

Public Health Implication of Drinking Water Sources Contaminated with *Giardia* spp. and *Cryptosporidium* spp. in South Ethiopia

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Abstract

Background: *Giardia duodenalis* and *Cryptosporidium* spp. are topmost causes of gastrointestinal diseases mainly in socio-economically disadvantaged regions. Understanding the molecular diversity and distribution of these parasites in water sources and the environmental variables that influence their prevalence is important to effectively control infections in at-risk populations, however, the state of knowledge about risks of these parasites associated with source water is little in Africa. This study aimed at characterizing risks of *Giardia duodenalis* and *Cryptosporidium* spp. from water sources in southern Ethiopia.

Methods: Twenty-one water samples (10L per source) were collected from five different types of drinking water sources. Sample was analyzed using immunofluorescence assay with fluorescein isothiocyanate-monoclonal antibodies and nested PCR; *tpi*-PCR was used for genotyping purpose in *Giardia duodenalis* isolates and *SSU-rRNA* and *gp60*-PCR in *Cryptosporidium* spp. isolates. Statistical analysis was performed using Spearman's rho correlation and independent-samples nonparametric test. The risk was characterized using quantitative microbial risk assessment.

Results: The immunofluorescence assay showed that cyst was detected in 81% (17/21) and oocyst in 71% (15/21) of the samples with mean concentrations of 64.6 cysts.10 L⁻¹ and 62.8 oocysts.10 L⁻¹. Genotypic analysis showed that *Giardia duodenalis* assemblage B was genotyped in 29.4% (5/17), assemblage A in 17.6% (3/17) and assemblages A+B in 52.9% (9/17) of the samples. *Cryptosporidium* spp. genotype was detected in 57.1% (12/21) of the samples; *Cryptosporidium parvum* in 38.1% (8/21), *Cryptosporidium hominis* in 14.3% (3/21) and *Cryptosporidium parvum* and *Cryptosporidium hominis* in 4.7% (1/21) of the samples. The average probability of illness from the water sources was 0.06 for *Giardia duodenalis* and 0.22 for *Cryptosporidium* spp.

Conclusions: The finding highlights the significance of water for transmission of *Giardia* spp. and *Cryptosporidium* spp. in southern Ethiopia. Genotypes detected in the samples indicate multiple sources likely contaminated the sources. Health risk that is inferred from the water sources to the public are significantly higher than most other reported findings. This study recommends the need of regular follow-up, improvement in water treatment facilities and setting appropriate legislation in areas where cycles of parasitic infection is linked to water sources.

Background

Giardia duodenalis (*G. duodenalis*) and *Cryptosporidium* spp. infect a wide range of hosts and are the topmost etiological agents of gastrointestinal diseases in humans [1,2,3,4,5]. *G. duodenalis* and *Cryptosporidium* spp. mainly *Cryptosporidium parvum* (*C. parvum*) and *Cryptosporidium hominis* (*C. hominis*) are also predominant genotypes reported from human isolates in Ethiopia [6,7] and other African countries [8,9,10,11]. Though *G. duodenalis* assemblages, such as assemblage C [12,13], F [14] and E [15] that rarely infect humans have been reported from human isolates, assemblages A and B are

the two genotypes known to infect humans in Ethiopia [14,16,17]. Sequence analyses of *G. duodenalis* isolates typed at various genetic markers, such as *tpi*, *GDH* and *βg* genes revealed isolates mostly belong to sub-assemblages All, AllI, BIII and BIV [7,18]. However, most assemblage B sequences varied than assemblage A sequences, and chromatograms displayed a number of double peaks [7,16,17,18].

Cryptosporidium spp. genotypes that belong to *C. parvum*, *C. hominis*, *C. viatorum*, *C. felis*, *C. meleagridis*, *C. canis* and *C. xiaoi* have been reported from human isolates in Ethiopia; *C. parvum* and *C. hominis* being predominant genotypes [6,7,18,19]. Sequence analysis of 13 isolates resulted in *C. parvum* sub-genotype that belong to zoonotic subtype family IIa and *C. hominis* subtype Ib [19]. Sequence analysis revealed high genetic diversity in *C. parvum* and *C. hominis*, including *C. parvum* zoonotic subtype families IIa and IIc and anthroponotic subtype families IIc, IIb, IIe and If-like, and *C. hominis* subtype families Id, Ie and Ib [6]. *C. parvum* IIc subtype family transmitting mainly through anthroponotic route is the most common in sub-Saharan Africa [8,20,21,22]. Other studies have reported higher prevalence of *C. parvum* subtype IIa and IIc in Ethiopia [6,19] and Egypt [23,24], which signifies the importance of zoonotic transmission in the region. In addition, sequence analysis of the single gp60 that tested positive for *Cryptosporidium* spp. revealed the presence of *C. hominis* subtype IbA9G3 [18].

G. duodenalis and *Cryptosporidium* spp. infections occur mainly through ingestion of cysts and oocysts contaminated with water, food, hand, environmental surfaces and faecal-oral route. These parasites infect about 5-10% of the world's population [25] and 200 million people per year worldwide [26,27], and are important causes of diarrheal diseases in children under five years [28] mainly in the tropics and socio-economically disadvantaged areas [29,30,31]. About 2.9 million *Cryptosporidium*-attributable cases occur per year in children in sub-Saharan Africa, being the second cause of diarrheal death after rotavirus in children younger than five years [28,32]. *C. parvum* and *C. hominis* cause more than 90% of human cryptosporidiosis cases [2,4]. The disease results in moderate to severe diarrhea, resulting prolonged courses, relapses and extra intestinal sequelae in up to 40% of the cases [33,34] and associated with increased mortality in African countries [35]. Likewise, more than one billion people are at risk of infection from *G. duodenalis* [3]; the higher frequency of infection is reported in developing countries and deprived communities in developed countries [27,29,30,36,37]. Because of the substantial burden imposed by *G. duodenalis* and *Cryptosporidium* spp. in developing countries, these parasites have been included in the Neglected Diseases Initiative of World Health Organization [26].

