limiting factors in both Afar and Amhara regions. Data of the study, indeed, revealed that poor water quality brought about by contamination of the water services as seen through thermotolerant coliforms mainly contributed to the non-functionality in the study areas (Fig 4). Monitoring drinking-water supplies safety implies the quality of the water needs substantial improvement like monitoring and recording of access to functional water services [39].

The growths of yellow colonies at 44 $^{o}$C for 24 hours cultured water samples using membrane filter (MF) technique in laboratory were considered that the basic water services were contaminated either with human or animal feces that prompt the water source's status to be a non-functional category of water services. The growths of the bacterial colonies were shown in (Fig 5).

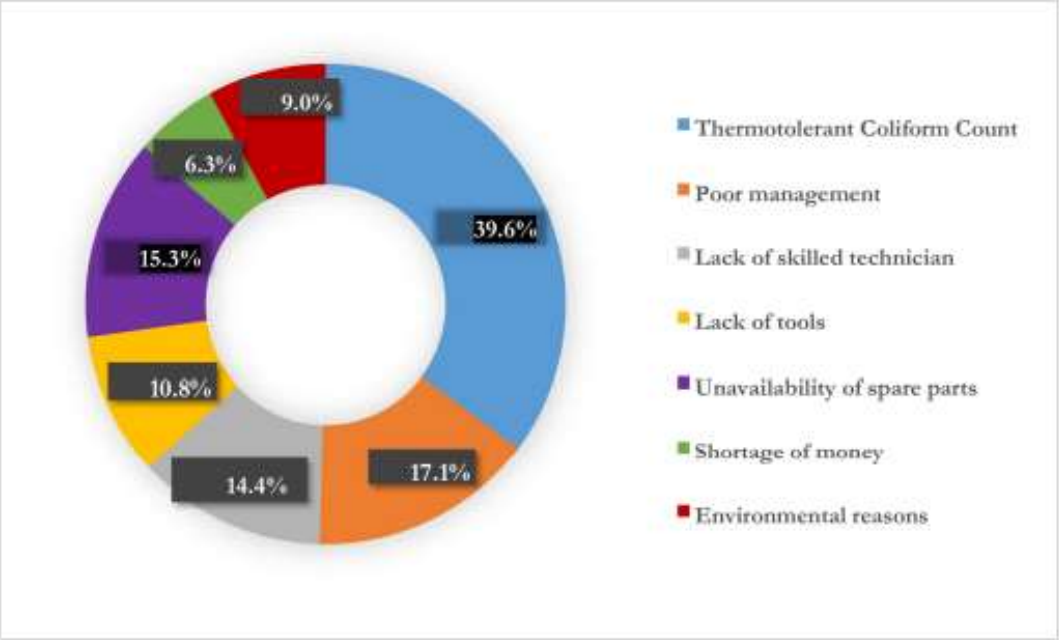


**Fig 4. Factors resulting in basic water services non-functionality in Amhara and Afar regions, Ethiopia.**

https://doi.org/10.1371/journal.pone.0248944.g004

Finally, including water quality as a parameter contributes to significant reductions of non-functionality of basic water services due to poor water quality by 34.2%. Hence, improving water microbiological water quality reduces the occurrence of morbidity and mortality due to non continues flow of water and contamination of water by disease causing micro-organisms such as TTC an indicator for diarrhea causing agents of Entrobactericeae.

## Water services management: Institutional findings of WASHCos in relation to water quality

Based on the responses of WASHCos', a significant number, 81 (73%) fully understood that the WASHCos are responsible and accountable to address water quality through water treatment/chlorination of the local water service. Twenty-three (20.7%) had partial knowledge and 7 (6.3%) did not know their duty and accountability. The significant number of WASHCos that understand their duty and accountability was also assured by the practice of WASHCos by taking over treatment of the basic water services largely (n = 43, 38.7%) (Table 3). Absence of clear role and responsibility of WASHCo membership might contribute to the loss of team cohesive work hindering the continues functioning of the water services. Almost all 110 of the WASHCos (99.1%) have reported obtaining capacity building trainings, which would contribute for those 60% of functional water services considering water quality. Overall, 108 (97.3%) WASHCos had taken training on management-related topics, 107 (96.4%) on operation and maintenance, 70 (63.1%) on sanitation and hygiene-water treatment/chlorination, 61 (55%) on child safeguarding (CSG) and 46 (41.4%) on gender. The data shows that several of them

**Fig 5. Growth of the thermotolerant microorganisms using membrane filter technique in water samples in Amhara and Afar regions of Ethiopia.**

https://doi.org/10.1371/journal.pone.0248944.g005

have taken two or more types of trainings (Table 7). Of the total water services and WASHCos, 20 (18.0%) were operating using tap attendants under the WASHCos structure that also contributes to the sanitation and hygiene promotion to address water treatment/chlorination. The

**Table 7. Results of institutional related factors in relation to water quality in Afar and Amhara regions of Ethiopia.**

| Variables | | Count | Percent |
|---|---|---|---|
| Knowledge on duty and accountability | Yes | 81 | 73.0 |
| | Partially | 23 | 20.7 |
| | No | 7 | 6.3 |
| Capacity building training for WASHCos | Yes | 111 | 100.0 |
| | No | 0 | 0.0 |
| Capacity training on management | Yes | 109 | 98.2 |
| | No | 2 | 1.8 |
| Capacity training on operation & maintenance | Yes | 108 | 97.3 |
| | No | 3 | 2.7 |
| Capacity training on sanitation and hygiene-water quality | Yes | 71 | 64.0 |
| | No | 40 | 36.0 |
| Capacity training on gender | Yes | 47 | 42.3 |
| | No | 64 | 57.7 |
| Capacity training on CSG | Yes | 61 | 55.0 |
| | No | 50 | 45.0 |
| Capacity training on other topics | Yes | 29 | 26.1 |
| | No | 82 | 73.9 |
| Tap attendants for water point-hygiene promoters (water quality) | Yes | 20 | 18.0 |
| | No | 91 | 82.0 |

https://doi.org/10.1371/journal.pone.0248944.t007

**Table 8. Results of institutional related factors for basic water functionality in Afar and Amhara regions of Ethiopia.**

| Variables | | Frequency | Percent |
|---|---|---|---|
| WASHCos recorded minutes | Yes | 54 | 48.6 |
| | No | 57 | 51.4 |
| Rate of meeting interval | Weekly | 3 | 2.7 |
| | Every two weeks | 6 | 5.4 |
| | Monthly | 40 | 36.0 |
| | Quarterly | 4 | 3.6 |
| | Other | 1 | 0.9 |
| WASHCos access to spare parts | Contractors leftover spare parts | 5 | 4.5 |
| | INGOs/NGOs | 9 | 8.1 |
| | None | 20 | 18.0 |
| | Woreda Market | 19 | 17.1 |
| | Woreda Water Office | 43 | 38.7 |
| | Zone Market | 15 | 13.5 |
| Trend for community gathering & discussion with WASHCos | Yes | 74 | 66.7 |
| | No | 37 | 33.3 |
| Accountability of WASHCos and reporting requirement | Yes | 52 | 46.8 |
| | No | 59 | 53.2 |
| Documentation of WASHCos -Financial/management manual | Yes | 25 | 22.5 |
| | No | 86 | 77.5 |
| Documentation of WASHCos- Administrative/bylaw manual | Yes | 57 | 51.4 |
| | No | 54 | 48.6 |
| Documentation of WASHCos-Minutes | Yes | 54 | 48.6 |
| | No | 57 | 51.4 |
| Documentation of WASHCos—Beneficiaries list | Yes | 86 | 77.5 |
| | No | 25 | 22.5 |
| Documentation of WASHCos-Savings account | Yes | 67 | 60.4 |
| | No | 44 | 39.6 |

https://doi.org/10.1371/journal.pone.0248944.t008

roles of the tap attendants include among others: opening the water point on time, ensuring proper queuing by users on the basis of first come first serve, collecting water fees on the spot of the water point and handover to the cashier of the WASHCo members, cleaning the public water point areas and promotion of sanitation and hygiene. In turn, tap attendants were paid on monthly bases by the WASHCos for rendering such water services (Table 7).

Several of WASHCos 43 (38.7%) both in Afar and Amhara regions were found to access spare parts from Woreda Water Offices (WWOs), a few 19 (17.1%) from Woreda markets and some others 15 (13.5%) from Zonal markets. Even though, providing of spare parts for WASHCos by private contractors and NGOs are not a common practice, it was interesting to find out that 9 (8.1%) and 5 (4.5%) WASHCos used to get spare parts from international non-governmental organizations (INGO)/NGOs and Contractors leftover spares, respectively. Indeed, a non-negligible number 20 (18%) of WASHCos were not accessing spare part at all. Of those WASHCos who accessed spare parts, 49 (44.1%) had established controlling mechanisms on how the spare parts are being used as part of material management. Documentation practice of WASHCos was also considered in this study using WASHCos interview, FGDs and records review. As a result, beneficiaries' lists by 86 (77.5%), saving accounts of bank by 67 (60.4%), administrative-bylaw of manuals by 57 (51.4%) and meeting minutes by 54 (48.6%) of WASHCos were documented (Table 8).

