CrossMark

ORIGINAL PAPER

Detection by PCR of pathogenic protozoa in raw and drinkable water samples in Colombia

Jessica Triviño-Valencia[1] · Fabiana Lora[1] · Juan David Zuluaga[1] ·
Jorge E. Gomez-Marin[1]

Abstract We evaluated the presence of DNA of *Giardia*, *Toxoplasma*, and *Cryptosporidium* by PCR, and of *Giardia* and *Cryptosporidium* genera by immunofluorescence antibody test (IFAT), in water samples, before, during, and after plant treatment for drinkable water. We applied this method in 38 samples of 10 l of water taken from each of the water treatment steps and in 8 samples taken at home (only for *Toxoplasma* PCR) in Quindio region in Colombia. There were 8 positive samples for *Cryptosporidium parvum* (21 %), 4 for *Cryptosporidium hominis* (10.5 %), 27 for *Toxoplasma gondii* (58.6 %), 2 for *Giardia duodenalis* assemblage A (5.2 %), and 5 for *G. duodenalis* assemblage B (13.1 %). By IFAT, 23 % were positive for *Giardia* and 21 % for *Cryptosporidium*. An almost perfect agreement was found between IFAT and combined results of PCR, by *Kappa* composite proportion analysis. PCR positive samples were significantly more frequent in untreated raw water for *C. parvum* ($p = 0.02$). High mean of fecal coliforms, high pH values, and low mean of chlorine residuals were strongly correlated with PCR positivity for *G. duodenalis* assemblage B. High pH value was correlated with PCR positivity for *C. parvum*. Phylogenetic analysis of DNA sequences was possible, showing water and human clinical sequences for *Toxoplasma* within the same phylogenetic group for B1 repeated sequence. PCR assay is complementary to IFAT assay for monitoring of protozoa in raw and drinkable water, enabling species identification and to look for phylogenetic analysis in protozoa from human and environmental sources.

✉ Jorge E. Gomez-Marin
gepamol2@uniquindio.edu.co

Jessica Triviño-Valencia
jeca1424@hotmail.com

Fabiana Lora
flora@uniquindio.edu.co

Juan David Zuluaga
expelius@gmail.com

[1] Grupo GEPAMOL, Centro de Investigaciones Biomédicas, Facultad de Ciencias de la Salud, Universidad del Quindio, Av. Bolivar 12N, Armenia, Quindio, Colombia

Introduction

Waterborne protozoa are a major challenge for drinking water treatment. *Cryptosporidium* and *Giardia* are the most frequently reported waterborne protozoa, but *Toxoplasma*, *Cyclospora*, and *Entamoeba hystolitica* are also of importance by their impact on global health (Baldursson and Karanis 2011). Some of these parasites have gained more attention recently. For example, the recent Global Enteric Multicenter Study, which analyzed more than 9000 children with diarrhea, found that *Cryptosporidium*, an organism previously thought to be restricted to the immunocompromised, was an important cause of diarrhea, especially in children younger than 2 years (Kotloff et al. 2013). Most considered the infection by *Giardia*, the most common intestinal parasite of humans in many countries, as of little harm for human health; however, prospective studies have changed this view. *Giardia* infection in early childhood was associated with poor cognitive function and failure to thrive (Berkman et al. 2002). In a rigorous meta-analysis, *Giardia duodenalis* was positively linked to persistent diarrhea (Muhsen and Levine 2012), and in another study, it was found that giardiasis was a strong predictor of stunted growth (Botero-Garcés et al. 2009). *Toxoplasma*

infection in humans is also a significant cause of morbidity as cause of congenital infection and of opportunistic infections in immunocompromised patients (Gomez-Marin 2010), in whose cases the waterborne route has been suggested to be the major source of *Toxoplasma* infection (Lopez-Castillo et al. 2005).

Protozoa resist traditional chlorinated water treatment and are found at the different steps of water treatment (Karanis et al. 1998; Alarcón et al. 2005; Castro-Hermida et al. 2015). Here we report the DNA detection of pathogen protozoa in raw and treated water obtained after formaline/ether concentration method. This technique enables us to determine the protozoa presence in many water samples in the Quindio region of Colombia and its concordance with detection by immunofluorescence antibody test (IFAT) for *Giardia* and *Cryptosporidium*.

Materials and methods

Cyst and oocyst parasite reference strains

Waterborne, Inc., Clinical and Environmental Parasitology Products (New Orleans, LA, USA) purchased purified *Cryptosporidium parvum* oocyst and *G. duodenalis* cyst preparations and kept them at 4 °C until use. This reagent is a mixture of *G. duodenalis* cysts and *C. parvum* oocysts in a mixed aldehyde buffer. The concentration of this suspension was counted by hemocytometer (McCuin and Clancy 2003).