G. duodenalis and *Cryptosporidium* spp. transmit through multiple ways: direct transmission route through animal to human or person to person and indirect transmission route through materials contaminated with cysts and oocysts [38,39,40]. However, *Cryptosporidium* spp. and *Giardia* spp. are the most common parasitic protozoa that use water as a main vehicle of transmission [2,19,41]. Their cyst and oocyst withstand a range of environmental conditions and survive for long periods mainly in moist environment and variety of water sources [42,43,44,45], and resist disinfectants used in most water treatment schemes [1,46,47,48]. From 381 worldwide outbreaks (documented from 2011 – 2016) attributed to waterborne transmission of parasitic protozoa, *Cryptosporidium* spp. (63% of the outbreaks) and *Giardia* spp. (37% of the outbreaks) were the most common etiological agents that caused the

outbreaks solely [49]. Climate change and population growth are also predicted to increase prevalence of the parasites in water sources [35].

Despite the significant importance of water for transmission of parasites, more than 300 million people in sub-Saharan Africa have poor access to safe water [50,51]. Poverty coupled to inadequate water treatment facilities, poor hygiene and sanitation, and lack of education is a limiting factor to access safe water that predisposes people to waterborne giardiasis and cryptosporidiosis [31,37]. Availability of data on water quality and microbial contamination level of water sources complying with microbial contamination legislation and guideline stipulated by regulatory bodies helps to minimize risks to the public. Though previous studies have provided important epidemiological information in Ethiopia, health risks associated with water sources are less appreciated and remain a public health defiance mainly in the at-risk population, worsening socio-economic conditions of peoples in the region [37,52,53]. The present study aimed at assessing and characterizing the risks of *G. duodenalis* and *Cryptosporidium* spp. infections associated with drinking water sources in southern Ethiopia.

Materials And Methods

Study area and design

This study was done from February to May 2016 in southern Ethiopia that consists of 13 administrative zones, covering about 10% of the landmass of Ethiopia. It is located in 4°43'–8°58' N latitude and 34°88'–39°14' E longitude with an altitude range of 376–4207 m above sea level. The annual rainfall and temperature range of 500–2200 mm and 15–30°C, respectively. Drinking water sources in the region range from treated to untreated sources of municipal/tap, unprotected spring, protected spring and wells, and river/stream water. Chlorination was the only techniques used to treat municipal/tap water source, whereas the other sources were untreated. Spring water is a natural discharge point of subterranean water at the surface of the ground. Unprotected spring is often unprotected or poorly protected that is open to the atmosphere, whereas protected spring is protected by providing a concrete headwall or spring box around the eye of the spring (where water emerges) that prevents direct contamination of the water. The protective cover usually overlies the excavated area and the area is fenced for some distance to prevent direct access by humans and animals. River is a natural stream of water of considerable volume, larger than a brook or creek, and flows downwards until it reaches its end, crossing land, hills and plains in its journey. Stream is a natural body of running water flowing in a natural channel as distinct from a canal; contains water at least part of the year.

The study area was selected using multi-stage cluster sampling technique; seven zones from the region and three districts from each zone were selected. Health risks were characterized using 21 different water sources that were selected from each district and 576 individuals that were using the water sources. Participants were selected based on a single proportion formula, considering design effect 1.5. According to the sample sizes allocated to each site, households were selected using systematic sampling technique, with an individual per household randomly selected. Children < 2 years and individuals who

did not often use the water sources for drinking purpose were excluded, as the present study aimed at characterizing risks of *G. duodenalis* and *Cryptosporidium* spp. infection associated to drinking water sources. Participants' information was gathered using a semi-structured questionnaire and water samples were analyzed quantitatively using immunofluorescence assay (IFA) and qualitatively (nested-PCR), and risks were characterized using quantitative microbial risk assessment (QMRA).

Stock suspension preparation and enumeration

Human stool samples from patients infected with *G. duodenalis*, *Cryptosporidium* spp. were obtained from Arba Minch hospital, southern Ethiopia. The samples were suspended with distilled water and filtered through a 0.5-mm sieve and concentrated by centrifugation at 1050 g for 10 min and the supernatant was decanted. Cysts and oocysts were isolated using percoll-sucrose gradient (specific gravity: 1.09–1.10) and centrifugation at 1050 g for 10 min. Stock suspensions having concentrations of 10^3 to 10^4 cysts or oocysts per liter were prepared using reagent water (MilliQ, Millipore) with 0.01% Tween 20. Enumeration was performed using haemocytometer chamber count (Bright-Line, Reichert, Buffalo, NY), to achieve the optimal counting accuracy, 10 different chambers were counted for each cyst/oocyst suspension. Spiking suspensions having 10^2 to 10^3 concentrations of each cyst and oocyst per liter were prepared by dilution using a drop count procedure. Droplets (10-20 μ l) from the stock suspensions were pipetted onto the edge of a microscope slide and counted using bright light microscopy (bright field illumination, Nikon, Japan) at 250X and 400X magnifications for cysts and oocysts, respectively. The counts were replicated for three times and the concentrations of the organisms were established. In addition, the same volumes of the suspensions were directly transferred into well slides, stained with FITC-MAb (Waterborne Inc., New Orleans) and enumerated using IFA.

Recovery efficiency and detection limit

Initial recovery efficiency of the method was achieved by spiking known number of cysts/oocysts into a 10 L reagent water and counting the recovered parasites in the spiked sample, in triplicate. Concentration of cysts/oocysts in each sample, the recovery efficiency and detection limit of the method were calculated using equations 1, 2 and 3, respectively.

$$\text{Equation 1. } (Oo)\text{cysts conc.} = \frac{\text{Number of (oo)cysts detected in sample by IFA}}{\text{Recovery efficiency for sample batch} \times \text{Sample volume}}$$

$$\text{Equation 2. } \text{Rec. eff (\%)} = \frac{\text{Number of (oo)cysts detected in the positive control}}{\text{Number of (oo)cysts seeded to positive control}} \times (100)$$

$$\text{Equation 3. } \text{Limit of detection} = \frac{1}{\text{Recovery efficiency} \times \text{Sample volume}}$$