**Table 9. The levels of cooperation of WASHCo members in Amhara and Afar regions in Ethiopia.**

| Variables | Response | Yes | Percent |
|---|---|---|---|
| Teamwork of WASHCos | Low | 23 | 20.7 |
| | Fair | 31 | 27.9 |
| | Good | 31 | 27.9 |
| | Very good | 9 | 8.1 |
| | Excellent | 17 | 15.3 |

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Overall, the degree of cooperation, teamwork and socialization 54 of the WASHCos (48.6%) were categorized as low and fair (Table 9). These group dynamics deserve special attention for the success of executing their roles, duties and accountabilities properly. On the other hand, water user communities resulted in a deliberate damage of water structures because of a disagreement between upper and downstream water users with respect to water sharing for domestic, animal drinking and irrigation purposes. Further, the damage of water structures for one or other reasons might contribute to water contamination due to runoff that contains human or animal wastes.

## Water services management: Environmental findings

Water services non-functionality was seen from environmental perspective as it is one of the factors that could contribute to low performance of water services (Table 4). Accordingly, the result shows that environmental factors that contributed to the non-functionality of water services were indicated by 10 (9%) of the study participants. Environmental factors include climate change impacts such as flood, natural land slide, thunder, water yield reduction, high windstorm and shorter rainy seasons. Flood and natural land slide might contribute for the contamination of damaged or improperly constructed basic water services [40].

Rainfall variability as a result of climate change affects many spring water services in Amhara region and rainwater dependent technology types in Afar region. This study finding was reinforced by other study findings that more than 30% of water supplies [41] were non-functional as a result of seasonal problems, which is noted also in a study conducted in urban Ethiopia [42]. Moreover, environmental indicators had statistically significant relationship with functionality in one of the most recent studies [19] conducted in rural Ethiopia. This variation could be due to inconsistent list of environmental factors considered by different researchers. For instance, the later study focused on the awareness of the community on environmental-related adaptation measures while our study emphasized the actually occurred environmental related factors affecting functionality. Relatively, the RPS from motorized boreholes or shallow wells provide consistent water services apart from its dependency on the lifting device; submersible pump and generator functionality as the buffer aquifer storage capacity remain constant. Therefore, during water supply feasibility assessment and designing, use of local climate information to reduce non-functionality of basic water services is very important.

## Water services management: Financial findings of WASHCos

The main sources of finance, water fee collection trend, mode of payments of the water fee, practice of use of available finance for operation and maintenance of water services and the overall financial management methods are variables included under the financial factors of functionality of basic water services in our study. According to the large majority of respondents (n = 97, 87.4%), the main source of finance for the WASHCos was water fees collected

from users. Our result is higher than the result of a study [19] where 49.2% (n = 102) water users collect water fee in similar socio-economic status to our study area, rural areas of Ethiopia for day-to-day operation and minor maintenance. This high level of water fee payment observed in this study might be explained by the variations in motivation and capacity building works provided for WASHCos. Based on our finding, the minimum and maximum amount of money available in the bank or microfinance per WASHCos was nil and 370,000.00 ETB or (USD$0.00–11,200), respectively.

Overall, the total sum of the 111 WASHCo's finance was 742,275.00 ETB or (USD $22,490.00) with a mean value of 6,691.66 ETB or (USD$203.00). The mode of payment of water fee was predominantly 61 (55%) by WASHCos on a monthly basis. The study reveals that nearly one-third of WASHCos and water services (n = 35, 31.5%), didn't start water fee collections. This was however lower than data of another study that reported 53% (n = 357) did not pay water fees [20]. In fact, the better fee collection attained by the participants of the present study might be attributed to strong motivation and capacity building efforts of the WASHCo's. To overcome the ever-increasing basic water operation and maintenance costs, there is a need to encourage water services that lack collections to commence fee payments. Likewise, capacity building that included water quality-water treatment/chlorination, hygiene promotion and other incentive should continue for an improved maintenance of those, which are already practicing fee collection.

The study showed that among those who used to collect water fees, the maximum amount is 50.00 ETB (USD$1.51) per household (HH) per month with a mean value of 4.70 ETB or (USD$0.14) and standard deviation of 8.00 ETB or (USD$0.24). This finding is partly similar to the result of a study [23] that reported 3.2 ETB (USD$0.10) per household per month with standard deviation of 1.8 ETB (USD$0.06). Regarding the water fee collections within the custody of committee members 32 (28.8%) WASHCos were found to hold the allowable limits (<2,000.00ETB) for day-to-day O&M as per the agreed bylaw. Some WASHCos has never spent on O&M whereas, some other WASHCos had an expenditure of a maximum of 100,000.00 ETB or (USD$3,030.00) for O&M. The total sum of expenditure used for O&M by WASHCos were 473,808.00 ETB or (USD$14,360.00) with a mean amount of 4,307.35 ETB or (USD$130.00) and standard deviation of 13,977.75 ETB or (USD$425.00). Nine (8.1%) of WASHCos were used cash amount up to 2,000.00 ETB or (USD$60.00).

Moreover, there were total of 17 WASHCos (15.3%), which used cash amounting to greater than 2,000.00 ETB or (>USD$60.00) (Table 10). Effective mechanism of implementation of the basic water services tariff, supported with live business plan depending on the nature and technology types and the capital incurred during construction of the technologies, as well as including water quality improving activities is fundamental for functional basic water services and its benefit over time.

## Discussion

This research suggests that if the goal of SDG 6.1 is to be achieved "universal access to safe drinking water for all" water quality parameters must be mandatory, not optional, as the current definitions state. This research suggests two potential pathways for monitoring global and country-level progress toward SDG 6.1 and access to clean drinking water. Both pathways emphasize the importance of water quality in understanding existing water services available to people around the world. Since the presence of TC and TTC is associated with negative health outcomes in the form of mortality and morbidity, it is essential that water quality is isolated as a variable to be addressed through the application of chlorine by maintaining the 'pH' and turbidity of the basic water that provides operational and verification of monitoring water

**Table 10. Results of financial sources and its management in Amhara and Afar regions of Ethiopia.**

| Financial factors | | Count | Percent |
|---|---|---|---|
| Sources of finance or budget | Users | 99 | 89.2 |
| | No sources | 12 | 10.8 |
| Mode of payment of water fee | Per-Container | 5 | 4.5 |
| | Daily | 3 | 2.7 |
| | Monthly | 61 | 55.0 |
| | Free | 23 | 20.7 |
| | Other | 19 | 17.1 |
| Amount of water fee per HH per month | Not set | 36 | 32.4 |
| | 0.10–5.00 | 56 | 50.5 |
| | >5–10.00 | 7 | 6.3 |
| | >10 | 12 | 10.8 |
| Water fee at committee members hand | None | 76 | 68.5 |
| | 1.00–2000.00 | 32 | 28.8 |
| | > 2000.00 | 3 | 2.7 |
| Water fee not collected | Fee collected | 107 | 96.4 |
| | Not collected | 4 | 3.6 |
| Cash used for operation and maintenance | None | 85 | 76.6 |
| | Up to 2,000.00 | 9 | 8.1 |
| | > 2000.00 | 17 | 15.3 |

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quality parameters. Currently, water services classified as "basic" may lack nearby accessibility, potability, *or* availability. This means that multiple variables are collapsed through a classification of water services as "basic." While additional detail has been captured by defining basic water services as functional or non-functional, this does little to provide actionable knowledge about the potability of water providing through basic water services. Water quality provides the strongest metric toward achieving SDG 6.1 goal of "safe drinking water" since achieving accessibility and/or availability water of poor quality does little to address the mortality and morbidity experienced by millions around the globe

In the first suggested pathway, a water service must offer non-contaminated drinking water to be classified as "functional." In other words, this suggested revision places a water quality parameter *inside* the definition of "functional" or "non-functional" water services. In current definitions, classification as functional or non-functional is based upon whether or not the water service operates to deliver water at the time of survey. See Table 11 for the suggested revisions to the SDG definitions.

A second potential pathway for improvement would be to alter the Drinking Water Ladders used in SDG Water Monitoring. In its current form, too many variables are collapsed into the definition of "basic water" services—policymakers and interested stakeholders do not know if the issue is water quality, availability, or accessibility. Adding a rung that specifies water quality with nearby (not on premises) accessibility will allow for better clarity and data capture around precisely how to target improvement programming, policies, and development schemes. See Table 12 for the suggested additional "safe basic water service" rung to be placed below "safely managed water service" but above "basic water service."

