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Monitoring of Microbial Contamination of Groundwater in the Upper Choluteca River Basin, Honduras

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Abstract: Water can act as a vector for several microbes with significant pathogenic potential for both humans and animals. Waterborne infections are a critical public health concern as they cause more than 3.4 million deaths annually. Total and thermotolerant coliforms and intestinal enterococci have traditionally been used to assess the quality and suitability of drinking water. The aim of this study was to evaluate the microbiological quality of groundwater from six sub-basins located in the upper Choluteca River basin in Honduras and to determine the *E. coli* phylogroups isolated in these samples. Our findings show high rates of fecal contamination, which suggests that the groundwater in the basin is unsafe for human consumption. Phylogroups B1 and D were the most frequent among 99 *E. coli* isolates, while C and F were the least frequent phylogroups. Measures must be taken to raise awareness about sanitation and good practices for the management of household waste as well as the waste generated by agro-industrial activity and livestock.

Keywords: fecal contamination; *Escherichia coli*; phylogroups; groundwater; Choluteca River basin; Honduras

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1. Introduction

"Water is life" is certainly one of the most recognizable sayings ever [1]. However, when water is a factor in the spread of numerous diseases with significant fatality rates, this saying becomes meaningless [2]. Water can spread viruses, bacteria, helminths, and protozoa with high human and animal pathogenic potential [3,4]. The main waterborne infectious diseases are cholera, bacillary dysentery, typhoid fever, viral and bacterial gastroenteritis, leptospirosis, amoebic dysentery, cryptosporidiosis, giardiasis, balantidiasis, viral hepatitis, and poliomyelitis [3–6]. Water-borne pathogens are usually present in human and/or animal feces, and they reach groundwater or surface water sources largely through leaching, septic tank breaches, sewage, and industrial waste. These pathogens may later reach sources of communal water supplies [7,8]. Poor hygiene habits, bad governance, quickly expanding economies with high population density, housing with poor sanitation, and a lack of access to drinking water are some of the key factors that favor the transmission of infectious diseases through water [9–12].

The World Health Organization (WHO) estimates that more than 3.4 million people worldwide die each year as a result of diseases associated with water [13]. Waterborne infections in the United States of America cause more than 120,000 hospitalizations, 7000 deaths, and around 7 million new cases of gastroenteritis each year [14]. The situation is far worse in low- and middle-income nations, where nearly 500,000 children under the age of five die from diarrhea directly from consuming contaminated water [15]. In 2018, the United Nations World Report on the Development of Water Resources was released. This report

updated the water availability situation and provided estimates for 2050 [16], highlighting that nearly half of the world's population lives in regions that experience water scarcity for at least one month of the year and warning of the considerable reduction in surface water supplies that has taken place in recent decades. Furthermore, it is anticipated that by 2050, this percentage will rise to 57%. This report also emphasizes the widespread chemical and biological contamination of water that is now occurring, particularly in Africa and Latin America [16].

The depletion of surface water resources and the increase in demand for water by the growing population worldwide have led to the search for alternatives that can mitigate the scarcity. If managed appropriately, groundwater represents a promising resource [17]. Exploiting groundwater has grown in importance as a factor of socioeconomic development [16,18]. Groundwater is the main source of drinking water for half of the world's population [18,19], and accounts for about 40% of the irrigation water used on 100 million hectares of arable land [20]. However, both natural and anthropogenic sources of contamination, such as atmospheric contamination, effluent discharges, chemical products used in agriculture, soil erosion, and microbial contamination because of inadequate sanitation practices, may have a negative impact on the quality of groundwater [21–24].

One of the major health risks is fecal contamination of water bodies that are intended for human consumption since they may contain pathogenic microorganisms that endanger people's health [3,5]. The safety of water for human consumption must therefore be determined, and its quality must be assessed [25]. The presence and count of total coliforms (TC), thermotolerant coliforms (TtC), and intestinal *Enterococcus* (formerly fecal *Streptococcus*), have long been used to monitor and certify the quality and acceptability of water intended for consumption [25–27]. These indicators are widely used due to their invariable presence in the intestine and feces of warm-blooded animals, so their presence in any body of water has a high predictive value as an index of fecal contamination and the presence of pathogenic microorganisms [3,28–30]. Thus, water for human consumption should under no circumstances contain microorganisms that indicate fecal contamination [2,25,27].