The inter-assay recovery efficiency of the method was also assessed to reveal the variation in the different water samples. The effect of matrix on recovery efficiency of the method was determined using

spiked environmental water samples that were collected from the same sources. To assure the absence of contamination throughout the analytical procedure, unspiked reagent water sample was analyzed accordingly. Two water samples with the same volume of water were collected from the same source; One sample for pathogen analysis and the other for recovery efficiency analysis. Spiking experiment per sample was carried out in triplicate and percent recovery (REC) was calculated according to [54], (equation 4). The number of cysts/oocysts in the water samples per 10 L were calculated, (equation 5).

$$\text{Equation 4. } (REC) = \frac{(N-NSAMPLE)/CC}{0.5ml/PV} \times (100)$$

Where N is the number of cysts/oocysts counted in the spiked water sample, $NSAMPLE$ is number of cysts/oocysts counted in non-spiked environmental sample, CC is the number of cysts/oocysts spiked and PV is the analyzed pellet volume per sample.

$$\text{Equation 5. } (Oo)cysts_{per\ 10\ L} = \frac{10}{VE} \times \frac{100}{REC_{avg}} \times (NSAMPLE)$$

Where VE is volume examined, REC_{avg} is average recovery of cysts/oocysts, and $NSAMPLE$ is number of cysts/oocysts found in the unseeded water. When no cysts/oocysts were found, a value (detection limit) of each water type was used was used for $NSAMPLE$.

Water sample collection

Ten-liter water samples were collected from each source from points representing the nature of water sources. Samples were collected using a clean and sterilized plastic bottle that were washed three times with hot and distilled water. Prior to sample collection, the sample bottles were partially filled, rinsed three times with the same water and drained from the sampler. Sand and other debris were avoided by carefully collecting water rinsing into the water in low- turbidity and the bottles were filled to collect ten-liter water sample. Piped water samples were collected from reservoirs while stream/river water samples were collected at the edges that had low-flow turbidity and debris. The samples were immediately placed in a lightproof insulated box containing ice-packs with water to ensure rapid cooling and preserve the state of the water and shipped to the laboratory, Arba Minch University and overnight stored in a refrigerator at 4°C until processed. Water turbidity and free chlorine were measured immediately on the time of sample arrival, within 24 h of sample collection. Water turbidity was measured using a Hach 2100N turbidimeter (Hach, Loveland, CO, USA) and expressed in nephelometric turbidity units (NTU). Free chlorine in municipal water was measured using DPD chlorine test kit (La- Motte, Chestertown, MD, USA) and expressed in milligram per liter.

Water sample processing

Sample filtration, elution, concentration, application on slide and drying were carried out within 24 hours of collection. The samples were filtered through 142-mm-diameter with 3.0µm-pore-size cellulose nitrate (Sartorius) membrane. The water sample was drawn through the membrane under negative pressure and the membrane was scraped with a smooth-edged plasticine molder and rinsed with 0.1% Tween 80 (T80). The membrane was rinsed repeatedly by turning it for three times until it became clean and the eluate was collected in a clean and sterilized plastic dish and transferred to a sterile 50 ml centrifuge tube. The dish was rinsed with 0.1% T80, pooled with the resuspended pellet, centrifuged at 5000 *g* for 10 min. All supernatant of a sample was aspirated to leave the pellet. The pellet was resuspended with 0.1% T80 and transferred into a fresh sterile centrifuge tube. The emptied tube was rinsed with 0.1% T80, the washes were pooled with the resuspended pellet, centrifuged at 5000 *g* for 10 min and the supernatant was decanted. Pellets of the same sample were collected together and 0.5 ml of each aliquot was used for detection and enumeration. The remaining portions were stored at –20°C for genotypic analysis.

Pathogen detection and enumeration

Cysts and oocysts were enumerated using IFA and haemocytometer chamber. The IFA was done by transferring 10-µl aliquot of each sample, in triplicate. About 10 µl aliquot of each sample was directly transferred into separate slides, air-dried at 55°C and placed in a dark humid chamber, and incubated at room temperature for 30 min with combined fluorescein isothiocyanate–monoclonal antibodies (FITC-Mab) (Aqua-Glo G/C kit; Waterborne, Inc., New Orleans, LA). The slides were removed from the chamber, the condensation was let evaporated, and one drop of wash buffer was applied to each well according to the manufacturer's instructions. The slides were washed in PBS containing T80 (0.01% [vol/vol]; PBST), air-dried at 55°C and tilted on a clean paper towel, and the excess detection reagent was aspirated using a clean Pasteur pipette. The haemocytometer count was also performed by applying 1 µl of the final pellet; five counts were performed per test.

The wells were counterstained each with 50 µl of 4', 6-diamidino-2-phenylindole (DAPI) staining solution (Sigma, St. Louis, MO) at 0.4 µg/ml and stood at room temperature for 2 min. A drop of the wash buffer was re-applied to each well, and the slides were tilted and processed as mentioned above. Mounting medium was added to each well and covered using cover slip. Excess mounting fluid was removed from edges of the coverslip using soft tissue and the edges were sealed onto the slides using clear nail polish and examined using epifluorescence. The FITC-Mab staining, DAPI and Differential interference contrast (DIC) examinations were conducted according to the manufacturer instruction. Shape, size and internal morphological features for each apple-green fluorescing object that met the size and shape characteristics of cysts and oocysts per pellet were enumerated. Depending on the recovery rate and detection limit of the method (see 'Recovery efficiency and detection limit'), the total estimated numbers were calculated based on the analyzed fraction of pellet and total volume of filtered water. The percentage of samples positive in DAPI fluorescence examination and *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts viable in DIC examination was analyzed. Cysts and oocysts that exhibited typical FA fluorescence, typical size and shape, nothing atypical on DAPI fluorescence and nothing atypical on DIC microscopy were considered as positive results.