This study does have limitations. As this study design was an observational cross-sectional, it was possible to determine if there were association between variables but not causal effects or inferences. Local government structures such as Zonal and Woreda Water Offices were not included in the study, which could help to substantiate findings through the triangulation of

**Table 11. Suggested revisions to basic water services—Adding water quality parameters to functional and non-functional classifications.**

| | Functional Basic Water Service | Non-functional Basic Water Service |
|---|---|---|
| **SDG Definition** | If the water from improved source does not meet any **one** of the three criteria (source is accessible on premises, water is available on demand, water is free from contamination and a round trip to collect water takes 30 minutes or less including queuing. | If the water service from improved source fails to fully operate or only partially operates at time of survey. |
| | *Water quality optional* | *No water quality priority parameter considered* |
| **Suggested Revision** | If the water from improved source is free from contamination of priority parameters (TC and TTC/*E. coli*), **and** it is not on premise **or** available on demand, **and** a round trip to collect water takes 30 minutes or less including queuing. | If water service is fails to fully operate or only partially operates at time of survey **or** is not free from priority contaminants (TC and TTC/*E. coli*). |
| | *Priority water quality parameter mandatory* | *Priority water quality parameter mandatory* |

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data from different sources. Future research could include these additions or expand this research design to new regions and woredas that experience less food insecurity.

## Conclusions

This research suggests that if the goal of SDG 6.1 is to be achieved "universal access to safe drinking water" water quality parameters must be mandatory, not optional, as the current definitions state. The findings suggest two potential pathways for improving data collection around water services, water quality, and determining the functionality of basic water services —including water quality as a parameter of "functionality" of a water service or by adding a rung titled "safe basic water service" on the Drinking Water Ladder for monitoring.

Our findings from Amhara and Afar regions of Ethiopia emphasize the importance of water quality to be added to the definition of basic water services in global development

**Table 12. Suggested revisions to drinking water ladder rungs: Adding "Safe Basic Water Service"**

| Ladder Rungs | Rung Definition |
|---|---|
| **Safely managed water service** | Drinking water from a source that is (1) accessible on premises **and** (2) free from contamination **and** (3) available on demand. |
| **Safe basic water service** | Drinking water from an improved source that is (1) accessible nearby so that a round trip to collect water takes 30 minutes or less including queuing **and** (2) free from contamination. |
| | If water is operationally available on demand, at time of survey, then it may be classified as functional. |
| | If water is not operationally available on demand, at time of survey, then it may be classified as non-functional. |
| **Basic water service** | If an improved source does not meet any **one** of the three criteria **and** a round trip to collect water is *less than* 30 minutes. |
| | If water is operationally available on demand, at time of survey, then it may be classified as functional. |
| | If water is not operationally available on demand, at time of survey, then it may be classified as non-functional. |
| **Limited water service** | If an improved source does not meet any **one** of the three criteria **and** a round trip to collect water *exceeds* 30 minutes. |
| **Unimproved water service** | Drinking water from an unprotected spring or dug well. |
| **Surface water** | Drinking water taken directly from a river, lake, pond, canal, irrigation channel or other. |

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indicators. 38 (34.23%) of "functional" water services are reclassified as "non-functional" water services when we include water quality results—meaning this water is not "safe drinking water." Speaking more broadly, for water and development specialists focused on achieving the Sustainable Development Goal 6 regarding global achievement of "clean water and sanitation," our findings from Ethiopia suggest that additional research in other developmental contexts should be undertaken to assess the percentage of basic water services that, when water quality is taken into account, are found to be non-functional. Further, our findings around WASHCos demonstrate that research and developmental programming in other countries would do well to consider strengthening local management of water services and train local managerial committees in the importance of water quality and methods for water quality treatment.

## Supporting information

**S1 File. Survey questionnaire and FDG checklist.**
(PDF)

**S2 File. Laboratory protocol for bacteriological and physico-chemical technique in water sample analysis.**
(PDF)

**S3 File. Data of functionality and water quality assessment for basic water.**
(XLS)

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This study was written during the lockdown period of state of emergency as a result of the Novel Coronavirus Disease (COVID-19) pandemic occurred in more than 200 territories. Therefore, we would like this study to be in memorial of those above 8.3 million morbidity cases and above 450 thousand of mortalities or deaths caused by COVID-19 globally up to the date of first submission.

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**Action:** As part of the dissemination strategy, we have shared the research finding for about WASH sector of Policymakers-Government (MoWIE & MoH), Bilateral & Multilateral donors (DFID, ADB, UNICEF, WB, EU), NGOs/Development Partners (CO-WASH, WAE, SNV, PLAN, SCI, WVI), Universities & research Institutes-Scholars and Water Management Bodies. UNICEF taken the initiative to pilot and share the lessons for the JMP for revision consideration.

**Annex-4. Article published: Scoping Rivew Protocol-titled 'PCR-based detection of pathogens in improved water sources: a scoping review protocol of the evidence in low- and middle-income countries**

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BMJ Open

# PCR-based detection of pathogens in improved water sources: a scoping review protocol of the evidence in low-income and middle-income countries

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## ABSTRACT

**Introduction** Occurrence of diverse human enteric bacterial, viral and protozoal pathogens in improved drinking water because of pathogenic microbial contamination is of increasing public health concern, particularly in low-income and middle-income countries (LMICs). Detecting microbial pathogens in water supplies comprehensively and accurately is beneficial to ensure the safety of water in LMICs where water contamination is a major concern. Application of PCR-based methods in detecting the microbial quality of water provides more accurate, sensitive and rapid outcomes over conventional methods of microbial identification and quantification. Therefore, exploring water quality outcomes generated through PCR-based methods is important to better understand the status and monitor progress towards internationally set goals for LMICs. This scoping review aims to map the existing evidence on the magnitude and characteristics of diarrhoeagenic pathogens as detected by PCR-based methods in improved water sources within the context of LMICs.

**Methods and analysis** This study will be undertaken in line with the Joanna Briggs Institute (JBI) methodology for scoping reviews. We will consider the available publications covering PCR-based microbial water quality assessment of improved drinking water sources in LMICs. Searches will be undertaken in PubMed/Medline, Scopus, Web of Science, JBI, Cochrane Library and Google Scholar. A grey literature search will be conducted in Google and ProQuest.

**Ethics and dissemination** The College of Natural and Computational Science Institution Review Board of Addis Ababa University gave formal ethical approval to this study protocol. The findings of this study will be disseminated to the concerned body through peer-reviewed publications, presentations and summaries.

## STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ Three review authors will work independently to select studies, extract data and assess quality.
- ⇒ To produce more comprehensive results on pathogen detection using PCR in improved water sources, this study will collect data from a variety of sources.
- ⇒ Only studies written in English will be included in this scoping review.

## INTRODUCTION

Genetic diversity of human enteric bacterial,[1 2] viral[3–6] and protozoal[7] genomes in water is increasing and becoming complicated as a result of contamination of improved water at the sources, collection and home storage that creates increasing public health threats in low-income and middle-income countries (LMICs). Detecting microbial pathogens in water supplies comprehensively and accurately is beneficial to ensure the safety of water in LMICs where water contamination is a major concern.[8] Traditional techniques of using pure cultures grown in labs to characterise microbial water quality have limitations due to false-positive results[9–13] and inability to identify non-culturable microbes since only <1% of natural microorganisms are culturable.[14 15] The causing agent of up to 40% of diarrhoea cases cannot be identified using the culture method.[16] Water-related pathogens were known to cause persistent or acute diarrhoea in humans, dehydration-related diarrhoea in infants and children, traveller's diarrhoea, watery diarrhoea lasting up to a week, and bloody diarrhoea or dysentery.[17] Very limited attention has been paid to the non-culturable but harmful microorganisms[18 19] such as enteric toxin *Escherichia coli*[18] and *Legionella pneumophila*.[20] These are causative agents for diarrhoea and pneumonia which are particularly common in LMICs. A method to characterise microbial quality that provides a relatively unbiased picture on the species type, quantity, distribution and functionality of pathogenic microorganisms in water supply within the context of LMICs is essential. This can help to monitor the progress towards achieving the UN Sustainable

Development Goal target 6.1 (SDG6) such as safely managed drinking water services (SMDWS).[21]

For successful monitoring of SMDWS, it is important to draw on reliable water quality outcomes generated using effective methods of characterising a variety of pathogens in water samples, beyond bacterial presence/absence and colony count.[22] Fresh perspectives on the molecular technique of PCR were first described in 1985 by a scientist named Mullis Kary,[23] followed by the sequence-based molecular method that was first applied for drinking water quality assessment in 2003.[14]

PCR is a simple chain reaction method applied for genome-wide analysis through a three-step procedure involving information coding of nucleic acids (DNA/RNA, mRNA) and protein. The methods are powerful molecular techniques that can be used for a range of applications, from detecting up to quantification of bacterial, viral and protozoal pathogens in small quantities (1–2 µL) of water samples.[24] Furthermore, PCR, compared with conventional methods, increases the overall detection frequency by 4% of the bacterial enteropathogens,[25] measures specific gene expressions more rapidly,[26] and detects enteric viruses and enterococci, which can appear in the form of viable but non-culturable pathogens quantitatively and rapidly in water samples.[27] Moreover, some PCR techniques can distinguish viable cells from dead cells.[24]