Escherichia coli has been recognized as one of the most robust indicators of fecal contamination among the available biomarkers [25,28–30]. To better understand the role played by the presence of *E. coli* in water, the determination of its phylogenetic distribution has been recommended as a complementary analysis [26]. Most *E. coli* isolates can be phylogenetically classified into eight groups (A, B1, B2, C, D, E, F, and G) and five cryptic clades (I–V) [31,32]. Human pathogenic strains have been associated with phylogroups B2 and D, whereas commensal and antibiotic-resistant bacteria have been linked to phylogroups A, B1, and G [31,32]. On the other hand, B2 has been recognized as the predominant phylogroup in human feces, while the intestinal microbiota of animals is dominated by the B1 phylogroup. In addition, *E. coli* isolates considered "naturalized", that is, intestinal isolates that have adapted to natural environments over time, are mainly grouped into cryptic clades [26].

The sustainable development agenda proposed for 2030 commits to "leaving no one behind", seeking universal and equitable access to drinking water, thus promoting socioeconomic development, and achieving the full realization of human rights throughout the world. To achieve this goal, periodic monitoring and surveillance of water quality are essential [33]. Assessing groundwater quality is crucial for low- and middle-income countries like Honduras to preserve public health and manage water resources sustainably [33]. There is scarce information available about the quality of groundwater in Honduras. This study aimed to analyze the microbiological quality of groundwater in six sub-basins situated in the upper Choluteca River basin and to determine the phylogenetic distribution of the isolated *E. coli* strains.

2. Materials and Methods

2.1. Sampling Sites and Sample Collection

The study area is located in the central-southern region of the country, with a transition climate of dry forest, and is delimited by the upper part of the Choluteca river basin

(Figure 1), encompassing the capital city, Tegucigalpa. The Choluteca River basin is made up of six sub-basins: "Choluteca Alta" (with an extension of 39.3% of the total area), "Yeguare" (17.8%), "Río del Hombre" (15.45%), "San José" (16.03%), "Guacerique" (8.28%), and "Río Chiquito" (2.91%), with a total extension of 2942 km^2. Throughout the year, the climate in this region has a distinct bimodal pattern, with a dry season lasting from November to May and a rainy season lasting from May to October. The monthly precipitation fluctuates between 4.6 and 167.4 mm on average, with a monthly average of 72.7 mm and extreme monthly values ranging from 0 to 490.7 mm. The basin is inhabited by about 1.5 million people, which make up more than 14% of the nation's total population [34].

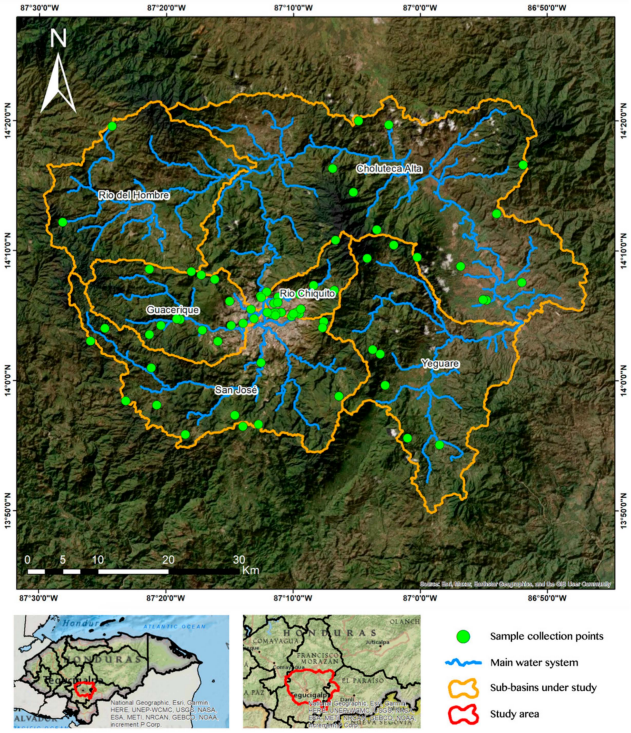

Figure 1. Map showing the geographic location of the sub-basins under study, along with sampling sites. Scale 1:300,000, projected coordinate system UTM WGS 84 ellipsoid, horizontal datum WGS 84 zone 16 N.

From October 2019 to April 2022, water samples were collected at 99 randomly selected locations in six sub-basins in 14 municipalities (Figure 1). The number of samples collected in each sub-basin was distributed as follows: Río Chiquito (n = 30), Choluteca alta (n = 22), Guacerique (n = 18), San José (n = 15), Yeguare (n = 10), Río del Hombre (n = 4) (Figure 1). The water samples were collected following the indications of the Standard Methods for the Examination of Water and Wastewater, 23rd Edition [27]. For each sampling site, approximately 500 mL of water was collected in sterile plastic bags, and all samples were transported in refrigerated boxes at 4 °C until analysis.