***Giardia duodenalis* and *Cryptosporidium* spp. genotyping**

DNA extraction

Genomic DNA extraction was performed for all 21 drinking water samples. Portions of each pellet obtained from the respective samples (see 'Water sample processing' section) were suspended by vortexing, and 300 µl of the sample was transferred directly into a 1.5 ml centrifuge tube and centrifuged for 2 minutes at 13,000 rpm and the supernatant was discarded. The sediment was re-suspended in 300 µl sterile distilled water and centrifuged at the same conditions three times repeatedly. The extraction was carried out using five freeze-thaw (-70°C) cycles followed by QIAamp DNA Mini isolate kit (Qiagen, Germany) in accordance with the original protocol. Then, the samples (200 µL each) were washed seven times with 1 mL of PBS (pH= 7.3) and centrifuged at 14000 g for 1 min. The pellets collected in the preceding steps were re-suspended in 1400 µl of buffer ASL, heated to 95°C for 10 min, then vortexed, and centrifuged at 15000 g for 1 min. The digestion process with proteinase K was elongated to 30 min at 70°C. DNA yields of each sample were determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm. DNA purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. DNA length was also determined by pulsed-field gel electrophoresis through an agarose gel, and the genotyping process was performed accordingly.

***Giardia duodenalis* genotyping**

The genotypes were determined using the *tpi* gene based nested PCR, as it is a good phylogenetic marker for molecular analysis [55]. According to a previous work [56], primary and secondary primers that amplify 618 and 557 bp of the gene were used, respectively. In the primary PCR amplification, external forward primer – TPI-FW1 (5'-CAGAAAATAAATIATGCCTGCTC-3') and external reverse primer TPI-RV1 (5'-CAAACCTTITCCGCAAACC-3') were used. The primary PCR amplification was carried out in 50 µl reaction mixture containing 5 µl of DreamTaq™ Buffer (10x), 1 µl of dNTPs mix (10 µM each), 1 µl of GI-TPI-FW1 and 1 µl of GI-TPI-RV1 (10 µM each), 0.25 µl of (5 Unit) Dream Taq DNA polymerase, 0.25 µg/µl of genomic DNA and nuclease free water. The reaction was carried out in 30 cycles, each consisting of 95°C for 45 seconds, 55 °C for 45 seconds and 72 °C for 1minute with an initial hot start at 95 °C for 5 minutes and a final extension for 7 minutes. In the secondary PCR amplification, the internal forward primer – TPI-FW2 (5'-CCCTTCATCGGIGGTA ACTTCAA-3') and internal reverse primer TPI-RV2 (5'-ACATGGACITCCTCTGCCTGCTC-3') were used. The PCR reaction was carried out similar to the primary PCR reaction, except using 4 µl of the primary PCR product and prolonging the final extension to 10 minutes. The genotype of *G. duodenalis* was detected and genotyped using 1.5% gel electrophoresis analysis of the final PCR amplicons. In every activity, both positive control (*G. duodenalis* DNA) and negative control (nuclease free water) were used.

The final positive PCR products were sequenced using the corresponding primers used for PCR amplification. Sequencing was carried out using a capillary sequencer, 3730xl DNA Analyzer (Applied Biosystems, CA, USA), in combination with ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits

(Applied Biosystems, CA, USA). The pre- and post-sequencing processes were automated on a robotic platform consisting of Biomek[®] FX and NX instruments (Beckman Coulter, Fullerton, CA, USA). The chromatograms and sequences generated from this study were viewed and assembled using the BioEdit Sequence Alignment Editor Program version 7.2.5 (www.mbio.ncsu.edu/bioedit/bioedit.html). The consensus sequences were compared with sequences registered in GenBank, using the basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/blast). Genotyping of sub-assemblages was determined based on sequence homology (100% identity) of the isolates with sequences in GenBank. Representative sequences of assemblages A (KT728546.1), B (EU781015.1), C (AY228641.1), D (DQ246216.1), E (EU272157.1), F (AF069558.1) and G (AY228640.1) were included for comparison analysis. In addition, sub-assemblage A reference sequences - 07JTPI (KT728546.1), AIIGRW (KF963577.1), BSWAll (KF963567.1) and BRW All (KF963573.1) - and sub-assemblage B reference sequences - BI (HQ397719.2), BIV (LO2116.1), BV (HQ666895.1), BVII (HQ666897.1), Hole H13 (KT948108.1), Swemon 200 (EU781015.1) and HS98 (KC632554.1) - were included. For further genetic variation analysis, *G. muris* (AF069565.1), *G. ardeae* (AF069564.1) and *G. microti* (AY228649.1) were also included.

***Cryptosporidium* spp. genotyping**

The *SSU*-rRNA and *gp60* genes of *Cryptosporidium* spp. were amplified using nested PCR protocol. In the primary PCR of *SSU*-rRNA gene, external primers – CR-SSU-FW1 (5'–TTCTAGAGCTAATACATGCG–3') and CR-SSU-RV1 (5'–CCCATTCCTTCGAAACAGGA–3') that amplify 1,323 bp fragment of the gene were used. In the secondary PCR, internal primers – CR-SSU-FW2 (5'–GGAAGGGTTGTATTTATTAGATAAAG–3') and CR-SSU-RV2 (5'–CTCATAAGGTGCTGAAGGAGTA–3') that amplify 852 bp fragment of the gene were used. The primary PCR amplification of *SSU*-rRNA gene was carried out in 50 µl reaction mixture containing 5 µl 10X Dream Taq buffer, 1 µl dNTPs mix (10 µM each), 1 µl CR-SSU- FW1 and 1 µl CR-SSU-RV1 (10 µM each), 0.25 µl of (5 Unit) Dream Taq DNA polymerase, 0.25 µg/µl of genomic DNA and nuclease free water. In the primary PCR reaction, 30 cycles were carried out, each consisting of 94 °C for 45s, 55 °C for 45s and 72 °C for 1 min., with an initial hot start at 94 °C for 3 min and a final extension for 7 min. In the secondary PCR reaction, 4 µl of the primary PCR product will be used and the cycles will be increased to 35; whereas, the other PCR conditions were similar to the primary reaction.

In the primary PCR amplification of *gp60* gene, external primers – CR-GP60-FW1 (5'–TTACTCTCCGTTATAGTCTCC–3') and CR-GP60-RV1 (5'–GGAAGGAACGATGTATCTGA–3') that amplify 915 bp fragment of the gene were used. In the secondary PCR, internal primers – CR-GP60-FW2 (5'–TCCGCTGTATTCTCAGCC–3') and CR-GP60-RV2 (5'–GCAGAGGAACCAGCATC–3') that amplify 869 bp of the gene fragment were used. Except a different annealing temperature (54°C), the PCR amplifications of *gp60* gene was analogous to *SSU*-rRNA gene amplification. The genotypes were determined using 1.5% gel electrophoresis: 5 µl of the final PCR product with 2 µl 6X loading dye at 110 V for 55 min were used. During each PCR reaction, known DNA and nuclease free water were used as positive and negative controls. In addition, the primers were checked based on gene sequences available from GenBank, the primers were also pretested and the PCR conditions were optimized. *Cryptosporidium* spp. isolates were

only typed at the genus level; genotypic analysis of *C. hominis* and *C. parvum* isolates at sub-type level was not done.