Formalin-ether concentration method for water samples

We collected a volume of 10 l by samples in plastic sterile recipient and then divided in aliquots of 500 ml. We centrifuged each for 5 min at 1.600g. We discarded supernatant from each tube and then vortexed each tube and collected them all into one tube, attaining approximately a total volume of 15 ml. We centrifuged this tube at 1600g for 3 min and then discarded the supernatant, and completed pellet was until 5 ml in sterile saline solution. Then, we added 5 ml of formalin solution 10 % and 3 ml of diethyl ether 99 % (Sigma, USA). We sealed and rigorously shook the tube to bring the diethyl ether in contact with all parts of the sediment and performed a new centrifugation at 1000g for 2 min. After that, four different layers formed, as follows: (i) a sediment at the bottom, (ii) saline, (iii) water debris, and (iv) diethyl ether on the top. We decanted the upper three layers so that only the sediment remained in the tube. We resuspended this layer in 500 μl of 0.85 % sodium chloride and subsequently placed 100 μl on slide for immunofluorescence and 300 μl for DNA extraction procedures as described below.

DNA extraction method

The Wizard Genomic DNA Purification Kit (Promega, USA) with mechanical lysis with zirconium bead was applied for DNA extraction in water eluates. This method in previous assays in our hands, with reference strains, showed the best recovery performance. We centrifuged 300 μl of sediment eluates at 13,500g for 6 min at 4 °C and resuspended pellet in 20 μl of isoamilic alcohol and 900 μl of DNAazol lysis buffer and incubated at room temperature for 5 min. Then, we added 0.3 g of 0.5-mm zirconia beads (BioSpec product Inc., USA) and followed by shaking in a mini-bead beater (Stratech UK) for 1 min and put on ice for 1 min; we repeated this step five times. After centrifugation at 13,500g for 6 min, we transferred supernatant to a new tube and mixed it with 300 μl of nuclear lysis buffer, vortexed it for 20 s, and maintained it at 37 °C for 30 min. Then, we added 100 μl of protein precipitation lysis, vortexed for 20 s, and put tube on ice for 5 min. Subsequently, we centrifuged sample at 13,500g 2 min and transferred supernatant to another tube with 300 μl of isopropanolol, mixed gently four times, and centrifuged at 13,500g for 2 min and discarded supernatant and dissolved pellet in Tris-EDTA buffer.

Reference strains for DNA analysis

The reference strains we used as controls for sensitivity and specificity of PCR reactions were as follows: WBC6 from *Giardia* assemblage A1, *Toxoplasma* oocyst ME49 strain (clonal type II), cat 19, TX 586 collected June, 2014 and kindly donated by Dr. Dubey (USDA, Belstville). *Cryptosporidium hominis* DNA was kindly gifted by Dr. Lucy Robertson (NMBU, Oslo, Norway) and *C. parvum* DNA from cattle by Dr. Jesus Cortés (Universidad Nacional de Colombia) and Dr. Santín-Durán from the Environmental Microbial and Food Safety Lab ANRI/ARS/USDA (MD, USA).

PCR assays

To detect *Toxoplasma* DNA, we used conventional nested PCR for this study as described previously (Ponce and Gomez-Marin 2003). Briefly, this test amplified a 97-bp fragment of the B1 gene of *Toxoplasma*, which is tandemly arrayed, 35-fold repetitive (GenBank accession number AF179871). The primers for first PCR were Toxo N1 (694 to 714 nt) 5′-GGAACTGCATCCGTTCATGAG-3′ and Toxo C1 (887 to 868 nt) 5′-TCTTTAAAGCGTTCGTGGTC-3′. The second PCR was performed with the primers Toxo N2 (757 and 776 nt) 5′-TGCATAGGTTGCCAGTCACTG-3′ and Toxo C2 (853 and 831 nt) 5′-GGCGACCAATCTGCGAATACACC-3′. All primers were synthetized by Invitrogen Corporation (USA). The first amplification protocol consisted of one initial stage of denaturation for 5 min at 94 °C, followed by 40 cycles of amplification, and 1 cycle consisting of 1 min at 94 °C for DNA

denaturation, 1 min of annealing at 53 °C, and 1 min of extension at 72 °C. Subsequently, we performed an additional step of 10 min of final extension at 72 °C. We made the second PCR with the product of the first amplification, consisting of one initial stage of denaturation for 5 min at 94 °C, followed by 14 cycles of amplification, and 1 cycle consisting of 1 min at 94 °C for DNA denaturation, 1 min of annealing at 53 °C, and extension at 72 °C by 30 s. Then, we performed an additional step of 10 min of final extension at 72 °C. We used 1.5 % agarose gel electrophoresis to analyze PCR products, which were positive or negative. Positive control was DNA from the *Toxoplasma* control strains and negative control was distilled water in the presence of primers.

Giardia DNA was detected by amplifying specific sequences for *Giardia* assemblage A and B of the glutamate dehydrogenase (GDH) gene (Lebbad et al. 2011). Sequences of primers for assemblage A were forward 5′-CCGCGAGATCGGGTACCTGTA-3′ and reverse 5′-GCCGGAGACAGAGACACCG-3′ (637 bp) and for *Giardia* assemblage B were forward 5′-ATCCTTAAGTTCCTCGGC-3′ and reverse 5′-ATCGGTTATCTGTTTGGAC-3′ (232 bp). This assay aimed to establish the presence of *Giardia* for specific genotypes. The PCR mix was the same for assemblage A and B and consisted of 12.5 μl of GoTaq Green Master Mix (Promega, USA) containing deoxyribonucleoside triphosphates 0.2 mM, MgCL 25 mM, Taq polymerase 0.05 U of primers, and 3 μl of DNA in a final volume of 25 μl. For assemblage A, amplification cycles were as follows: denaturation at 94 °C for 5 min; then 38 cycles comprising 1 min at 94 °C, annealing 61 °C for 1 min, and 1 min at 72 °C; followed by a final extension of 5 min at 72 °C. For assemblage B, amplification cycles were as follows: denaturation at 94 °C for 5 min; then 38 cycles comprising 1 min at 94 °C, annealing 49 °C for 1 min, and 1 min at 72 °C; followed by a final extension of 5 min at 72 °C. This PCR in previous assays in our hands for assemblage A had sensitivity of 65.5 fg/μl and for assemblage B of 6.5 fg/μl.