2.2. Hydrogeological Conditions

The Choluteca River originates in the central zone of Honduras. It makes its way through the departments of Francisco Morazán, El Paraíso, and Choluteca, flowing into the Gulf of Fonseca. Its first tributaries are formed on the slopes of the Yerbabuena mountain in the municipality of Lepaterique, with the name Quebrada del Tigre, which, upon confluence with Quebrada Grande, takes the name Río Grande or San José. At the height of the city of Tegucigalpa, the Choluteca River is the result of three rivers: the Jacaleapa, which rises in the mountains of Azacualpa; the Río Grande, which rises in the Cerro de Hula; and the Guacerique River, which rises in the mountains of Yerbabuena. The Choluteca River

receives the Chiquito River when it reaches Tegucigalpa. The Chiquito River is born in the mountains of the municipality of San Juancito. The Chiquito River originates near Tegucigalpa, and the lithology of the region displays a high density of fractures with significant potential as a source of water. With strong porosity but low permeability, other zones with thick tuffs contain aquifers with constrained potential.

2.3. Determination of Fecal Contamination Indicators

The water samples were processed within the first 6 h after collection. The isolation and count of Total Coliforms (TC), Thermotolerant Coliforms (TtC) and intestinal *Enterococcus* (IE) were carried out using the membrane filtration technique according to the guidelines of the Standard Methods for the Examination of Water and Wastewater [27]. Under sterile conditions, 100 mL of water was collected from each sampling location and filtered through cellulose nitrate membranes with a pore size of 0.45 µm and a diameter of 47 mm (Millipore Inc.®, Burlington, MA, USA). Once the samples were filtered, the membranes were transferred to m-Endo LES agar Petri dishes (Criterion™, New York, NY, USA) for the isolation of TC; to mFC agar (Criterion™) for TtC; and to m-Enterococcus agar (Acumedia®, San Bernardino, CA, USA) for the search for IE. The incubation conditions were 24 h at 37 °C to determine the presence of TC and 24 h at 44.5 °C for TtC, while the m-Enterococcus cultures were incubated at 37 °C for 48 h. The number of Colony-Forming Units (CFU)/100 mL was calculated following the incubation period. According to the manufacturer's recommendations for each of the three media, the isolates were assessed based on the color of the colonies. Dark red colonies with a metallic luster were interpreted as TC in m-Endo LES agar; blue colonies in the mFC medium were considered TtC; and any red colony was interpreted as *Enterococccus* spp. on m-Enterococcus agar.

2.4. Phenotypic Identification of Escherichia coli

Colonies presumptively identified as *E. coli* on mFC agar (Criterion™) were taken randomly from each plate and cultured on blood agar and MacConkey agar (Thermo Scientific™ Oxoid™, Waltham, MA, USA), and incubated at 37 °C for 18–24 h. A presumptive identification was made based on traditional biochemical tests: indole production, mobility, Voges Proskauer, and Simmons citrate. All the biotypes that showed any of the two following patterns: (+ + − −), (− + − −) were then confirmed using the API 20 E identification system® (bioMérieux, Marcy-l'Etoile, France). Bacteria identified as *E. coli* were inoculated into Brain Heart Infusion (BHI) broth (Millipore®, Sigmaaldrich, Darmstadt, Germany) with 20% glycerol and stored at −80 °C for further studies.

2.5. DNA Extraction and Identification of Phylogenetic Groups

For DNA extraction, *E. coli* strains were inoculated in Luria-Bertani liquid medium and incubated for 24 h at 37 °C. Subsequently, the genomic DNA was extracted using the Wizard Genomic DNA Purification kit® (Promega, Madison, WI, USA), according to the manufacturer's instructions. Finally, the DNA was suspended in 100 µL of elution buffer. DNA was stored at −20 °C until use.

For the identification of phylogenetic groups, the methodology previously described by Clermont et al. (2013) and Clermont et al. (2019) was used [31,32]. This technique relies on a quadruplex PCR reaction to amplify the *arpA*, *chuA*, *yjaA*, and *TspE4.C2* genes; however, in this study, the *arpA*, *chuA*, and *yjaA* genes were amplified by a triplex PCR, and *TspE4.C2* was amplified separately. Briefly, both reactions were carried out in a final volume of 20 µL composed of 10 µL of 2× PCR Master Mix (Promega Corp., Madison, WI, USA), 3 µL of DNA, 0.2 µL of each of the following primers: chuA.1b, chuA.2 and yjaA.1b, yjA.2b; and 0.4 µL of the primers aceK.f y arpA.r. For the uniplex PCR, 0.4 µL of the following primers were used: tspE4C2.1b and tspE4C2.2b. All primers were used at a concentration of 10 µM. The amplification program for both reactions was as follows: an initial denaturation of 94 °C for 4 min, followed by 30 cycles at 94 °C for 20 s, an annealing step of 59 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 5 min.