The advances in pathogenic microbial testing methods improves our understanding about newly emerging waterborne diarrhoeal pathogens. For example, reports showed an increasing number of different microbial genotypes[28] that can cause diseases. Water quality results obtained through PCR methods would give a better picture in providing sensitive and rapid results[29] and can be applied as a microbial source-tracking tool for generating more reliable information, which in turn contributes to addressing the SDG target for water supply in LMICs. Despite its importance, the use of PCR in the identification and quantification of water pathogens has not been taken up by the LMICs. On the other hand, the disadvantages of PCR-based water quality methods for environmental water samples, which may not be the case with samples from improved sources, are: (1) Need to isolate the microorganisms due to the dilution effect (low concentration of microorganisms per volume) or require a culture step to increase the number of microorganisms in the sample. (2) Pollutants can accumulate along with the target microorganisms for environmental water, which may not be the case for water samples from improved water. (3) Membrane clogging during filtration of environmental water samples, which is not the case with water samples from improved water sources. (4) Loss of target cells and/or nucleic acids during the various water sample preparation steps (concentration, extraction, purification), which can result in the inefficient concentration of the target microorganisms to be detected and consequent false-negative results. (5) Lengthy and complex procedures; and (6) Inhibitors are difficult to remove and are not eliminated (some have the same solubility properties as nucleic acids).[30]

The different types of waterborne pathogens detected using PCR methods, as well as their magnitude in terms of rates or concentrations in various developed countries have been outlined. In the USA, *E. coli* O157:H7 was found in drinking water at a concentration of 30–40 cells per 100 mL.[29] In another study conducted in Florida, USA, *Cryptosporidium* rates were 45%, poliovirus rates were 55% and *Giardia* rates were 41%.[7] *E. coli* cells were detected at a mean concentration of 310 cells per 100 mL isolated from rainwater tanks in Australia.[31]

This review will discuss current and emerging advanced techniques for comprehensively and accurately assessing pathogenic microbial water quality that authorities in LMICs can use. The purpose of this scoping review, therefore, is to map the existing evidence on the magnitude and characteristics of diarrhoeagenic pathogens detected by PCR-based methods in improved water sources in the context of LMICs.

## REVIEW QUESTION

The scoping review will be guided by the question, 'What are the types, magnitude and concentrations of waterborne pathogens detected in improved water samples from LMICs through the PCR method?'

## METHODS AND ANALYSIS

### Study design and protocol

The research question is best addressed through a scoping review, as it is broad and aims to map all the available evidence on the topic. This scoping review will be undertaken in line with the Joanna Briggs Institute (JBI) methodology.[32] Briefly, the first step in the scoping review is defining the review question using the Participant (Drinking Water Sources), Concept (PCR-based Microbial Quality), and Context (LMICs) or the Population, Concept and Context (PCC) framework. The PCC inclusion criteria which are discussed in detail below will be used for structuring the search strategy. After selecting the relevant studies to be included in the review, data will be extracted using a prepared data extraction excel spreadsheet tool standardised and presented in a tabular/diagrammatic form and a narrative summary in line with the scope of the review.

### Inclusion criteria

#### Types of water sources

For this review, we will consider studies that included PCR-based microbial water quality assessment of improved drinking water sources. We will use the standard definition of 'improved drinking water source' as it is defined by the WHO/UNICEF Joint Monitoring Programme, as a water source that, by nature of its construction, is adequately protected from outside contamination, particularly faecal matter.[33] This definition includes piped water

in a dwelling, plot or yard, and other improved sources such as public taps or standpipes, tube wells or boreholes, protected dug wells, protected springs, and rainwater collection. This review will not include water quality data about the following 'un-improved' water sources: unprotected dug well, unprotected spring, cart with small tank/drum, tanker truck and surface water (river, dam, lake, pond, stream, canal, irrigation channels). Although it is categorised as 'un-improved' in the above definition, bottled water will be considered in this review as the reason for its categorisation as 'un-improved' (ie, in relation to 'water quantity') is not relevant for the focus of this review which is on 'water quality'.

### Concept

The overarching concept of interest for this scoping review is the microbial quality of drinking water determined by PCR-based methods. Accordingly, this review will consider studies that identified, quantified and characterised pathogens in drinking water using PCR techniques such as digital PCR and real-time quantitative PCR (qPCR). Employed PCR detection methods and detection capacity, type of detected pathogens, detected gene numbers/copies, types and associated waterborne diseases will be explored.

### Context

This review will include studies conducted within the context of LMICs. The World Bank classification of economies according to 2019 gross national income per capita will be used to identify these countries.[34]

## Types of studies

All peer-reviewed studies that characterise diarrhoeagenic pathogens in improved water sources using PCR techniques and conducted in LMICs will be included in this review. These may include quantitative and/or mixed-method studies, cross-sectional studies, and intervention/experimental studies. Conference items/abstracts will not be considered in this review to avoid potential duplication.

## Search strategy

The literature search strategy consists of a three-stage process and will be structured using the PCC framework in line with the JBI scoping review methodology.[35] In the first stage, text words contained in the titles and abstracts of the articles will be reviewed. In the second stage, a fully comprehensive search will be undertaken using the following search terms: (/Improved drinking water sources) AND (/PCR-based microbial quality) AND (/Detection OR Identification OR Characterisation) AND (/Pathogenic microbes) (/Low- and middle-income countries) (see online additional file 1) for PubMed. A full comprehensive search will be undertaken in the following databases: PubMed/Medline, Scopus, Web of Science, JBI, Cochrane Library and Google Scholar. Relevant index terms will be identified and a search grid will be developed for each database with guidance of a research librarian. Only peer-reviewed full-text studies conducted in LMICs (ie, the Context) and published in English will be included. Studies that are not peer-reviewed and literature reviews will be excluded. In the third and final stage, the reference lists of the included studies will be examined to identify additional sources.

## Study selection

All identified citations will be exported to Mendeley Desktop reference management software V.1.19.4 (Mendeley, Elsevier, the Netherlands). After removing duplicates, the titles and abstracts of the identified studies will be reviewed by three independent reviewers (STG, NES and YMT). The independent reviewers will create a single online Mendeley software reference management account for data extractors to share articles. The documents without abstracts will be screened at the full-text level. Then, the full texts of selected studies will be retrieved and assessed in detail against the inclusion criteria by the same reviewers. Any disagreement between the three reviewers during the screening stage will be resolved through discussion or by the involvement of a fourth reviewer. For the screening of articles at full-text level, rejection of an article will be decided by the review team on the suggestion of the first reader. At each stage, the number of studies excluded and the reasons for exclusion will be archived and reported in the final scoping review. The results of the search strategy and screening process will be reported in full in the final scoping review report and presented in a Preferred Reporting Items for Systematic Reviews and Meta-analyses flow diagram.[36]

## Data extraction

Data will be extracted by three independent reviewers (STG, NES and YMT) from studies included in the review using a prepared data extraction excel spreadsheet tool. The spreadsheet will be prepared, piloted for feasibility and modified based on the results of the pilot test. For each study, authors' name, place and year of publication, study period, date of search, the country/region of the study, type of water source, water treatment technique, if any, sample type, data on sample size, types of PCR techniques used, the result of included studies: detected type of pathogen/s, amount, detection capacity and associated waterborne diseases will be extracted. We will also include the indirect or direct viability data comprising culturable faecal indicator microbes such as *E. coli*, *enterococci* or other effects on free chlorine and total chlorine levels to understand the potential risk of positive detections. Viable but non-cultivable microorganisms that may be present as a result of environmental stresses or water treatment processes are not detected by culture-based methods and can therefore lead to false-negative assessments of *E. coli* in water samples.[31] SRG will reconcile the extracted data to avoid missing important information or including irrelevant information. The data extraction form will be pretested and revised as necessary during the review process. We will apply the dMIQE2020 guideline

with a simplified and modified minimum information for publication of quantitative digital PCR experiments (dMIQE) table to determine the quality of the data reported in the studies to be collected. The application of this method helps in assessing the number of nucleic acids, including molecular weight and calculations when using mass.[37] Similarly, we will use the minimum information for publication of quantitative Real-Time PCR experiment (MIQE) checklist for the real-time qPCR data.[38] The reviewers will contact the authors of primary studies for missing data and clarifications if required; such inclusions will be reported in the final scoping review. A particular study will be excluded if there is no response from the author(s).

## Collecting, analysing and reporting the results

Following data extraction by independent reviewers, the studies will be classified based on the type of waterborne pathogen detected by PCR in improved water sources, detection methods and the level of contamination investigated, the setting, and the study method, as well as significant findings (see online additional file 2). We will create a useful summary that identifies and maps important evidence on the magnitude and characteristics of pathogens identified by PCR in improved water sources by combining all relevant findings from multiple sources. The data will be managed using a narrative synthesis approach, with the aim of summarising and explaining the findings of these studies. This will include a numerical summary of all of the review's findings, thematic analysis of qualitative data and summary statistics of participatory studies to summarise outcomes.