Cryptosporidium DNA species was detected by amplifying specific sequences for *C. hominis* from the 60-kDa glycoprotein (gp60) gene (Ebner et al. 2015), forward 5′-

AGAAAGAACTGTAGAGGGC-3′ and reverse 3′-GAGTAGTAGTTGCTGTGGAC-′5 (213 bp), for *C. parvum* from the 60-kDa glycoprotein (gp60) gene, forward 5′-TAGTGAAGACGATGGCCAAA-3′ and reverse 5′-CAGCTGGGGTACCTTCTC-3′ (149 bp).The PCR mix was the same for *C. hominis* and *C. parvum* and consisted of 12.5 μl of GoTaq Green Master Mix (Promega, USA) containing deoxyribonucleoside triphosphates 0.2 mM, MgCL 25 mM, Taq polymerase 0.05 U of primers, and 3 μl of DNA in a final volume of 25 μl. For *C. hominis*, amplification cycles were as follows: denaturation at 94 °C for 5 min; then 38 cycles comprising 1 min at 94 °C, annealing 47 °C for 1 min, and 1 min at 72 °C; followed by a final extension of 5 min at 72 °C. For *C. parvum*, amplification cycles were as follows: denaturation at 94 °C for 5 min; then 38 cycles comprising 1 min at 94 °C, annealing 52.5 °C for 1 min, and 1 min at 72 °C; followed by a final extension of 5 min at 72 °C. This PCR in previous assays in our hands for *C. parvum* had sensitivity of 49.3 fg/μl and for *C. hominis* of 50 fg/μl.

We also included control for contamination during DNA extraction to control carryover contamination and consisted it in a tube without template but containing all reagents for DNA extraction and filled with the same pipette thereafter water samples. To avoid contamination, we took several measures, such as separate space to set up PCRs (pre-PCR room) separate filter tips and pipettes, and various negative controls (no DNA, DNA extraction contamination controls). In addition, we tested some negative samples by adding control protozoa DNA to detect inhibitors.

The SensoQuestLabCycler machine (USA) performed all PCR amplifications.

Immunofluorescence detection of *Giardia* and *Cryptosporidium* (IFAT test)

The Aqua-Glo™ kit catalog number A100FLK (Waterborne Inc., USA) was used to detect the cyst and oocyst stages of these parasites in particles isolated from water utilizing the principle of direct immunofluorescence antibody test (IFAT). The antibody reagent consists of a mixture of fluorescein-

Table 1 Number and percent (%) of PCR positive assays for each protozoa by site of sampling

Site of sampling	*Cryptosporidium parvum*	*Cryptosporidium hominis*	*Toxoplasma gondii*	*Giardia duodenalis* assemblage A	*Giardia duodenalis* assemblage B
Surface water, before plant ($n=13$)	5	2	10	1	5
Plant ($n=5$)	1	1	2	0	1
Network ($n=20$)	2	1	12	1	2
Home ($n=8$)	ND	ND	3	ND	ND
Number of PCR positives/total samples (% positives)	8/38 (21.0 %)	4/38 (10.5 %)	27/46 (58.6 %)	2/38 (5.2 %)	5/38 (13.1 %)

ND not done

labeled mouse monoclonal antibodies made to cyst and oocyst outer wall antigenic sites (epitopes) of *G. lamblia* and *C. parvum*. This reagent is genus-specific and will bind only to the cysts and oocysts of these two parasites if they are present. The reagent shows varying degrees of cross-reactivity with cysts and oocysts of other species of *Giardia* and *Cryptosporidium*. The cysts and/or oocysts will appear bright apple green when viewed under a fluorescence microscope using the appropriate filters for fluorescein. Procedures, controls, and results interpretation followed manufacturers' recommendations. Lecture of slides was done with an Evos FL Auto cells System microscope (Life Technologies, USA). Three microscopists read the slides. This method did not test home samples.

Study area

Samples were collected before, during, and after treatment in one drinking water treatment plant in Quindio region (4° 32′ 00″ N, 75° 42′) located in the area known as "Eje Cafetero" (Coffee Axis), because it is here that most of the world famous Colombian coffee is produced. This area is located within the country's central mountain range. The drinking water treatment plant obtains the water from river and performs treatment with coagulation, flocculation, sedimentation, filtration, and disinfection by chlorination. In total, 38 samples were taken in different water treatment steps, including raw water source areas, in plant treatment (reaction tank, settling pond, sand filtration, and the clear water tank) and in system of distribution (network). In addition, we took eight samples at home only for *Toxoplasm*a DNA detection, sampling 10 l at each point. Recipients were previously sterilized. The recipients were immersed 1 m at each point in surface source and in plant treatment points.