When the results of the previous reactions were not conclusive enough to discriminate between phylogroups A and C, B2 and G, D and E, F and G, and between clade I and group E, the presence of the genes *arpA*, *trpA*, *ybgD*, *cfaB* was evaluated according to the algorithm proposed by Clermort et al., 2019 [32]. A uniplex PCR was performed to determine the presence of each gene: 10 µL of PCR Master Mix 2× (Promega Corp.), 0.2 µL of each primer, and 3 µL of DNA in a final volume of 20 µL. The amplification of the *arpA* and *trpA* genes was carried out under the following conditions: 94 °C for 4 min, 30 cycles of 94 °C for 20 s, 57 °C for 30 s, 72 °C for 30 s, and a final extension step of 72 °C for 5 min. On the other hand, to determine the presence of phylogroups F and G, the amplification reactions for the *cfaB* and *ybgD* genes were performed with the following program: an initial denaturation cycle of 94 °C for 4 min, followed by 35 cycles of denaturation. Then, 94 °C for 1 min, an annealing step of 57 °C for 1 min, 72 °C for 1 min, and a final extension step of 72 °C for 5 min. Amplification products are visualized on 2% agarose gels stained with ethidium bromide.

The sequences of the primers used in this study are described in Supplementary Table S1. Figure 2 shows the band patterns and an in silico analysis for each of the phylogroups described in this study as described by Clermont et al. (2013) and Clermont et al. (2019) [31,32].

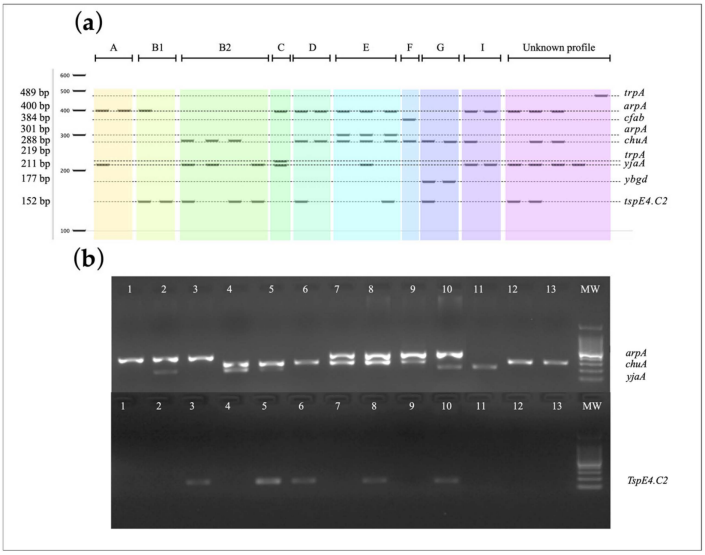

Figure 2. (**a**) In silico analysis showing band patterns per gene to classify the 8 phylogroups and clade I of *E. coli*; (**b**) PCR products from the triplex (**top**) and uniplex (**bottom**) assays of the phylogroups characterized in this study. Lane 1, phylogroup A; lane 2, phylogroups A or C; lane 3, phylogroup B1; lane 4, phylogroup B2; lane 5, phylogroup B2; lane 6, phylogroups B2 or G; lane 7, phylogroups E or D; lane 8, phylogroups E or D; lane 9, clade I or phylogroup E; lane 10, unknown; lane 11, clade I; lane 12, phylogroup F; lane 13, phylogroup F or G. MW: 100 bp DNA ladder.

2.6. PCR Product Sequencing

At least two amplicons from each gene were chosen at random to be sequenced on both flanks using the same primers that were used to amplify the molecular markers and in accordance with Psomagen's (https://lims.psomagen.com/, accessed on 12 February 2023) instructions to verify that each phylogroup was correctly amplified. The quality of the sequences was analyzed with Geneious Prime Software® 2023.2. The database of the NCBI platform was consulted to confirm the identity of the sequences using the BLAST tool. The sequences were compared with accessions deposited in GenBank, recording the result with the highest percentage of similarity. The sequences obtained in this study were deposited in GenBank, and accession numbers were assigned (Table 1).

Table 1. Amplicon size in base pairs of the molecular markers used to classify *E. coli* phylogroups and accession numbers assigned by GenBank.