## Patient and public involvement

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

## Ethics and dissemination

The College of Natural and Computational Science Institution Review Board (CNS-IRB) of Addis Ababa University gave formal ethical approval to this study protocol. As one of the objectives in the fulfilment of the PhD title 'Identification and characterization of major diarrheagenic bacteria in improved water supply systems and consuming community in South Wollo, Ethiopia', this study protocol was approved by the CNS-IRB, Addis Ababa University Review Board (award/grant number=CNSDO/729/10/2018) dated 24 July 2018. The findings of this study will be disseminated to the concerned body through a peer-reviewed publication, presentations, and summaries, email and social media.

**Contributors** STG deliberated on the subject of the study, participated in its design and coordination, was involved in data extraction, critically appraised all papers selected for inclusion in the review, drafted the protocol, and mainstreamed and included feedback from other team members. NES and YMT developed the research methods and supported the first drafting of the manuscript. AFD and SRG conducted the review, and read and shaped the manuscript. All authors contributed to the drafting of the protocol, and have read, reviewed and approved the final manuscript.

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**Competing interests** None declared.

**Patient and public involvement** Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

**Patient consent for publication** Not applicable.

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**Annex-5. Published Article-titled 'Diarrheagenic toxins in stool correlate to drinking water from improved water sources in Ethiopia'.** https://www.editorialmanager.com/envc/default1.aspx

**Annex-5.1. Antibiotic microbial resistance (AMR) and biochemical tests**

**1.1.1. Biochemical tests from stool samples: At AHRI national laboratory.**

Selected biochemical tests were performed before performing the PCR tests for the stool samples. Accordingly, the sulfur, indole, motility (S.I.M.) test and the gas production test using S.I.M. medium-IVD-Ref-610181 tests were carried out after culturing the inoculates on Trypticasein Soy Agar (TSA) from Conda, category number 1068.00 from Farm Europe of 40 g per liter at 37 $^0$C for 24 hours for a total of 390 isolates from a total of 248 samples.

Indole was produced as a result of the degradation of the amino acid tryptophan hydrolyzed with tryptophanase upon the addition of three drops of Kovac's Indole Reagent (Cat. no. 49431AR). S.I.M medium contains ferrous ammonium sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide. Hydrogen sulfide production is detected when ferrous sulfide, a black precipitate, is produced as a result of ferrous ammonium sulfate reacting with $H_2S$ gas (see the Figure 11 below).

As a result of the biochemical tests, out of the total of 390 isolates, a total of 352 isolates were classified as *E. coli* species (A-D), 23 isolates were Shigella or *E. coli* species (A-D), 14 isolates were *Kelebsela pneumonia* and one isolate was Providence stewarti (these species isolates were subject to further confirmatory analysis).

**Figure 14. The pictures show that the result of the consecutive biochemical tests.**

### 5.1.2. Antibiotic microbial resistance for NLF

We conducted a disc diffusion susceptibility test for non-lactose fermenter (NLF) bacteria. We cultured the isolates using Mueller-Hinton-Agar (MHA) ref: 225250 of

batch number 6209540 with a ratio of 38 g for 1 liter at 37 $^0$C for 24 hours. Using the bacteria solution, the cultured bacteria were inoculated to the level of the turbidity standard of 0.5 McFarland equivalent. The suspensions of the test bacteria were smeared on the entire area of the Petri-dish with Mueller-Hinton-Agar using sterile cotton-tipped swabs. Four types of antibiotic discs (Cefotaxime 30 µg (CTX30), Meropenem 10µg (MEM10), Ceftazidime 30 µg (CF30) and Imipenem 10 µg (IPM10)) were placed on the smeared the Petri-dish appropriately using sterile forceps and incubated at 37 $^0$C for 24 hours. The zone of inhibited diameters of the Petri-dishes were measured in units of millimeter. The results are shown in the below Table 16.

**Table 17** Antibiotic Microbial Resistance test result for non-lactose fermenter bacteria

| Sample code | Antibiotic Discs | Measurement in mm | Result from the standard (CLSI) in mm | | | Conclusion |
|---|---|---|---|---|---|---|
| | | | Susceptible (S) | Intermediate (I) | Resistant ® | |
| THS34-2 | CTX30 | 27 | ≥ 21 | 18–20 | ≤ 17 | Susceptible |
| | MEM10 | 25 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 9 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | IPM10 | 24.5 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| THS29 | CTX30 | 26.5 | ≥ 21 | 18–20 | ≤ 17 | Susceptible |
| | MEM10 | 25 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 11 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | IPM10 | 25 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| KHS27 | CTX30 | 28 | ≥ 21 | 18–20 | ≤ 17 | Susceptible |
| | MEM10 | 32 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 12 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | IPM10 | 33 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| ZAHS21 | CTX30 | 10 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | MEM10 | 23 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 1 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | IPM10 | 26 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| KaHS19 | CTX30 | 30 | ≥ 21 | 18–20 | ≤ 17 | Susceptible |
| | MEM10 | 31 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 17 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | IPM10 | 28.5 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| MHS16 | CTX30 | 24 | ≥ 21 | 18–20 | ≤ 17 | Susceptible |
| | MEM10 | 26 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 8 | ≥ 21 | 18–20 | ≤ 17 | Resistant |

| | | | | | | |
|---|---|---|---|---|---|---|
| | IPM10 | 28 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| THS54 | CTX30 | 30 | ≥ 21 | 18–20 | ≤ 17 | Susceptible |
| | MEM10 | 34 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |
| | CF30 | 16 | ≥ 21 | 18–20 | ≤ 17 | Resistant |
| | IPM10 | 35 | ≥ 23 | 20–22 | ≤ 19 | Susceptible |

The AMR test result shows that of the seven stool samples tested for AMR, all seven stool samples were resistant to Cefotaxime 30 µg (CF30). Seven stool samples were susceptible to the three drugs (CTX30, MEM10, IPM10) in the Cephalosporin/Clavulanate disc versus the zone of diameter greater than the standard of the Clinical and Laboratory Standard Institute (CLSI) which are a positive producer of extended spectrum beta-lactamase (ESBL).


**Figure 15.** These pictures show the AMR test carried out for the selected few samples of stool.

## Annex-6. Applied lab protocol

**Quick-Start Protocol**

**April 2018**

**QIAamp® DNA Mini Kit**

1. Cut filter paper (47mm diameter) into small pieces and place in a 1.5 ml microcentrifuge tube.
2. Add 1ml Buffer ASL
3. Mix by vortexing at 14,000rpm for 2 minutes.
4. Boil or heat the suspension at 70°C in water bath until completely lysed 5 minutes.
5. Vortex for 15 seconds.
6. Centrifuge the sample at full speed (14,000rpm) for 1 minute to pellet the particles in the filter.
7. Pipet 1.2ml of the supernatant into a new 2ml micro-centrifuge tube and discard the pellet.
8. Add 1 inhibitEX tablet or buffer and vortex and centrifuge the sample at full speed for 3 minutes.
9. Centrifuge the sample at full speed for 3 minutes.
10. Pipet 15 µl proteinase K into a new and empty 1.5ml micro-centrifuge tube.
11. Pipet 200 µl supernatant from the sample and add to the micro-centrifuge tube that contains proteinase K.
12. Add 200 µl Buffer AL. Mix thoroughly by vortexing for 15 s.
13. Boil or heat the suspension at 70°C for 10 min.
14. Add 200 µl ethanol (96–100%). Vortex for 15 s. Briefly centrifuge the tube to remove drops from the lid.
15. Briefly centrifuge the tube at full speed (14,000rpm) for 1 minute to remove drops from the lid.
16. Label the lid of a new QIAamp spin column and place it into a 2ml collection tube.
17. Pipet completely the mixture onto the QIAamp Mini spin column (in a 2 ml collection tube).
18. Centrifuge at 14,000 rpm for 1 min.
19. Carefully open the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW1. Centrifuge at 14,000 rpm for 1 min. Discard the flow-through and collection tube.
20. Carefully open the QIAamp Mini spin column in a new 2 ml collection tube and add 500 µl Buffer AW2. Centrifuge at full speed 14,000 rpm for 3 min. Discard the flow-through and collection tube.
21. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube and centrifuge at full speed for 1 min. This eliminates the chance of possible Buffer AW2 carryover.
22. Transfer the QIAamp spin column into a new, labelled 1.5 ml micro-centrifuge tube.
23. Pipet 150 µl Buffer AE directly onto the QIAamp membrane. Close the cap

24. Incubate at room temperature (25degree centigrade) for 1 minutes
25. Centrifuge at 14,000rpm for 1 minute to elute DNA
26. Remove the filter and close the cap that contains DNA for PCR.