DNA sequencing and phylogenetic analysis

For sequencing, PCR products were gel-purified from low-melt agarose gels, followed by recovery using the Wizard

Table 2 Number and percent (%) of positive for *Cryptosporidium* and *Giardia* IFAT genus specific assays by site of sampling

Site of sampling	*Cryptosporidium* sp.	*Giardia* sp.
Surface water, before plant (*n* = 13)	6	5
Plant (*n* = 5)	1	2
Network (*n* = 20)	1	2
Number of IFAT positives/total samples (% positives)	8/38 (21 %)	9/38 (23.6 %)

Table 3 Correlation between positivity of PCR for each protozoa and physicochemical characteristics of water

Physicochemical characteristics	*Cryptosporidium parvum*	*Cryptosporidium hominis*	*Toxoplasma gondii*	*Giardia duodenalis* assamblage A	*Giardia duodenalis* assamblage B
Mean turbidity (NTU) in PCR negative versus positive samples	0.8 versus 0.7 (p = 0.71)	0.8 versus 0.9 (p = 0.16)	0.78 versus 0.90 (p = 0.73)	0.86 versus 0.85 (p = 0.49)	0.84 versus 0.9 (p = 0.15)
Mean temperature (°C) in PCR negative versus positive samples	19.2 versus 18.6 (p = 0.64)	19.0 versus 19.2 (p = 0.73)	19.6 versus 18.8 (p = 0.2)	19.1 versus 18.0 (p = 0.8)	19.4 versus 17.7 (p = 0.15)
Mean fecal coliforms UFC/100 ml in PCR negative versus positive samples	89 versus 111 (p = 0.28)	80 versus 202 (p = 0.08)	37.1 versus 126 (p = 0.18)	96.6 versus 40 (p = 0.97)	65.8 versus 217 (p = 0.006)
Mean chlorine residual (mg/l) in PCR negative versus positive samples	0.98 versus 0.67 (p = 0.6)	0.97 versus 0.47 (p = 0.6)	1.1 versus 0.77 (p = 0.11)	0.9 versus 0.9 (p = 0.86)	1.0 versus 0.17 (p = 0.006)
Mean pH value in PCR negative versus positive samples	7.3 versus 7.7 (p = 0.048)	7.4 versus 7.5 (p = 0.5)	7.3 versus 7.4 (p = 0.4)	7.4 versus 7.7 (p = 0.2)	7.3 versus 7.9 (p = 0.001)

*p < 0.05

PCR Prep kit (Promega, WI). Sequencing followed BigDye® terminator cycling conditions by using the normal automatic service of Macrogen (Korea) in 3730XL DNA sequencer with the same primers of the PCR amplifications. We removed reads containing more than 10 % 'N's; (ii) more than 50 % bases having low quality value (Phred score ≤5). We aligned sequences with Clustal W and derived phylogenetic trees using maximum likelihood phylogeny test in Molecular Evolutionary Genetics Analysis (MEGA) software, Version 6 (available at: http://www.megasoftware.net/). Calculations were made with Kimura 2 –parameter model in 2000 bootstrap replicates. Tree inference was made over initial neighbor joining trees with strong branch swap filter.

Statistical analysis

Results are median [min-max] or mean±standard deviation for continuous variables and N (%) for categorical variables. We analyzed differences in proportions using the chi-square test or Fisher's exact test when appropriate. We compared differences in means using Student's t test or nonparametric test if not normally distributed, with the statistical package software SPSS, version 14 (SPSS Inc. Chicago, USA). We used Epi-Info to perform stratified analysis (Centers for Disease Control and Prevention [http://www.cdc.gov/epiinfo/]). Values below $p < 0.05$ were statistically significant. Test results for PCR and IFAT for *Giardia* and *Cryptosporidium* were classified into two-by-two contingency tables, and the concordance between the tests was determined by κ statistics. Composite *Kappa* proportion and confidence intervals for proportions were calculated in the website VassarStats (http://vassarstats.net/kappa.html). Interpretation of agreement according to the results of the composite *Kappa* proportion was as follows: <0 less than chance agreement, 0.01–0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, and 0.81–0.99 almost perfect agreement (Viera and Garrett 2005).

Results

Results of PCR assays by each protozoon

In total, we studied 46 samples (Table 1). Thirteen samples were from surface raw water and 33 from treated water taken at plant ($n = 5$), network ($n = 20$), and home ($n = 8$). Significantly, more samples were positive for PCR for protozoa in untreated (surface and plant) than in treated water (network and home), with corrected Mantel Haenzel estimate of $p = 0.0018$. When the frequency was analyzed by each protozoa, PCR positive assay was significantly more frequent in untreated raw water for *C. parvum* ($p = 0.02$). When physicochemical characteristics of water were correlated with PCR positivity for each protozoa (Table 2), high mean fecal coliforms and pH values and low mean of chlorine residual were strongly correlated with PCR positivity for *G. duodenalis* assemblage B. Also, high pH value was correlated with PCR positivity for *C. parvum* (Table 2).