Code	Gene	PCR Product (bp)	GenBank Accession Number
E01	*arpA*	400	OQ571720
E03	*arpA*	400	OQ571721
E04	*chuA*	288	OQ571722
E05	*chuA*	288	OQ571723
E06	*chuA*	288	OQ571724
E07	*yjaA*	211	OQ571725
E09	*yjaA*	211	OQ571726
E10	*TspE4.C2*	152	OQ571727
E11	*arpA*	301	OQ571728
E13	*trpA*	489	OQ571729
E14	*trpA*	489	OQ571730
E15	*trpA*	489	OQ571731
E16	*yjaA*	211	OQ571732
E28	*ybgD*	177	OQ571733
E30	*cfaB*	384	OQ57173

3. Results and Discussion

3.1. Microbiological Analysis

Microbial contamination indicators are important parameters to determine the suitability of water for human consumption [2]. In this study, we assessed the levels of total coliforms (TC), thermotolerant coliforms (TtC), and intestinal *Enterococcus* (IE) in 99 groundwater sources located along the Choluteca River basin. The counts of TC, TtC, and IE by sub-basin and municipality are shown in Table 2.

The presence of TC was observed in 100% of the water samples. TC counts ranged from 2 CFU/100 mL to 6×10^4 CFU/100 mL. The highest TC count was observed in a sample from the "Choluteca Alta" sub-basin located in the Villa de San Francisco municipality, which was collected in the dry season of 2020. Similarly, two samples from the "Río Chiquito" sub-basin located in the municipality of the Central District ranked second with the highest number of TC, with counts of 4.2×10^4 CFU/100 mL. On the other hand, two samples from the "Choluteca Alta" and "Yaguare" sub-basins, which are in the municipalities of Morocelí and Valle de Ángeles, respectively, showed low counts close to 2 CFU/100 mL.

Coliform bacteria include several genera of aerobic and facultative anaerobic Gram-negative bacilli that are non-spore-forming and capable of lactose fermentation, acid production, and gas production when incubated at 35–37 °C [25,27]. Major coliform genera include *Escherichia*, *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Serratia* [25,27]. It has been common practice to detect the presence of TC in water to assess whether it is safe for consumption and recreational use. Coliforms are found in the intestines of animals and humans, as well as in the environment. For this reason, they are no longer considered a good indicator of fecal contamination, and there is not always a direct relationship between TC and pathogenic bacteria [25,35]. Even though TC are no longer a reliable indicator of fecal contamination, their detection allows for the assessment of the general hygienic condition of water supplies [2]. The presence of TC in groundwater indicates contamination by wastewater discharges, decomposing matter, and especially organic waste. This organic waste is associated with faulty or absent septic systems, sewage leaks, or sewage systems in bad condition. It's also important to emphasize the improper management of agricultural and livestock waste around the sample sites [25,30,35,36].

As TC are not the most reliable indicator of fecal contamination, TtC detection is crucial in identifying whether fecal contamination exists in bodies of water that are used for human consumption [2]. The TtC is a subgroup of the TC capable of growing at temperatures higher than other species of this group, at an optimal growth temperature of 44–46 °C [2,25,27]. *E. coli* has been found to be the species that is most frequently isolated within TtC, accounting for up to 95% of isolates [2,37]. For this reason, the detection of *E. coli* in bodies of water, particularly groundwater, is considered a good indicator of fecal contamination [38], and its absence helps determine the suitability of

water for human consumption [2,27,38]. The results of the thermotolerant bacterial culture showed that 94% (n = 93) of the samples contained TtC. The TtC count ranged from 1 CFU/100 mL to 2.2 × 10^4 CFU/100 mL. The locations with the highest counts were a site in Lepaterique and another in the Central District. Six samples did not reveal the presence of TtC. These samples were collected at points located in the municipalities of Morocelí, Ojojona, and the Central District. The TtC count should be zero in accordance with WHO recommendations for drinking water quality and the technical standard for Honduras' drinking water quality [2,25,39]. Additionally, any bacteria that are thought to be of fecal origin should not be present in water that is intended for human consumption. [2,39]. Hence, our findings reveal a worrying truth.