## Annex-7. Survey data collection questionnaire

### RT[1]-Characterization of major diarrheagenic bacteria in IWS[2] and CC[3], Ethiopia

Good morning/ good afternoon. My name is (please tell your full name). I am part of a team who are studying the characterization of major diarrheagenic bacteria in improved water supply systems and the consuming community, in South Wollo, which is in your community. Your local leaders granted us permission to conduct this study. You were selected to answer some of our questions as you visited the health facility in the study period. If you agree to participate in the study, I will ask you questions about your drinking water, eating habits, and collect samples of the water that you are drinking for the purpose of laboratory examination. The interview will take approximately 15 minutes. Aim/Objective: This research will focus on determining the concentration, characteristics and the level of risk of exposure to diarrheagenic bacteria related to improved drinking water services and the consuming communities. In addition, it will contribute to the identification of pathotype, genotype and characterization of species and strain types of waterborne bacterial disease using state of the art technology detection of pathogenic organisms or molecular techniques so as to assist in the program by delivering information for the local practitioners working in prevention, diagnosis and control of bacteria that cause diarrhea.

Volunteer to participate

(•) Yes ( ) No

G1: Thank you for your time!

## Group

***Annex-I: Tools for case and sample result report - Diarrhea Case Report and Stool Sample Recording Template by Woreda***

Section A: Socio-Demographic Questions

A1: Name of technical Person obtaining consent

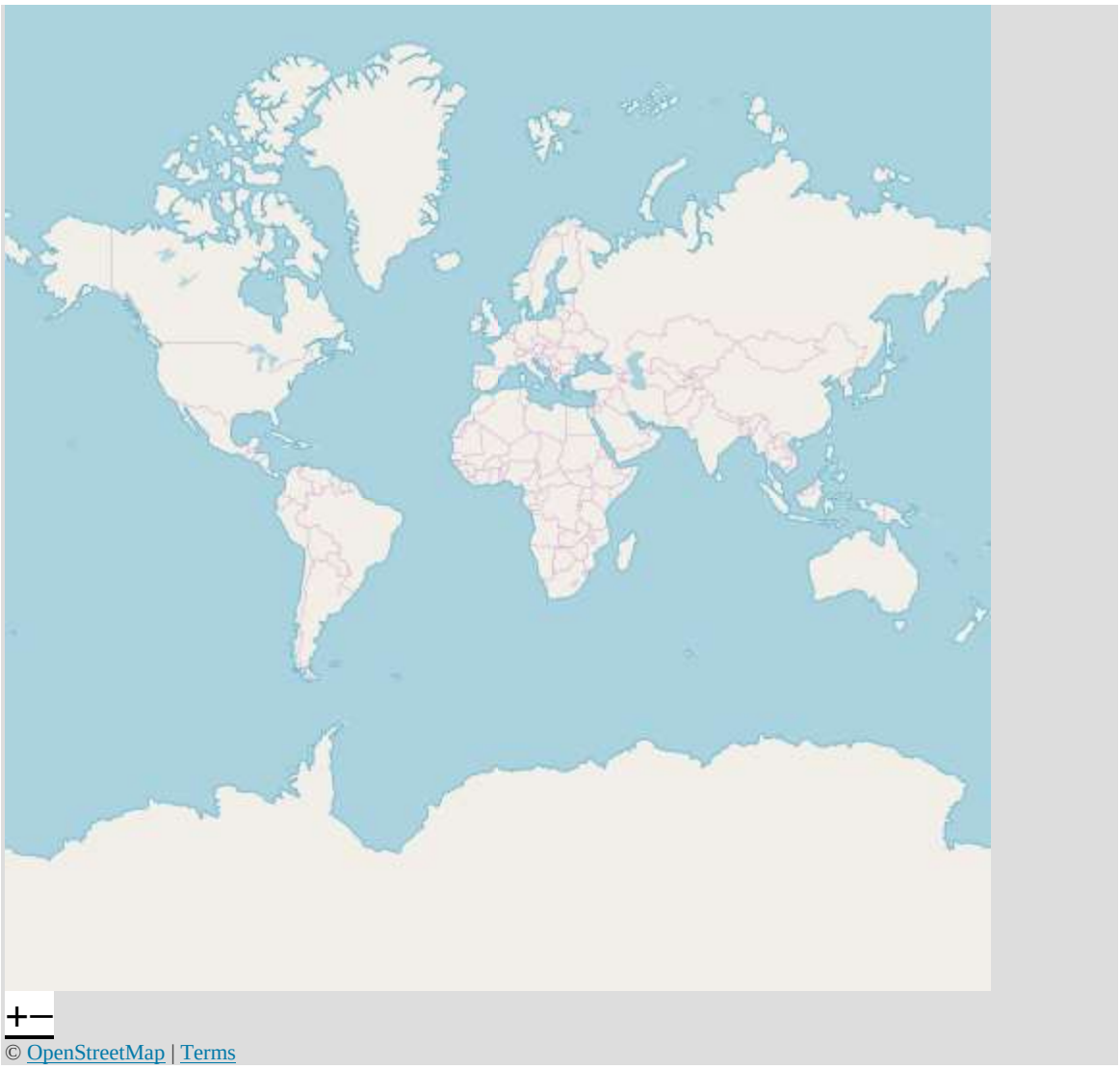
A2: Name of health facility

A3: Type of health facility

( ) Hospital ( ) Health Centre ( ) Health Post

A4: GPS Location of the health facility* *Please use an approximation of 8 meters*

[1] RT – Research Tool
[2] IWS - Improved Water Supply
[3] CC – Consuming Community




latitude (x.y °) longitude (x.y °) altitude (m) accuracy (m)

A5: Name of interviewer

A6: Visit Date and Time

A7: Card number/code of interviewee/Respondent

A8: Age (in Year)*_E.g. If a person is 23 years and 11 months, please insert age is equal to 23. If a child is less than one year please insert age is equal to Zero_

A9: Does the child have a guardian?*

Yes No

G2: This child is excluded since he/she do not have a guardian

## Group

A10: Phone number of the case with other phone number options*

A11: Name of the woreda the case came from

○ Dessie Zuria ○ Kutaber ○ Wuraelu ○ Legehida ○ Tenta ○ Mekedela

A12: Name of the village the case came from

A13: Sex

○ Female ○ Male

***Annex-2: The following questions are exclusion points that terminate the case not to included in the study.***

B1: Have you eaten any raw food in the last one month?**Raw food: Any uncooked animal or plant origin food*

◉ Yes ○ No ○ I don't know

G3: This person is excluded since he/she ate raw food during the last one month

**Group**

B2: MUAC[4] if pregnant and postpartum women (with infants less than 6 months) measurement less than 23cm.

◉ Yes ○ No

B3: MUAC for children less than 5-year measurement less than 13cm.*

◉ Yes ○ No

B4: MUAC for children between 5 to 18 years' measurement less than 18cm.

◉ Yes ○ No

B5: MUAC for Adults greater than or equal to 18 years' measurement less than 25cm

◉ Yes ○ No

G4: This person is excluded since his or her MUAC size is less than standard cut-off point

**Group**

B6: Have you traveled outside your permanent area in the last two weeks? *

◉ Yes ○ No

G5: This person is excluded since he/she traveled outside of his/her permanent residence in the last two weeks?

**Group**

***Annex-3: Objective-1: E. coli toxin examinations:***

C1: Age of the case

C2: Sex of the case

C3: Woreda of the case where to live

[4] MUAC – Mid-Upper Arm Circumference

C4: Diarrheal Type Using WHO classification code by history (sign and symptom) and microscopy examas reported by health facilities? *

- Enteroaggregative *E. coli* (EAEC)-104
- Enteropathogenic *E. coli* (EPEC): O127:H6E2348/69 – 105
- Diffusely Adherent *E. coli* (DAEC): - 105
- Entrotoxigenic *E. coli* (ETEC) or Verocytotoxigenic *E. coli* (VTEC) -106
- Shiga Toxin producing *E. coli* (STEC) – 106
- Enterohemoregic *E. coli* (EHEC) – 106
- Entroinvesive *E. coli* (EIEC) – 106
- Other

C5: Please indicate classification Other than mentioned above*

Take stool sample

**G6: Thank you for your participation!**

C6: Please indicate classification Other than mentioned above*

***Annex-5: Examination result of improved water sources***

E1: What sources (water technology type) did your drinking water come from?**If more than a source: please use the information of the most closest to the household and/or currently used*

Piped connection to yard/household Public stand/tap Borehole Protected Shallow Well Protected dug well Protected on spot spring Protected gravity spring Rain water Other

E2: Please mention the water technology type other than listed above*

E3: Name of the water source come from/observed*

E4: Can you tell me all the ways you know to make water safer to drink in your home?*

Boiling Liquid chlorine Chlorine tablets Coagulant/flocculent Solar disinfection Ceramic filter Bio Sand filter Membrane filter Cloth filter Other Do not know Option 12

E5: Other reason for making water safer to drink at your home?*

E6: Do you make the water safer to drink at your home?*

Yes No Do not know

E7: If yes, how did you make the water safer to drink?*

Boiling Liquid chlorine Chlorine tablets Coagulant/flocculent Solar disinfection Ceramic filter Bio sand filter Membrane filter Cloth filter Settling Other

E8: If you don't take measures to make water safer to drink at your home, what was the reason?*

E9: Did your water treated at the source?