Results of *Giardia* and *Cryptosporidium* IFAT and correlation with PCR results

There were 8 samples positives from 38 tested (21 %) by IFAT for *Cryptosporidium* and 9 samples positive of 38 (23 %) by *Giardia* IFAT test (Table 3). Some physicochemical characteristics of water were significantly correlated with positivity by IFAT. Positivity by IFAT for *Giardia* was correlated with higher mean of fecal coliforms and high water pH. Samples positive by IFAT for *Cryptosporidium* were significantly associated to higher mean of turbidity, low temperature, and low residual chlorine concentration (Table 4).

Because the IFAT test is genus but not species specific, the percent of agreement as indicated by *Kappa* index, between the IFAT and PCR assays for *Cryptosporidium*, was calculated by adding the results of PCR for *C. parvum* and *C. hominis* and, for *Giardia*, by adding the results of PCR for *G. duodenalis* assemblage A and

Table 4 Correlation between positivity of *Cryptosporidium* sp. and *Giardia* sp. IFAT genus specific assays and physicochemical characteristics of water

Physicochemical characteristics	*Cryptosporidium* sp.	*Giardia* sp.
Mean turbidity (NTU) in IFAT negative versus positive samples	0.8 versus 0.9 ($p = 0.016$)*	0.8 versus 0.9 ($p = 0.053$)
Mean temperature °C in IFAT negative versus positive samples	19.4 versus 17.6 ($p = 0.04$)*	19.2 versus 18 ($p = 0.95$)
Mean fecal coliforms UFC/100 ml in IFAT negative versus positive samples	84 versus 123 ($p = 0.11$)	77 versus 156 ($p = 0.008$)*
Mean chlorine residual mg/l in IFAT negative versus positive samples	1.1 versus 0.16 ($p = 0.0034$)*	1.0 versus 0.5 ($p = 0.12$)
Mean pH value in IFAT negative versus positive samples	7.3 versus 7.8 ($p = 0.009$)*	7.3 versus 7.7 ($p = 0.02$)*

IFAT immunofluorescence antibody test

*$p < 0.05$

Table 5 Composite *Kappa* proportion agreement between results of *Cryptosporidium* sp. and *Giardia* sp. IFAT genus-specific assays and results of PCR assays

| Test compared	*Kappa* composite agreement proportion (confidence interval)	Interpretation
IFAT for *Cryptosporidium* and combined results of PCR assays for *C. parvum* and *C. hominis*	0.92 (0.65–0.91)	Almost perfect agreement
IFAT for *Giardia* and combined results of PCR assays for *G. duodenalis* assemblage A and assemblage B	0.97 (0.65–0.91)	Almost perfect agreement
IFAT for *Cryptosporidium* and PCR assays for *C. parvum*	0.62 (0.62–0.89)	Substantial agreement
IFAT for *Cryptosporidium* and PCR assays for *C. hominis*	0.89 (0.68–0.93)	Almost perfect agreement
IFAT for *Giardia* and PCR assays for *G. duodenalis* assemblage A	0.80 (0.6–0.9)	Substantial agreement
IFAT for *Giardia* and PCR assays for *G. duodenalis* assemblage B	0.94 (0.6–0.89)	Almost perfect agreement

assemblage B. This resulted in almost perfect agreement (Table 5). When the *Kappa* index was calculated separately for each PCR species-specific result, the composite *Kappa* proportion of agreement was almost perfect between IFAT for *Cryptosporidium* and PCR for *C. hominis*, IFAT for *Giardia* and PCR result for *G. duodenalis* assemblage B, substantial between IFAT for *Cryptosporidium* and PCR for *C. parvum*, and IFAT for *Giardia* and PCR for *G. duodenalis* assemblage B (Table 5). Within positive samples by IFAT for *Cryptosporidium*, three of them were also positive by *C. hominis* PCR assay and four by *C. parvum* PCR assay. Only one sample was positive by PCR for *C. hominis* but not by IFAT and four were positive for *C. parvum* PCR assay but not for IFAT for *Cryptosporidium*. For *Giardia* IFAT test, there were 9 samples positive of 38 (23 %) and 2 of them were also positive by *G. duodenalis* PCR for

assemblage A and 4 positive by PCR for *G. duodenalis* assemblage B. Three samples were positive by PCR for *G. duodenalis* assemblage B but not by IFAT *Giardia* test. Only one sample of raw water taken before plant was positive by IFAT and positive, by PCR, either for *C. parvum* as *C. hominis*. Similar situation was found in another raw sample, positive by IFAT for *Giardia* and positive either by PCR for *G. duodenalis* assemblage A as *G. duodenalis* assemblage B.

Results of sequencing and phylogenetic analysis of amplified sequences

Quality of amplification was good to obtain sequencing in 27 DNA amplified sequences for *Toxoplasma*, in 9 of *C. parvum*, in 2 of *C. hominis*, and in 1 of *G. duodenalis* assemblage A.