Furthermore, the presence of intestinal *Enterococcus* (IE) is a second supplementary indicator that shows proof of fecal contamination in water [2,25]. IE was present in 73.4% (n = 73) of the samples analyzed. The highest value of IE was 2.24 × 10^4 CFU/100 mL in a sample collected in the "San José" sub-basin located in the municipality of Santa Ana. The distribution of TC, TtC, and IE counts by sub-basin is shown in Figure 3. It is worth noting that the TtC / IE ratio can be quite helpful for figuring out where the contamination originated. It has been suggested that the amounts of *E. coli* and/or TtC and *Enterococcus* shed by humans are significantly different from those shed by animals [40–43]. In this study, we evaluated the source of microbiological contamination using the relationship between TtC and IE in accordance with what was proposed by Geldreich et al., 1969 [41]. Briefly, if the TtC/IE ratio was >4.0, this would suggest human-derived contamination, but when the TtC/IE ratio was <0.7, it would suggest contamination of animal origin. On the other hand, when the TtC/IE ratio is in a range between 0.7 and 4.0, it would be considered mixed contamination [41]. Following this parameter, the source of contamination was determined for those sites whose IE counts were greater than or equal to 1 CFU/100 mL. Of the 99 samples analyzed, 73.7% (n = 73) had counts greater than or equal to 1 CFU/100 mL. The analysis of the source of microbiological contamination is shown in Table 2. In 47.9% of the 73 samples that were analyzed, contamination of animal origin was found. Mixed contamination was detected in 27.3% of the samples, and human contamination was observed in 24.6% of the samples.

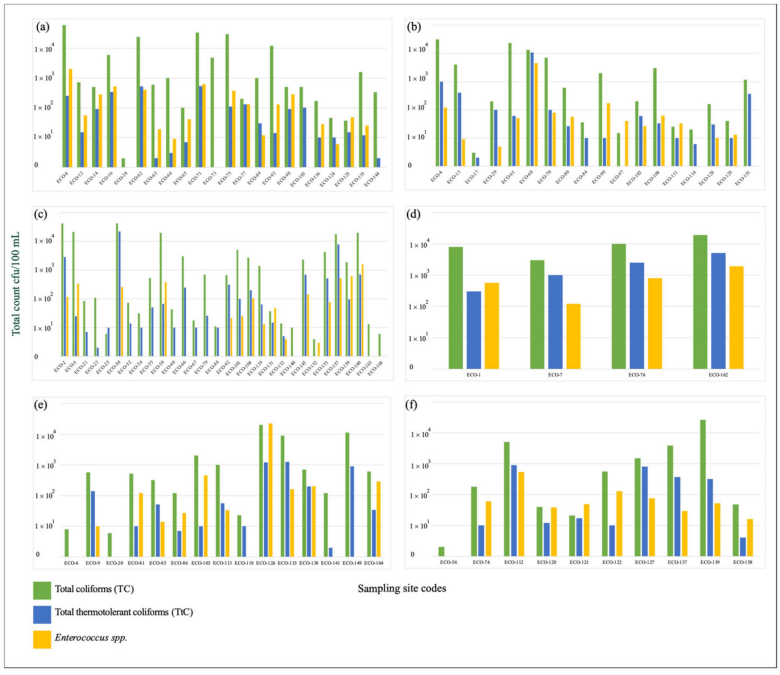

Figure 3. Counts of total coliforms (green bars), thermotolerant coliforms (blue bars), and intestinal *Enteroccocus* (yellow bars) by sampling point distributed by sub-basin: (**a**) Choluteca Alta, (**b**) Guacerique, (**c**) Río Chiquito, (**d**) Río del Hombre, (**e**) San José, and (**f**) Yeguare.

Table 2. Counts of Total Coliforms (TC), Thermotolerant Coliforms (TtC), and Intestinal *Enterococcus* (IE) and the result of the analysis of the TtC/IE ratio in the Choluteca River basin of Honduras.