Yes No I don't know

E10: Are the drinking water storage containers' lids or covers present and in place? Yes/No

E11: Are the drinking water storage containers sanitary and free from cracks? Yes/No

E12: Amount of water you are consumed in a day (time in hrs_____) in liters (ask guardian for children) _____

E13: Number of glasses of water you drunk yesterday including nights (24 hours) _____

E13: Is the area around the source properly fenced? Yes/No

E14: Observed animal waste near the houses and/or water sources Yes/No

E15: Do animals (Poultry, shoats, goats etc) share house with human? Yes/No

E16: If no, distance of animal cadges from the house in meters_____

E17: Observed human waste near the houses and/or water sources Yes/No

E18: Water Sample number/code

E19: Woreda of the water sample taken

Dessie Zuria Kutaber Legehida Tenta Mekdella Wereillu

E20: Village of the water sample taken

E21: Date of the water sample taken

E22: GPS of the water sampled


latitude (x.y °) longitude (x.y °) altitude (m) accuracy (m)

Take photo while sampling the water

E23: Lab result of PH in the water sample
E24: Lab result of turbidity in the water sample
E25: Lab result of residual chlorine in the water sample
E26: Lab result of *E. coli* in the water sample (colony count per 100 ml sample) using MF
E27: Laboratory result in the water sample using PCR in base pair*

- Enteroaggregative *E. coli* Heat Stable 1 (EAST1)
- Shiga-like toxin 1 (Stx1)
- Shiga-like toxin two (Stx2)
- Heat-stable(*Sta*)
- Heat-liable (*LT*)
- Other

E28: Please indicate classification other than mentioned above*

Take photo during lab analysis

## Annex 8 Article dissemination strategy

### Greetings,

My name is Shibabaw Tadesse Gemeda, and I am writing to share my WASH-related research, as it may be relevant for your professional work. You can learn additional details about my qualifications below my email signature.

My research is relevant for bilateral and multilateral donors, which I understand you are a professional in this area, because it helps to understand the context of drinking water quality in LMICs such as Ethiopia and recommends that water quality be included in the drinking water basic ladder definition.

My research is relevant for international and national WASH actors, which I understand you are a professional in this area, because it adds value to the development and revision of organizational water quality strategies and fills gaps in the definition of improved water supply classification.

My research is relevant for universities and research institutes, which I understand you are a professional in this area, because it lays the groundwork for future additional research to assess whether or not "functional" basic water services provide safe drinking water to users.

My research is relevant for civil society organizations (CSOs), which I understand you are working in this area, because it provides firsthand information to policymakers to assist them in properly addressing water quality in the drinking water ladder.

My research is relevant for water management bodies, which I understand you are a professional in this area, because it improves the situation of water quality on the ground and places an emphasis on monitoring and reporting water quality in a consistent manner in order to meet the "clean water for all" initiative.

In the attached article (published online https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0248944 through PLOS One) (https://doi.org/10.1371/journal.pone.0248944), we demonstrated:

a) water *quality* (meaning clean and safe drinking water) is not considered in classifying "basic water services" in Ethiopia.
b) if we include water quality, **one-third** of "functional" water services are reclassified as "non-functional" water services—meaning this water is not "safe drinking water"

This means that Ethiopian statistics around access to water and water services do not properly capture the ***safety*** of water in our rural areas. If we collected this data, we could more effectively and efficiently target rural water services and WASHCo training. In other research (currently under review in BMC-Environmental Health and Preventive Medicine- https://www.researchsquare.com/article/rs-732568/private/timeline and BMC-Archives of Public Health- https://doi.org/10.21203/rs.3.rs-135520/v1), I demonstrate how poor water quality leads to increased diarrheal disease, which negatively impacts Ethiopian life and decreases our productivity as a nation.

Should you be interested, I am available to speak in more detail and perhaps provide a presentation and discussion facilitation in your workplace. I am interested to assist you in any way in improving Ethiopian WASH policies and practices.

Thank you kindly for your time and attention,

Shibabaw Tadesse
Phone: +251 911 870596
Email: shibabawt@gmail.com

**Biography**

I am a PhD candidate in Water and Health with a specialization in Public Health at Addis Ababa University, Ethiopian Institute of Water Resources. My dissertation research focuses on the 'Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia,'. **Methods** used were mixed and cross-sectional studies among households that use improved water sources and had a household member visit a health care facility due to diarrhea using MF and polymerase chain reaction (PCR). I received my MSc in Water and Environmental Management from Loughborough University, WEDC, in the United Kingdom.

I am passionate about understanding the role of water and sanitation in Ethiopia development to improve people life/health in accessing clean water services. I held various positions with WaterAid Ethiopia (WAE), Save the Children International (SCI) in WASH, Health and Nutrition, Food Security and Livelihood Projects, and a European Union-funded multisectoral project.

**Table 18.** Target groups and their email dresses for the dissemination of the research findings.

| Target groups | Email lists | Remarks |
|---|---|---|
| Policymakers-Government | MoWIE & MoH: girmaabiy@gmail.com, sedikmt2012@gmail.com, nejumamuktar@gmail.com, ilyas1933@gmail.com, yferej99@gmail.com, munibyonis2000@yahoo.com, ekram.redwan@moh.gov.et, tgwami@gmail.com, mecca.selman@gmail.com, geremewto@gmail.com, marualem75@girma.com, peerwnhoomod@gmail.com meseret.zelalem@moh.gov.et, habtamu.alemay@moh.gov.et | Ministry of Water, Irrigation and Energy & Ministry of Health |
| Bilateral & Multilateral donors | DFID, ADB, UNICEF, WB, EU: m-solomon@DFID.gov.uk, T.MENKIR@afdb.org, dgelan@unicef.org, jalvarezsala@unicef.org, htaifour@worldbank.org, omodpirwnho@yahoo.com, wfeleke@worldbank.org, kutanew@who.int, latiboni2008@gmail.com, jbevan@unicef.org, kgoyol@unicef.org, rwogayehu@worldbank.org, findlewamabebe@gmail.com, kmamo@unicef.org, simretyasabu@yahoo.com, kit.dyer@gmail.com, Ambachew.Amare@yahoo.com, awoke2015@gmail.com, pfeldman@fastmail.fm, k_jembere@yahoo.com, aberraaseffa2005@gmail.com, M.MOTTOLESE@sdh- | |

| | | |
|---|---|---|
| | et.eu<br>arto.suominen@gmail.com, FMusinguzi@water.org | |
| NGOs | CO-WASH, WAE, SNV, PLAN, SCI, WVI:<br>mbeyene@snv.org, TseguredaAbraham@wateraid.org, wedikebe@gmail.com, ibrahimdarbule@gmail.com, abera_endeshaw@yahoo.com, gulilat_12@yahoo.com, wondu98@gmail.com, annarmcnamara@gmail.com, endriasjob@yahoo.com, ktakele@yahoo.com, lydiazigomo@yahoo.co.uk, Marjaana.pekkola@formin.fi, Gezahegn.Tadesse@formin.fi, kalgetaneh@gmail.com, asratgg2014@gmail.com, asratkss@gmail.com, tayehabtie2014@gmail.com, tadmas34@yahoo.com, tesfayekebede78@yahoo.com, SJE0115@KOICA.GO.KR, desufanta@gmail.com, richardwilsonwater@gmail.com, kululemekonnen@amref.org, Abiyot.Geremew@amref.org, abiyou_worku@wvi.org, SintayehuM@care.org.et, Lemlem.zeleke@rescue.org, bebayehu3@gmail.com, abebayehu3@gmail.com, mbekele23@gmail.com, Dered2010@yahoo.com, Orhbn252006@gmail.com, Teferidej2011@gmail.com, Fetenesisay21@gmail.com, jimamok@gmail.com, sifantig@gmail.com, mesibaru@yahoo.com, tolossamersha@yahoo.com, asfawterefe.75@gmail.com, melkamu.jaleta@mwawater.org THunde@oxfam.org.uk, kmeseret@snv.org, hailedinku2011@yahoo.com, manaye12@yahoo.com sileshigobena@yahoo.com, Liz.palmer@savethechildren.org, weti20042000@yahoo.com, sdobsevage@wateraidamerica.org m_ayele@yahoo.com, shegawfentaye@yahoo.com, birhanugen@yahoo.com, bgenet@snv.org, | |
| Universities & research institutes-Scholars | meseret.dessalegne@aau.edu.et, farisa0013@gmail.com, workutefera2000@yahoo.com, yeroosaa@gmail.com, dessalegn423@gmail.com, dawit.hailu@ahri.gov.et, biruk_23@yahoo.com, yonatalmesfin.tefera@adelaide.edu.au, minsinas@yahoo.com, virginiecollinge@gmail.com, adanesew@yahoo.com, solx82@yahoo.com, Teshome.L.Yami-1@ou.edu, submission.engineering@eurorendezvous.org, hayurilvet@gmail.com, amverashi@gmail.com, asmenst13@gmail.com, bkyonas@gmail.com, taklilu3@gmail.com, wedc-publications@lboro.ac.uk | Dr. Meseret – EIWR, AAU-focal person will share for more than 100 students and teachers |
| Water Management Bodies | alex.ck07@gmail.com, tayeje@gmail.com, teklitberhane@gmail.com, deregemengistu@yahoo.com, amanmiltesh@yahoo.com, bahiruwma@gmail.com, abrish1899@gmail.com, getchtik@gmail.com, yarkhz@gmail.com, alemayehu.mekonen84@gmail.com, nigussiet34@gmail.com, addissosi@gmail.com, nwagesho@gmail.com, beshahnb@gmail.com, | |

| | | |
|---|---|---|
| | shewidem@gmail.com, yasabuberkneh@gmail.com, kedirgo@yahoo.com | |
| Facebook | 275 Friends | |
| LinkedIn | 171 Connections | |