Fig. 1 The evolutionary history for B1 genomic repeated sequence of *Toxoplasma gondii* was inferred by using the maximum likelihood method based on the Tamura-Nei model. The analysis involved 59 nucleotide sequences. Water samples have the prefix home, surface, or network, indicating where they were obtained. There were a total of 59 positions in the final dataset. Evolutionary analyses were conducted in MEGA6
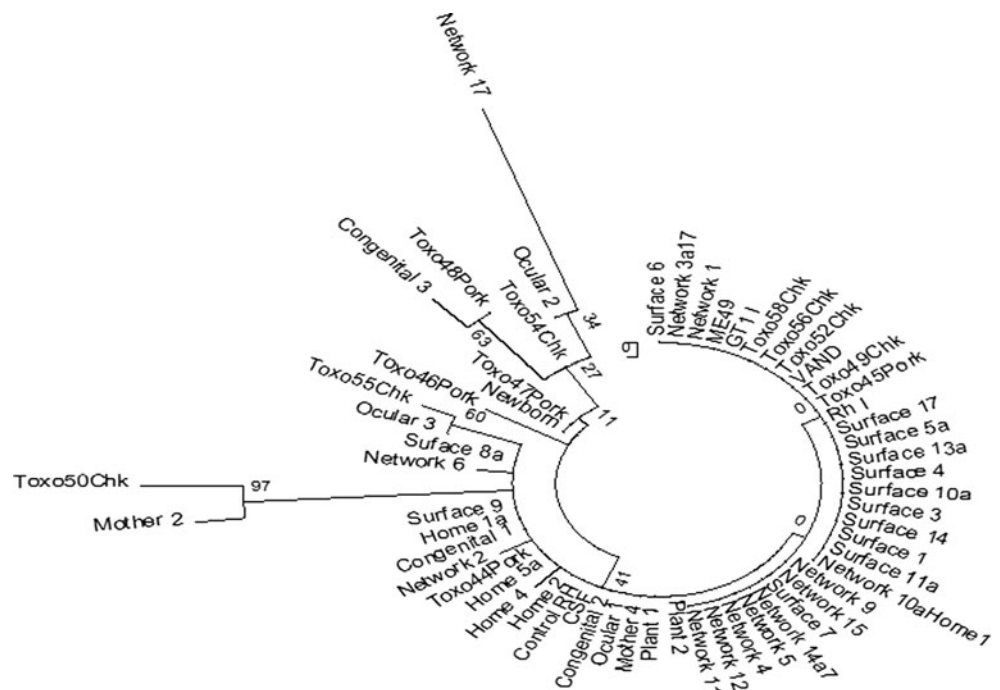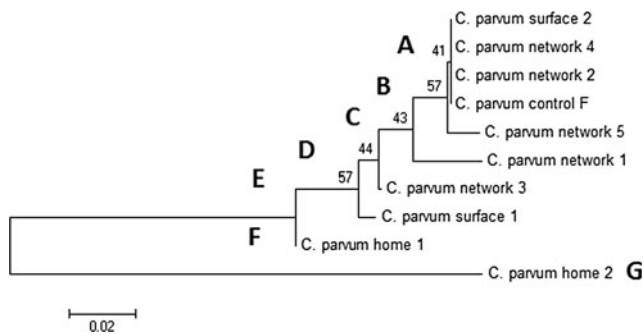
Fig. 2 The evolutionary history for 60-kDa glycoprotein (gp60) gene of *Cryptosporidium parvum* was inferred by using the maximum likelihood method based on the Jukes-Cantor model. The analysis involved ten nucleotide sequences. Codon positions included were first + second + third + noncoding. There were a total of 103 positions in the final dataset. Evolutionary analyses were conducted in MEGA6

The alignment of the same nucleotide region of sequences of *Toxoplasma* B1 region from 27 water samples was performed with sequences obtained from newborns with congenital infection ($n = 5$), ocular toxoplasmosis ($n = 3$), peripheral blood of mothers with recent acquired toxoplasmosis during pregnancy ($n = 2$), pork meat ($n = 4$), chicken meat ($n = 7$), and sequences reported in GenBank for the references strains Rh (clonal type I), GT1 (clonal type 1), ME49 (clonal type II), and VAND (atypical strain). Clinical and meat samples came from our previously published work (Alvarez et al. 2015; Franco-Hernandez et al. 2015). Maximum likelihood method revealed the phylogenetic history of this sequence, as described in Fig. 1. As expected for this region of the B1 sequence, most of samples were similar including water, clinical, and meat samples, with some divergent sequences. No particular groups were related with source of samples. The divergent sequences were either clinical as water samples.

For *C. parvum*, the phylogenetic analysis identified seven clusters within ten sequences, nine from the water samples of Quindio and one from the GenBank database (Fig. 2). The A

group included four sequences; the other groups had only one sequence each. No grouping was observed by source of sample.

For *Giardia* assemblage B and *C. hominis*, the number of sequences was only one or two sequences and only Clustal alignments are showed (Fig. 3). The *Giardia* sequence that was amplified in one sample at home was homologue in 98 % to the sequence of the *G. duodenalis* DH assemblage A2 isolate and 90 % to the sequence of the isolate P15 assemblage E, at GiardiaDB (http://giardiadb.org/).