Code	Sub-Basin	Coordinates		Municipality	Season/Year	TC CFU/100 mL	TtC CFU/100 mL	IE CFU/100 mL	Putative Source of Contamination According to TtC/IE Ratio
		Longitude	Latitude						
ECO-50	RC	87°11′14.443″ W	14°5′11.112″ N	DC	Rainy/2020	4.2×10^4	2.2×10^4	2.6×10^2	Human
ECO-68	GUA	87°11′22.05″ W	14°5′15.403″ N	DC	Rainy/2021	1.31×10^4	1.09×10^4	4.5×10^3	Mixed
ECO-157	RC	87°9′37.871″ W	14°5′19.319″ N	DC	Rainy/2020	1.7×10^4	7.90×10^3	5.2×10^2	Human
ECO-162	RH	87°28′8.722″ W	14°12′11.512″ N	LP	Rainy/2021	1.9×10^4	5.1×10^3	1.9×10^3	Mixed
ECO-2	RC	87°9′30.328″ W	14°5′10.308″ N	DC	Dry/2021	4.2×10^4	2.8×10^3	1.1×10^2	Human
ECO-76	RH	87°18′1.079″ W	14°8′24.116″ N	DC	Rainy/2021	1.0×10^4	2.5×10^3	8.0×10^2	Mixed
ECO-133	SJ	87°14′34.574″ W	13°57′22.331″ N	SA	Rainy/2020	9.0×10^3	1.2×10^3	1.6×10^2	Human
ECO-126	SJ	87°14′34.574″ W	13°57′22.331″ N	SA	Dry/2022	2.0×10^4	1.2×10^3	2.2×10^4	Animal
ECO-4	GUA	87°15′0.273″ W	14°6′7.35″ N	DC	Dry/2021	3.1×10^4	1.0×10^3	1.1×10^2	Human
ECO-7	RH	87°28′8.722″ W	14°12′11.512″ N	LP	Dry/2021	3.00×10^3	1.00×10^3	1.2×10^2	Human
ECO-112	YE	87°3′6.944″ W	14°2′4.316″ N	SAO	Rainy/2021	5.0×10^3	9.0×10^2	5.3×10^2	Mixed
ECO-149	SJ	87°6′22.6″ W	13°58′50.117″ N	TAT	Rainy/2020	1.1×10^4	9.0×10^2	0	N/A
ECO-127	YE	87°11′10.236″ W	14°6′27.805″ N	SAO	Rainy/2020	1.5×10^3	8.0×10^2	7.5×10^1	Human
ECO-160	RC	87°9′30.328″ W	14°5′10.308″ N	DC	Rainy/2020	2.0×10^4	7.0×10^2	1.6×10^3	Animal
ECO-145	RC	87°12′32.952″ W	14°6′31.676″ N	DC	Rainy/2020	2.3×10^3	6.8×10^2	1.4×10^2	Human
ECO-71	CA	87°6′53.126″ W	14°16′20.126″ N	DC	Rainy/2020	3.4×10^4	5.0×10^2	6.2×10^2	Mixed
ECO-62	CA	87°3′23.589″ W	14°11′38.021″ N	DC	Dry/2021	2.4×10^4	5.2×10^2	4.0×10^2	Mixed
ECO-153	RC	87°12′3.486″ W	14°6′50.971″ N	DC	Rainy/2020	4.3×10^3	5.1×10^2	7.6×10^1	Human
ECO-13	GUA	87°21′18.597″ W	14°8′35.467″ N	DC	Rainy/2021	4.0×10^3	4.0×10^2	9	Human
ECO-137	YE	87°4′9.258″ W	14°9′26.146″ N	VA	Rainy/2020	3.9×10^3	3.7×10^2	2.9×10^1	Human
ECO-151	GUA	87°20′24.475″ W	14°4′15.533″ N	DC	Rainy/2020	1.1×10^3	3.7×10^2	1	Human
ECO-16	CA	87°5′14.859″ W	14°14′31.612″ N	DC	Dry/2021	6.0×10^3	3.4×10^2	5.2×10^2	Animal
ECO-139	YE	87°3′6.944″ W	14°2′4.316″ N	SAO	Rainy/2020	2.6×10^4	3.2×10^2	5.2×10^1	Human
ECO-92	RC	87°10′54.804″ W	14°5′16.01″ N	DC	Dry/2021	6.7×10^2	3.1×10^2	2.2×10^1	Human
ECO-1	RH	87°24′16.359″ W	14°19′35.199″ N	DC	Dry/2021	8.0×10^3	3.0×10^2	5.6×10^2	Animal
ECO-8	CA	86°53′58.031″ W	14°12′52.217″ N	VSF	Dry/2020	6.0×10^4	2.5×10^2	2.0×10^3	Animal
ECO-66	RC	87°11′23.24″ W	14°5′3.13″ N	DC	Dry/2021	3.0×10^3	2.5×10^2	0	N/A
ECO-104	RC	87°10′54.804″ W	14°5′16.01″ N	DC	Rainy/2021	2.7×10^3	2.0×10^2	1.0×10^2	Mixed
ECO-138	SJ	87°24′49.15″ W	14°4′2.099″ N	LP	Rainy/2020	7.0×10^2	1.9×10^2	2.0×10^2	Mixed
ECO-9	SJ	87°23′9.855″ W	13°58′27.71″ N	DC	Dry/2021	5.7×10^2	1.4×10^2	1.0×10^2	Human
ECO-77	CA	86°51′52.369″ W	14°16′37.795″ N	CA	Rainy/2021	2.0×10^2	1.3×10^2	1.3×10^2	Mixed
ECO-75	CA	86°54′45.999″ W	14°6′12.513″ N	MO	Dry/2021	2.96×10^4	1.1×10^2	3.7×10^2	Animal
ECO-29	GUA	87°17′8.841″ W	14°3′54.693″ N	DC	Rainy/2021	2.0×10^2	1.0×10^2	5	Human
ECO-70	GUA	87°14′52.816″ W	14°4′16.653″ N	DC	Dry/2021	7.0×10^3	1.0×10^2	8.0×10^1	Mixed
ECO-101	RC	87°7′34.822″ W	14°4′34.016″ N	DC	Rainy/2021	5.0×10^3	1.0×10^2	2.5×10^1	Human
ECO-105	CA	86°51′52.369″ W	14°16′37.795″ N	CA	Dry/2021	5.0×10^2	1.0×10^2	0	N/A