Quantitative microbial risk assessment

Risks of the pathogens from drinking water sources were characterized using QMRA. Exposure of individuals to *G. duodenalis*, *C. parvum* and *C. hominis* infections was determined based on concentration of cysts/oocysts in the water samples, as previously described [57,58]. Individuals' exposure to each pathogen and the volume of water consumption through drinking exposure pathway per given amount of time, and water consumption behaviour of peoples (treatment practice before drinking) were gathered through a semi-structured questionnaire. Pathogen dose that are possibly ingested by an individual was computed as a product of volume of water consumed un-boiled or untreated in a given period and pathogen concentration in the water (equation 6). The daily dose of pathogen for treated water was computed according to [59], (equation 7). The probability of infection to each pathogen and its progress to illness were computed using an exponential dose-response model with parameters of an infection endpoint [60], (equation 8). Only cysts and oocysts exhibiting and meeting the criteria based on DIC examination were considered viable and used for analysis. As the cysts and oocysts detected in water samples may correspond to other species that are not infective to human, genotypic analysis was done to insure the presence of the respective species that are infective to humans.

$$\text{Equation 6. } Dose_{per\ day} = Pathogen_{conc./time} \times V_{consumed/day}$$

$$\text{Equation 7. } Dose = C_{per\ liter} \times \frac{1}{R} \times I \times 10^{-DR} \times V$$

Where C is concentration of pathogens in the water or partially processed water, R is recovery of the detection method, I is fraction of the detected pathogens that is capable of infection (viability), DR is decimal reduction factor ($DR = 0$ when concentration in the finished water is available), and V is daily individual consumption of the considered water.

$$\text{Equation 8. } P_{Infection} = 1 - \exp(-rD)$$

Where D is pathogen dose, and r is fraction of pathogens that survives to produce an infection

An exponential dose-response model for representative species of *G. duodenalis*, *C. parvum* and *C. hominis* was considered to avoid response variations for exposure to a variety of species within a genus. Dose-response model parameter, $r = 0.0199$ for *G. duodenalis* [61] and $r = 0.09$ for *Cryptosporidium* spp. [62,63] were employed. Two parameters: $r = 5.26 \times 10^{-3}$ for immunocompetent and $r = 0.354$ for immunocompromised sub-populations were also accounted for *C. parvum* [64]. The probability of daily

infection was extrapolated to yearly risk; consecutive exposures were assumed to be independent, then the probability of one or more infections for n exposures per year was supposed to be the corollary of 'n. For daily infections with values of $\ll 1$, infections per year were computed by multiplying the probability of daily infection and the number of days [60].

The probability of illness per given infection was computed using a morbidity fraction of each pathogen. Progression from infection to illness for *G. duodenalis*, *C. parvum* and *C. hominis* is ranged from 0.2 to 0.7 [60,65,66,67]. A morbidity fraction of one was used to compute the probability of illness to *Cryptosporidium* spp. infection for immunocompromised sub-populations [64]. The probability of morbidity was calculated according to [57,68], (equations 9). All the health outcomes of illness were considered as an endpoint and individuals were assumed to have at least one disease outcome.

$$\text{Equation 9. } P_{\text{Morbidity}} = P_{\text{Infection}} \times P_{\text{Ill/Inf}}$$

Where, $P_{\text{Infection}}$ is the probability of infection with a specific pathogen, $P_{\text{ill/inf}}$ is the probability of illness due to infection.

Questionnaire

The study participants' information was collected using semi-structured questionnaire, which was pretested on a small number of respondents that were chosen randomly in the population being studied. The respondents were interviewed and their responses were documented and the questions were rephrased and interviews were repeated, then each response was investigated and comparable responses were maintained. Once the basis and procedures of the study were understood and agreed upon, the participants (parents of children) signed the informed consent forms and participated in the study. Then, the study subjects age, sex, type(s) of drinking water source(s), amount or volume water used for consumption per day, water consumption days per month and types of water treatment techniques or mechanisms used by individuals were collected (see Annex VA and VB).

Statistical analysis

Nonparametric Spearman's rho correlation coefficient was computed to assess how the occurrence rate of *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts associated with the types of water sources and physicochemical parameters of the sources. Spearman's rho was preferred as it is suitable for data that are not distributed normally, moreover outliers have less of an effect on this statistical method. Correlation coefficients range in value from -1 (a perfect negative relationship), $+1$ (a perfect positive relationship) and a value of 0 (no linear relationship) were used to indicate the relationships of variables. In addition, independent-samples (Kruskal-Wallis) nonparametric test was performed to compare the distribution of cysts and oocysts across the types of water sources. In the analysis, $P < 0.05$ were considered statistically significant. The analysis were performed with the IBM* SPSS* Statistics for Windows, version 23 software (SPSS Inc., Chicago, IL, USA).

Results

Recovery efficiency and detection limit of the method

Inter-assay recovery efficiency of the method showed variations in the types of water samples that were analyzed during the study. Percent recovery of the method was found in the range of 14 to 31% for cysts (mean: 22.4%, SD: 5.248) and 13 to 25% for oocysts (mean: 19.7%, SD: 3.985). The highest average percent recoveries of cysts and oocysts were achieved in river/stream and protected water samples and the lowest in unprotected spring water samples, respectively.