Discussion

We found a high prevalence of positive PCR and IFAT samples for pathogenic protozoa in Colombian raw and drinkable water. We applied both assays for *Giardia* and *Cryptosporidium*, PCR and IFAT, to the same samples, and although they attained almost perfect agreement, some important discordances appeared, explainable in two different reasons; one is that IFAT is genus specific and PCR is species specific; therefore, IFAT can detect *Cryptosporidium* species or *Giardia* species that are not detected by PCR. Also, it is well known that PCR can be less sensitive than IFAT, because the wall of the cysts and oocysts of protozoa is a difficult target for DNA extraction procedures and by the presence of PCR inhibitors in environmental samples, as described in similar studies (Karanis et al. 2006; Castro-Hermida et al. 2015). This can explain why in some samples positive for IFAT, PCR was negative. In our study, this was the case for two samples for *Cryptosporidium* and in four samples for *Giardia*. But, the opposite situation is noteworthy. In the present study, we found three samples for *Giardia* negative by IFAT and PCR positive and five samples positive for *Cryptosporidium* by PCR and negative by IFAT. We think that this can be explained by good performance of our DNA method of

Fig. 3 Clustal alignment for polymorphic sites for **a** 60-kDa glycoprotein (gp60) gene sequence of *C. hominis* at Genbank and sequences amplified at network samples; **b** NADPH-dependent glutamate dehydrogenase gene of *G. intestinalis* with the sequence VANC/89/UBC/37

assemblage B obtained at Genbank. *Periods* denote agreement with the consensus sequence. *Dashes* denote insertions and deletions (INDELS) in the nucleotide sequence

extraction giving best sensitivity than IFAT to detect presence of protozoa in some samples. In consequence, the negative result in PCR in samples with positive IFAT would best correspond to presence of *Giardia* or *Cryptosporidium* different to that of *G. duodenalis* or *C. parvum* or *C. hominis*. The best agreement proportion between IFAT and PCR for *C. hominis* and *G. duodenalis* assemblage B relates to the greater frequency of these protozoa in the samples. In the central region of Colombia, a recent study in children showed that B assemblage was the predominant genotype in children's stool (Ramírez et al. 2015). Similar results of predominance of B genotype of *G. duodenalis* were found in water samples in Iran (Mahmoudi et al. 2015). The PCR for specific species has the limitation that it would overcome mixed occurrence of different species or genotypes. For that reason, metagenomics is warranted in future work (Prystajecky et al. 2015).

Although PCR does not demonstrate infectivity, it is a good indicator of the level of exposition of water for human consumption to pathogenic protozoa. The results for *Toxoplasma* are similar to those described by PCR in raw and treated water in Bulgaria of 48 % (Sotiriadou and Karanis 2008) and in Poland of 37.5 % (Sroka et al. 2006). Lower frequency of *Toxoplasma* PCR positive result appeared in the Northern Hemisphere, in countries such as Scotland, where by using real-time PCR, 8.7 % of 1411 samples were positive (Wells et al. 2015), and in France in Champagne region, where PCR assay was applied to 482 environmental water samples and *Toxoplasma* DNA appeared in 7.7 % of samples, including public drinking water samples (Aubert and Villena 2009). For *Giardia* and *Cryptosporidium*, our results are similar to studies in other countries of South America such as Brazil where, by using the same approach for *Giardia* and *Cryptosporidium*, it was found that 8.3 % of the samples of raw water were positive for *Giardia* by IFAT and 8.3 % positive by PCR. The presence of *G. duodenalis* and *C. parvum* correlated with turbidity (Almeida et al. 2015).

Phylogenetic analysis could only affect the locus amplified by PCR. To obtain epidemiological profiles of transmission and circulation of specific genotypes, one needs multi-locus analysis with a good amount and quality of DNA. This would help to define the sources and routes of transmission. At least for *Toxoplasma*, we grouped samples taken in treated water with clinical samples, suggesting water as potential source. The presence of DNA did not diminish as other protozoa did after chlorination. The Quindio region has elevated rates of toxoplasmosis. Epidemiological studies indicate that drinking of unboiled water is the most important risk associated with this infection and that drinking of bottled water reduces in the same proportion the risk for pregnant women (Lopez-Castillo et al. 2005).

In conclusion, our results confirm that PCR assay complements IFAT assay for monitoring of protozoa in raw and drinkable water, as proposed by other authors (Ma et al. 2014; Castro-Hermida et al. 2015). The sequencing of amplified regions enables species identification and phylogenetic analysis in protozoa from human and environmental sources, enabling identification of sources of contamination for drinkable water.

References

Alarcón M, Beltrán M, Cárdenas M, Campos M (2005) Recuento y determinación de viabilidad de *Giardia spp.* y *Cryptosporidium* spp. en aguas potables y residuales en la cuenca alta del rio Bogotá. Biomédica 25:353–365

Almeida JC, Martins FDC, Ferreira Neto JM, Santos MM, Garcia JL, Navarro IT, Kuroda EK, Freire RL (2015) Occurrence of *Cryptosporidium* spp. and *Giardia* spp. in a public water-treatment system, Paraná, Southern Brazil. Rev Bras Parasitol Vet 24:303–8

Alvarez C, de-la-Torre A, Vargas M, Herrera C, Uribe-Huertas LD, Lora F, Gómez-Marín JE (2015) Striking Divergence in *Toxoplasma* ROP16 Nucleotide Sequences From Human and Meat Samples. J Infect Dis 211:2006–2013

Aubert D, Villena I (2009) Detection of *Toxoplasma gondii* oocysts in water: proposition of a strategy and evaluation in Champagne-Ardenne Region, France. Mem Inst Oswaldo Cruz 104:290–5