**Table 19.** Summary of responses from the distributed groups.

| **Contacted persons** | **Responses** |
|---|---|
| Michael Negash-Project Manager/ Institutional Development Advisor<br><br>USAID Transform WASH Project<br><br>SNV Netherlands Development Organization | Congratulation! it is indeed an important area that needs all stakeholders' prior attention to meet the water related SDG. Your research findings can add value in this regard.<br>I will contact you for more info. It is also important to share it in different sector platforms and events.<br>Did you share by chance for Abiy (OWNP Coordinator) and Abireham (Sanitation Team Leader and National Coordinator) for their info and further dissemination through their networks, if you think that it is important? |
| Dr. Abebayhu Yilma | Thank for your email and info about your research work. I have a great appreciation for the work that you do. WASH is an interest area to work as it has huge impact on community wellbeing.<br><br>I'm happy to discuss with you further so let's keep in touch!! |
| Endrias Shobawe-WVI | I would like to congratulate you for the hard work you have done. As a PhD classmate, I can be a witness for how much you were dedicated for your research work. Your research will have a big value add for Ethiopian WASH program. |
| Tewodros Wondmneh - Monitoring, Evaluation, and Research Analysis Lead at Education Development Trust | Thanks for sharing the research brief findings. I liked it much. |
| Takele Kassa-RIPPLE | Congratulations!!! It is one of the important part of the Water sector which will add to the WaSH sector. |
| Birhanu Genet - WASH Adviser for Kunzila WASH Service Improvement Project<br>SNV Netherlands Development Organization | Congratulations!!! It is the result of your hard work.<br>This is very important data; I think good also to share the findings to sector actors in the platforms such as WASH MSF |
| Abiyou Worku \| Sanitation and Hygiene Coordinator \| World Vision Ethiopia | Congratulations!!! |
| Tseguereda Abraham-Head of Advocacy & Sector Strengthening-WaterAid Ethiopia | Congratulations Shibabaw,<br>Yes an important piece of work that we will be sharing internally. |

| | |
|---|---|
| Wndyfiraw Tadesse-CARE Ethiopia | Congratulations for your achievement and Thank you so much for sharing with us! |
| Takele HUNDE, Country WASH Coordinator<br><br>Oxfam \| Addis Ababa, Ethiopia | Congratulation for your hard work and achievement!!! As you have rightly indicated water quality has been given less attention, despite its huge impact on the public health and nation economy. I can say this is a significant contribution to the WASH and health sectors. |
| Asmamaw Abera - Debre Birhan University | Congratulations!!<br><br>Thank you for sharing! |
| Kebede Faris –Wollo University | Thank you for sharing. I am also happy and proud that you are pursuing your PhD.<br>Good Luck |
| Jorge Alvarez-Sala-UNICEF | Thank you very much for sending this interesting article. Your recommendation of setting up an intermediate service level between basic and safely managed is something that UNICEF has included in its Water Game Plan (attached).<br>I wish you all the best in your professional career. |
| Yared Legesse Tongi (MPH)<br>Lead Consultant: Development and Implementation of Water Safety Planning<br>Water Development Commission/MoWIE<br>Addis Ababa, Ethiopia | Many thanks for sharing this very important article on drinking water quality and the finding highly contributes to the government efforts to attain the water and health SDG targets. Hopefully, Water Development Commission and Regional Water and Health Bureaus would use the findings for planning and implementation monitoring of the drinking water services.<br>I will share the article with the staff of Water Development Commission (mainly with Water Supply Infrastructure Management Directorate) |
| Asmamaw Abeje | Well received, thanks. |
| Awoke Geto-EU Key Expert | I really thank you Shibabaw, for sharing your key article, focusing on drinking water quality which is one of the key SDGs indicators and your findings will have a contribution to the Ethiopian Government goal to attain the targets.<br>As a public professional, bringing such key articles on board and disseminating to the relevant national and regional actors of WASH and Water related subjects will be expected from us. Definitely I will further disseminate it to others for national contribution and use it for our SDH project too.<br>Keep the momentum for successful development and endeavor. |
| Waltaji Terfa Kutane<br>Climate Change and Health Technical Officer<br>WHO Country Office<br>Maputo, Mozambique | Thanks a lot for sharing this important information. Very relevant in line with SDGs6.1 monitoring and Ethiopia WASH Sector strategy Specially with ongoing Climate Resilient Water Safety Plans (Risk assessment and Management from source to mouth). I hope our colleagues in Ethiopia closely working with government such as Yared, Osman and others help in this regards,<br><br>Congratulation for the very insightful work and keep it up |
| Habtamu Alemay (BSC, MPH)<br>FMOH, HSSSD, Team coordinator | Congratulations!<br>Thank you for sharing this nice paper |

| | |
|---|---|
| Teshome Lemma (PhD)<br>Assistant Director, Hydrology and Water Security Program,<br>Research Scientist, Hydrology and Remote Sensing Laboratory Science.<br>School of Civil Engineering and Environmental Science<br>University of Oklahoma | I am so glad that you have already become a PhD candidate. I liked your great paper that you are publishing. |
| Aberra Aseffa (2nd year PhD student at CES, AAU (Sanitation and pollution stream)<br>WASH Cluster Regional IMO-UNICEF Oromia Field Office Team member | I would like to say congratulations to you on your publication of your dissertation work. I know you passed many ups and downs. Thank you very much and I hope I will share your findings with relevant WASH sectors in our Region. |
| Biruk Yeshitela<br>Armauer Hansen Research Institute<br>Jimma Road, ALERT campus<br>Addis Ababa, Ethiopia | Congratulations Shibabaw Keep it up!!! |
| Sophie Rubens<br>Editorial Team<br>Journal of Harmonized Research in Engineering | Greetings from Journal of Harmonized Research in Engineering. Thank you for prompt response. As you mentioned your article was submitted in another journal so Kindly send us another New manuscript you have<br>Awaiting for the valuable submission. |
| Mr. Ambachew Amare (EU –Project) | Congratulation! Thank you for sharing the online article on water quality, 'Biomarkers of Toxins in Improved Drinking Water and Diarrhea Patients of South Wollo Communities, Northern Ethiopia,' which has a public health importance in the WaSH sector'. It is very good resource document to health promotion and disease prevention. This is your professional contribution to your country and more. For sure, I will use the findings for our work and share the article to my contacts working on the related sector. Thank you again and keep moving! |
| Peter Feldman<br><br>Commugny, Switzerland<br><br>+41 76 460 0544 | Thanks for sending the link to, and summary of, this recent research. Just to join the chorus of many others who've also written – it's both disturbing to note the poor microbiological quality of 'basic' drinking waters in Amhara and Afar, but also encouraging that you and your colleagues have called attention to this important issue (water quality) which is a critical, but perhaps overlooked aspect of the drive to get more operational and convenient drinking water sources to all Ethiopians.<br><br>I hope the response to this research will include not only similar |

| | |
|---|---|
| | investigations in other regions of Ethiopia, but also in other countries in the region where a similar 'blind spot' may exist, in terms of monitoring and maintaining WQ standards in the drive to meet SDGs. And, it goes without saying that I hope this moves our government and other water sector colleagues forward in terms of crafting a scaled-up WQ monitoring and enforcement programme in Ethiopia.<br><br>It also might not be surprising to find that, with further investigation, these microbiological WQ problems are determined to be linked to the very real challenge of low access to 'basic' or 'safely managed' sanitation in the country, to leakage from unlined or poorly-lined pits from existing facilities, and/or to inadequate wastewater treatment and disposal practices.<br>And perhaps most importantly – to put into action a response effort to identify contamination sources that are impacting drinking water sources and to tackle that problem – including 1) issuing immediate public health notices to those affected communities/households (with clear recommendations for short-term WQ treatment measures needed to protect health); and 2) implementation of medium to long-term water treatment solutions in areas where there is persistent source contamination. |