Concentration of cysts/oocysts and correlation with water turbidity and free chlorine

Giardia spp. cysts and *Cryptosporidium* spp. oocysts were detected in drinking water sources in the region. The average concentrations of cysts and oocysts from different drinking water sources are indicated in Table 1. The highest average concentration of cysts and oocysts were detected in unprotected spring water samples and the lowest were found in river/stream and piped water samples. Independent-samples (Kruskal-Wallis) nonparametric tests showed distribution variation of cysts ($P=0.007$) and oocysts ($P=0.004$) across the different categories of the water sources. However, Spearman's rho correlation coefficient showed no correlation between the types of water sources and occurrences of cysts ($P=0.147$) and oocysts ($P=0.129$). Turbidity levels of the water samples ranged from 0.95-115 NTU (mean: 35.8095, SD: 38.56852); the highest value was observed in unprotected spring water samples and the lowest in piped water. The Spearman's rho correlation coefficient showed positive correlation between occurrence of cysts and water turbidity ($P=0.025$), while there was no correlation between oocysts and water turbidity ($P=0.130$). The concentration of free chlorine in piped water sample was found ≤ 0.55 mg. L⁻¹ (mean: 0.1950, SD: 0.20285), though it was not statistically computed, as it was measured for piped water only. The percentage/proportions of samples positive in DAPI fluorescence examination and cysts and oocysts viable in DIC examination is 91.4% and 90.6%, respectively. The average percentage of samples positive in DAPI and DIC examination is shown in Table 2.

Table 1. The occurrence and concentration of cysts and oocysts in drinking water samples

Water samples	No.	Positive samples (%)		Concentration (10. L ⁻¹)					
				Mean		Range (lower value is 0)		SD	
		Cyst	Oocyst	Cyst	Oocyst	Cyst	Oocyst	Cyst	Oocyst
Piped water	6	50.0	33.3	7.3	2.6	20.0	10.5	8.9	4.4
Protected spring	7	85.7	85.7	43.5	21.7	161.8	52.2	56.6	21.7
Unprotected spring	5	100	100	198.7	229.4	399.9	460.3	176.9	191.9
River/stream	3	66.7	33.4	4.9	1.5	11.1	4.5	5.6	2.6
Total	21	76.2	71.4	64.6	62.8	476.3	493.6	115.8	129.2

Table 2. Average percentage of samples positive in DAPI fluorescence examination and *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts viable in DIC examination

Water samples	No. of positive samples		Mean of samples positive in DAPI fluorescence examination and (oo)cysts viable in DIC examination (10. L ⁻¹)					
			DAPI fluorescence examination		DIC examination		Percent (%)	
	Cyst	Oocyst	Cyst	Oocyst	Cyst	Oocyst	Cyst	Oocyst
Piped water	3	2	9	3.5	7.3	2.6	81.1	74.3
Protected spring	6	6	52.5	28	43.5	21.7	82.8	77.5
Unprotected spring	5	5	210	248	198.7	229.4	94.6	92.5
River/stream	2	1	7	2	4.9	1.5	70	75
Total	16	14	69.6	70.4	63.6	63.8	91.4	90.6

Genotypes of *G. duodenalis* and *Cryptosporidium* spp.

G. duodenalis, *C. parvum* and *C. hominis* genotypes were detected in the samples. *G. duodenalis* was detected in 81% (17/21) of the water samples similar to the IFA, except variation in one sample that was negative in IFA. Assemblage specific genotyping analysis showed that *G. duodenalis* isolates in 29.4%

(5/17) of the samples belonged to assemblage B, 17.6% (3/17) belonged to assemblage A and 52.9% (9/17) belonged to combination of both assemblages. Among assemblage B genotypes in five water samples, three isolates were 100% identical to sub-assemblage HS98 (KC632554.1), one was identical to isolate Swemon 200 (EU781015.1) obtained from a vervet monkey in Sweden and one isolate was identical to sequence (KT948108.1). All assemblage B isolates were detected in unprotected spring water, except one in piped water. Assemblage A genotypes that were identified in three water samples belonged to sub-assemblage All and showed 100% identity with sequences registered in GenBank. Two isolates were simultaneously identical to BSW_mar'11 All (KF963567.1) and GRW_jul'11 All GRW (KF963577.1), and one isolate was identical to BRW_aug'11 All (KF963573.1). The mixed assemblage sequences showed 96-99% identity with sequences registered in GenBank. The chromatograms and sequences generated from this study were viewed, and double peaks were observed during chromatogram inspection in samples with mixed assemblages of A and B.

Cryptosporidium spp. genotypes were detected in 57.1% (12/21) of the water samples and showed some variation with the IFA. *C. parvum* was detected in 38.1% (8/21) of the samples, most of them were in unprotected and protected spring water sources. *C. hominis* was detected in 14.3% (3/21) of the samples and all were in protected spring water. Combination of *C. parvum* and *C. hominis* was detected in one sample. Two samples, each from piped and protected spring water, that were positive for IFA were negative for *C. parvum* and *C. hominis* nested-PCR genotyping analysis. Coexisted *C. parvum* and *G. duodenalis* were also detected in some samples.

Probability of infection and risk of illness

According to the information gathered through semi-structured questionnaire, 98.8% of the people had un-boiled water consumption habit. The average volume of water consumption per person per day was one-liter and frequency of consumption per month was 17 days (207 days/year). The average dose of cysts in all the water sources per one-liter volume of water ranged from 0.49 to 19.87 cysts L⁻¹ with an average dose of 6.46 cysts L⁻¹. The average dose of oocysts in the water sources per one-liter volume of water ranged from 0.15 to 22.94 oocysts L⁻¹ with an average dose of 6.28 oocysts L⁻¹. The highest dose of cysts and oocysts was observed in unprotected spring water sources and the lowest in river/stream water. The probability of infection was estimated based on the calculated doses of pathogens, and risk of illness per infection was determined using a morbidity fraction (0.5) of each pathogen (Table 3). The probability of infection and risk of illness to *Cryptosporidium* spp. according to immune status of sub-populations is shown in Table 4. All individuals were assumed to have at least one disease outcome.