Baldursson S, Karanis P (2011) Waterborne transmission of protozoan parasites: review of worldwide outbreaks—an update 2004–2010. Water Res 45:6603–6614

Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM (2002) Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet 359:564–571

Botero-Garcés JH, García-Montoya GM, Grisales-Patiño D, Aguirre-Acevedo DC, Alvarez-Uribe MC (2009) *Giardia intestinalis* and nutritional status in children participating in the complementary nutrition program, Antioquia, Colombia, May to October 2006. Revista do Instituto de Medicina Tropical de São Paulo 51:155–162

Castro-Hermida JA, González-Warleta M, Mezo M (2015) *Cryptosporidium* spp. and *Giardia duodenalis* as pathogenic contaminants of water in Galicia, Spain: the need for safe drinking water. Int J Hyg Environ Health 218:132–8

Ebner J, Koehler AV, Robertson G, Bradbury RS, Jex AR, Haydon SR, Stevens MA, Norton R, Joachim A, Gasser RB (2015) Genetic analysis of *Giardia* and *Cryptosporidium* from people in Northern Australia using PCR-based tools. Infect Genet Evol

Franco-Hernandez EN, Acosta A, Cortés-Vecino J, Gómez-Marín JE (2015) Survey for *Toxoplasma gondii* by PCR detection in meat for human consumption in Colombia. Parasitol Res

Gomez-Marin JE (2010) Protozoologia Medica, First. Manual Moderno, Bogota

Karanis P, Schoenen D, Seitz HM (1998) Distribution and removal of *Giardia* and *Cryptosporidium* in water supplies in Germany. Water Sci Technol 37:9–18

Karanis P, Sotiriadou I, Kartashev V, Kourenti C, Tsvetkova N, Stojanova K (2006) Occurrence of *Giardia* and *Cryptosporidium* in water supplies of Russia and Bulgaria. Environ Res 102:260–271

Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acácio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM (2013) Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382:209–22

Lebbad M, Petersson I, Karlsson L, Botero-Kleiven S, Andersson JO, Svenungsson B, Svärd SG (2011) Multilocus genotyping of human *Giardia* isolates suggests limited zoonotic transmission and association between assemblage B and flatulence in children. PLoS Negl Trop Dis 5:e1262

Lopez-Castillo CA, Diaz-Ramirez J, Gomez-Marin JE (2005) Risk factors for *Toxoplasma gondii* infection in pregnant women in Armenia, Colombia. Rev Salud Publica 7:180–190

Ma L, Sotiriadou I, Cai Q, Karanis G, Wang G, Wang G, Lu Y, Li X, Karanis P (2014) Detection of *Cryptosporidium* and *Giardia* in agricultural and water environments in the Qinghai area of China by IFT and PCR. Parasitol Res 113:3177–84

Mahmoudi MR, Nazemalhosseini-Mojarad E, Karanis P (2015) Genotyping of *Giardia lamblia* and *Entamoeba spp* from river waters in Iran. Parasitol Res 114:4565–70

McCuin RM, Clancy JL (2003) Modifications to United States Environmental Protection Agency methods 1622 and 1623 for detection of *Cryptosporidium* oocysts and *Giardia* cysts in water. Appl Environ Microbiol 69:267–74

Muhsen K, Levine MM (2012) A systematic review and meta-analysis of the association between *Giardia lamblia* and endemic pediatric diarrhea in developing countries. Clin Infect Dis 55(Suppl 4): S271–S293

Ponce N, Gomez-Marin JE (2003) Estandarización y validación clínica de la prueba de reacción en cadena de la polimerasa (PCR) para diagnóstico de toxoplasmosis cerebral en pacientes infectados por el VIH. Infectio 7:8–14

Prystajecky N, Tsui CK, Hsiao WW, Uyaguari-Diaz MI, Ho J, Tang P, Isaac-Renton J (2015) *Giardia* spp. are commonly found in mixed assemblages in surface water, as revealed by molecular and whole-genome characterization. Appl Environ Microbiol 81:4827–34

Ramírez JD, Heredia RD, Hernández C, León CM, Moncada LI, Reyes P, Pinilla AE, Lopez MC (2015) Molecular diagnosis and genotype analysis of *Giardia duodenalis* in asymptomatic children from a rural area in central Colombia. Infect Genet Evol 32:208–13

Sotiriadou I, Karanis P (2008) Evaluation of loop-mediated isothermal amplification for detection of *Toxoplasma gondii* in water samples and comparative findings by polymerase chain reaction and immunofluorescence test (IFT). Diagn Microbiol Infect Dis 62:357–65

Sroka J, Wójcik-Fatla A, Dutkiewicz J (2006) Occurrence of *Toxoplasma gondii* in water from wells located on farms. Ann Agric Environ Med 13:169–75

Viera AJ, Garrett JM (2005) Understanding interobserver agreement: the kappa statistic. Fam Med 37:360–3

Wells B, Shaw H, Innocent G, Guido S, Hotchkiss E, Parigi M, Opsteegh M, Green J, Gillespie S, Innes EA, Katzer F (2015) Molecular detection of *Toxoplasma gondii* in water samples from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water Res 87:175–181