Table 3. Average doses of pathogens and probability of infection per case and risk of illness per infection (water consumption per day per person was calculated to be one liter)

Water sources	<i>G. duodenalis</i>			<i>Cryptosporidium</i> spp.		
	Dose of cyst	Probability of infection $r=0.0199$	Probability of illness	Dose of oocyst	Probability of infection $r=0.09$	Probability of illness
Piped water	0.73	0.0144	0.0072	0.26	0.0231	0.0115
Protected spring	4.35	0.0829	0.0414	2.17	0.1774	0.0887
Unprotected spring	19.87	0.3266	0.1633	22.94	0.8731	0.4365
River/stream	0.49	0.0097	0.0048	0.15	0.0134	0.0067
Total average	6.46	0.1206	0.0603	6.28	0.4317	0.2158

Table 4. The probability of *Cryptosporidium* spp. infection and its progress to illness according to immune status of sub-populations

Water sources	Probability of infection		Probability of illness	
	Immuno-competent $r=5.26 \times 10^{-3}$	Immuno-compromised $r=0.354$	Immuno-competent $P_{ill/inf}=0.5$	Immuno-compromised $P_{ill/inf}=1.0$
Piped water	1.4×10^{-3}	8.8×10^{-2}	7.0×10^{-4}	8.8×10^{-2}
Protected spring	1.1×10^{-2}	5.4×10^{-1}	5.6×10^{-3}	5.4×10^{-1}
Unprotected spring	1.1×10^{-1}	9.9×10^{-1}	5.7×10^{-2}	9.9×10^{-1}
River/stream	8.0×10^{-4}	5.1×10^{-2}	4.0×10^{-4}	5.2×10^{-2}
Total average	3.2×10^{-2}	8.9×10^{-1}	1.6×10^{-2}	8.9×10^{-1}

Discussion

Environmental health is an indicator of human health. The concept of health is extremely tied up with the quality of the physical and socio-economic environment in which humans often depend on.

Environmental data based on scientific evidence provides a scientific basis to understand the health challenges and to infer the health status of humans living in the area playing a pivotal role for improvement of the public health. Although transmission of intestinal parasites through various ways have been reported, number of water-borne outbreaks have highlighted the importance of water as a main vehicle of *Giardia* spp. and *Cryptosporidium* spp. transmission. Infection is mostly occurring by exposure to drinking water contaminated with these pathogens [68,69,70]; excitingly, these parasites have been detected in packaged drinking water in Ghana [43,45]. This study is one of the few molecular studies conducted in Southern Ethiopia that provided data on molecular diversity of *G. duodenalis* and *Cryptosporidium* spp. in drinking water sources, and characterized morbidity risks of giardiasis and cryptosporidiosis quantitatively using IFA and qualitatively using nested-PCR genotypic analysis.

In the present study, *Giardia* spp. cyst was detected in 81% (17/21) of the water sources whereas *Cryptosporidium* spp. oocyst in 71.4% (15/21) of the water sources. Similar to the present finding, studies in northern Spain [71] and Sao Paulo, Brazil [72] found elevated concentration of cyst and oocyst in most of untreated and treated municipal drinking water samples from conventional and small-scale water treatment facilities. The present study found the highest concentration of cysts and oocysts in unprotected water sources while the lowest was found in river water samples. However, the highest concentration of both parasites was reported in river water samples [72]. Both parasites were also detected in natural and communal piped water in Uganda [31], tap and treated water storage tanks in Ethiopia [42], tap water and drinking water treatment plants in Egypt [44], well, spring, tap and river waters in Zimbabwe [73]. The majority of cryptosporidiosis cases in sub-Saharan Africa occur as a result of direct surface water consumption, and this transmission pathway could be responsible for an estimated 43.1 million cases annually, representing about 1.6 million DALYs [74]. The correlation between the occurrence of cysts and water turbidity is also consistent with studies done elsewhere [71,75].

The present findings revealed *G. duodenalis* (assemblages A and B) and *Cryptosporidium* spp. (*C. parvum* and *C. hominis*) are the main genotypes detected in drinking water sources. The findings showed that *Cryptosporidium* spp. (*C. parvum* and *C. hominis*) were detected in 71.4% (15/21) of the water sources. *G. duodenalis* assemblages A and B were detected in 81% (17/21) of the water sources. *G. duodenalis* isolates in 29.4% of the samples belong to Assemblage B, 17.6% belong to assemblage A and 52.9% belong to combination of assemblages A + B. The BLAST analysis showed higher genetic heterogeneity within assemblage B isolates than assemblage A isolates. All assemblage A isolates belong to sub-assemblage All, and isolates were 100% identical to reference sequences: BSW_mar'11 All (KF963567.1), GRW_jul'11 All GRW (KF963577.1) and BRW_aug'11 All (KF963573.1) registered in GenBank. Assemblage B were 100% identical to reference sequences HS98 (KC632554.1), Swemon 200 (EU781015.1) and isolate (KT948108.1). Despite the various loci used among studies, most assemblage A isolates typed at different genes (*tpi*, *GDH* and *βg* genes) belong to sub-assemblage All [29,3,50], which was also revealed in this study. Sub-assemblage All is predominantly reported from human isolates, though it is also found in animals [54], which indicates possibility of zoonotic transmission in the region. Similar to the present study, a study [7] that characterized 78 *G. duodenalis* isolates revealed the presence of sub-assemblages All, BIII and BIV in North-West Ethiopia, though there were discordant results on sub-

assemblage All/AIII and BIII/BIV in some isolates, and some differences regarding sub-assemblage B isolates. The presence of genetic heterogeneity within assemblage B isolates is supported by previous studies [5,37,55,57]. Similarly, a study [58] indicated sub-typing assemblage B isolates was more challenging than assemblage A isolates. Sequence analysis of 10 *G. duodenalis* isolates also revealed the presence of high genetic variation within assemblage B isolates, and chromatograms displayed a number of double peaks [7,16,17,18]. Mixed assemblages retrieved in the present study were relatively higher than each individual assemblage, which is supported by a study that reported higher prevalence of mixed assemblages in developing countries than in developed ones [54]. Most mixed sequences showed frequent double peaks in chromatograms and determining the origin was a challenging task. The sequences showed $\leq 99\%$ identity with sequences registered in GenBank, indicating that both assemblages in a sample were apparently divergent. The considerable number of mixed assemblages reflect complex circulation of the parasite in the region is likely. Similar to this notion, cross-species transmission of multiple *G. duodenalis* assemblages in areas where people, livestock and primates overlap in their use of habitat has been reported in Uganda [53].

The findings of present study revealed the predominance of *C. parvum* and *C. hominis* genotypes in south Ethiopia, which is also reported in other studies that have been done in different regions of the country [6,7,18,19]. However, these studies reported additional *Cryptosporidium* spp. that belong to *C. viatorum*, *C. felis*, *C. meleagridis*, *C. canis* and *C. xiaoi*. Sequence analyses of *Cryptosporidium* spp. isolates revealed the presence and genetic diversity of various subtypes, such as *C. parvum* zoonotic subtype families IIa and IIc and anthroponotic subtype families IIb, IIe and II-like, and *C. hominis* subtype families Id, Ie, Ib and IbA9G3 [6,18,22,24]. However, in the present study, only *Cryptosporidium* spp. *C. hominis* and *C. parvum* that mostly infect humans were taken into account, and the occurrence of the species was studied only at genus level.

According to the present findings, the average percentage risk of an individual to be infected with *G. duodenalis* and *Cryptosporidium* spp. (*C. parvum* and *C. hominis*) per day was 12.1% and 43.2%, respectively. The average risk of *C. parvum* infection were 3.2% for immunocompetent and 89.2% for immunocompromised sub-populations. The risks of illness per infection ranged from 0.48 to 16.33% for *G. duodenalis* and 0.67 to 43.65% for *Cryptosporidium* spp. (*C. parvum* and *C. hominis*). A study has also reported Ethiopia among four sub Saharan African countries with the highest number of cryptosporidiosis cases and together account for over 50% of all cases. The estimated annual number of cases attributable to *Cryptosporidium* spp. in consumed surface water 28.9 (35.3 \times 398.8) (\times 10,000 persons) and relative disease burden 41.0 (4.4 \times 49.7) DALYs/100,000 persons in Ethiopia and cases of illness in sub Saharan Africa. This study reveals the majority of cases are a result of direct surface water consumption and occur in the rural population [74]. Though the probability of infection was lower than the present finding, a study [48] similarly showed that the probability of *G. duodenalis* infection was close to the rates of acute diarrheic disease, $\leq 3\%$ for adults and 2–7 % for children in Brazil. The present finding also accord with other studies that reported high risks for *G. duodenalis* and *Cryptosporidium* spp. (*C. parvum* and *C. hominis*) infections from drinking water sources. A survey in 33 cities of China reported high burden of cryptosporidiosis in drinking water sources treated with conventional process. The overall

rate of diarrhea morbidity was 149 cases per 10⁵ populations; 2701 cases per 10⁵ immunocompromised and 148 cases per immunocompetent persons [76]. Higher health risk was reported for immune-deficient subpopulation than immunocompetent individuals [76,77]. This showed that the risk of infection is peculiarly high for immunocompromised individuals [28]. Similarly, a risk assessment carried out in rivers of China similarly showed high health risk of giardiasis through drinking and swimming pathway of exposures [68], which is much lower than the findings of the present study that used drinking as the only pathway of exposure. Africa apparently has the highest health risk that is almost double of the world average [51]. As water is a main vehicle of *Giardia* spp. and *Cryptosporidium* spp. transmissions, water treatment failure poses a significant risk to public health, as the parasites are resistant to most water treatment facilities [42,53,78]. The water quality in most developing countries mainly relies on a single microbial indicator and traditional treatment is primarily depended on chlorination, which are not adequate for pathogens that are resistant to such treatment facilities. Poor living condition that coincides with low accessibility to safe drinking water may associate with the highest health risk in most areas of the continent [28,68,70,77]. Therefore, the high disease burden in Ethiopia might be due to instability of water treatment process and less watershed protection, inadequate supply of safe water and poor environmental sanitation.

However, the present study has limitation on the period of sample collection. Samples were collected over a course of four months that leads to temporal/seasonal variation in water quality, especially in surface water due to precipitation and runoff. Children < 2 years and individuals who did not often use the water sources for drinking purpose were excluded, as well. In addition, the occurrence of *Cryptosporidium* spp. (*C. parvum* and *C. hominis*) was demonstrated only at genus level; sub-types were not included. QMRA also has its own limitation. These may have an impact on coverage of the study subjects and genetic diversity level of *Cryptosporidium* spp.

Conclusions

G. duodenalis and *Cryptosporidium* spp. are serious public health defiance in southern Ethiopia, being major contributors to drinking water sources contamination. This highlights the significance of water for transmission of these parasites in the region. *G. duodenalis* (assemblages A and B), *C. parvum* and *C. hominis* are the genotypes detected in the water sources. Zoonotic and anthroponotic genotypes that are detected in the water sources implies that multiple contaminat sources likely play a role for contamination of water sources in the region. The risks that are inferred from the water sources to the public are significantly higher than most other reported findings. Since high concentration of cysts and oocysts are detected in the water sources and large amount of peoples can be affected on a daily basis, there is a need to remedy and diminish the risks to public health. To enhance science-based decision and ensure sustainability of the environment and public health, careful planning and regular monitoring of water sources, application of adequate and appropriate water treatment facilities, and setting appropriate legislation and guideline is needed, basically in areas where cycles of parasitic infection is linked to water sources.

List Of Abbreviations

DAPI: 4', 6-diamidino-2-phenylindole; DPD: N, N-diethyl-p-phenylenediamine; DIC: Differential interference contrast; FITC-Mab: Fluorescein isothiocyanate–Monoclonal Antibodies; *gp60*: Glycoprotein 60; IFA: Immunofluorescence Assay; NTU: Nephelometric Turbidity Units; PCR: Polymerase Chain Reaction; QMRA: Quantitative Microbial Risk Assessment; REC: Percent Recovery; *SSU*-rRNA: Small Subunit Ribosomal RNA; T80: Tween 80; USEPA: United States Environmental Protection Agency.

Declarations

Ethical Approval and Consent to participate

The study protocol was reviewed and approved by Ethics Committee of Jimma University, Ethiopia.

Consent for publication

Not applicable.

Availability of supporting data

The data analyzed during this study are included in this manuscript. Data that are included in the manuscript will be available upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MD involved in all phases of the study; study design, data collection, laboratory works, analysis and writing the manuscript. ZM, STM, DS and LL supervised the study and revised the manuscript. All authors read and approved the final paper.

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