Table 2. *Cont.*

Code	Sub-Basin	Coordinates		Municipality	Season/Year	TC	TtC	IE	Putative Source of Contamination
		Longitude	Latitude			CFU/100 mL	CFU/100 mL	CFU/100 mL	According to TtC/IE Ratio
ECO-159	RC	87°6′48.804″ W	14°6′50.428″ N	DC	Rainy/2020	1.9×10^3	9.5×10^1	6.2×10^2	Animal
ECO-14	CA	87°4′50.448″ W	14°20′0.282″ N	TAL	Rainy/2021	5.0×10^2	9.0×10^1	2.8×10^2	Animal
ECO-98	CA	87°4′50.448″ W	14°20′0.282″ N	TAL	Rainy/2021	5.0×10^2	9.0×10^1	2.8×10^2	Animal
ECO-58	RC	87°11′22.05″ W	14°5′15.403″ N	DC	Rainy/2020	1.9×10^4	6.7×10^1	3.8×10^2	Animal
ECO-119	RC	87°6′48.804″ W	14°6′50.428″ N	SL	Rainy/2021	1.4×10^3	6.4×10^1	1.3×10^2	Human
ECO-61	GUA	87°19′9.788″ W	14°4′45.484″ N	DC	Dry/2021	2.3×10^4	6.0×10^1	5.0×10^1	Mixed
ECO-102	GUA	87°13′56.103″ W	14°4′25.433″ N	DC	Rainy/2021	2.0×10^2	6.0×10^1	2.6×10^1	Mixed
ECO-113	SJ	87°21′9.426″ W	14°1′0.002″ N	OJ	Rainy/2021	1.0×10^3	5.6×10^1	3.3×10^1	Mixed
ECO-85	SJ	87°21′9.426″ W	14°1′0.002″ N	OJ	Dry/2021	3.2×10^2	5.1×10^1	1.4×10^1	Mixed
ECO-55	RC	87°18′53.046″ W	14°4′44.041″ N	DC	Rainy/2021	5.3×10^2	5.0×10^1	0	N/A
ECO-164	SJ	87°25′56.462″ W	14°3′2.87″ N	LP	Rainy/2020	6.0×10^2	3.4×10^1	2.9×10^2	Animal
ECO-108	GUA	87°13′18.651″ W	14°5′30.667″ N	DC	Rainy/2021	3.0×10^3	3.3×10^1	6.2×10^1	Animal
ECO-89	CA	87°6′53.126″ W	14°16′20.126″ N	CA	Dry/2021	1.0×10^3	3.0×10^1	1.2×10^1	Human
ECO-128	GUA	87°19′9.788″ W	14°4′45.484″ N	DC	Dry/2022	1.6×10^2	3.0×10^1	1.0×10^1	Mixed
ECO-79	RC	87°9′29.49″ W	14°6′41.027″ N	DC	Rainy/2021	7.0×10^2	2.6×10^1	0	N/A
ECO-80	GUA	86°54′45.999″ W	14°6′12.513″ N	DC	Rainy/2021	6.0×10^2	2.6×10^1	5.7×10^1	Animal
ECO-5	RC	87°7′40.775″ W	14°4′5.857″ N	DC	Dry/2021	2.1×10^4	2.5×10^1	3.4×10^2	Animal
ECO-121	YE	87°2′3.604″ W	14°10′27.206″ N	VA	Dry/2022	2.1×10^1	1.7×10^1	4.9×10^1	Animal
ECO-12	CA	86°56′47.027″ W	14°8′48.662″ N	VSF	Rainy/2020	7.2×10^2	1.5×10^1	5.5×10^1	Animal
ECO-125	CA	87°3′23.589″ W	14°11′38.021″ N	DC	Dry/2022	3.7×10^1	1.5×10^1	4.8×10^1	Animal
ECO-131	RC	87°9′43.072″ W	14°5′16.712″ N	DC	Rainy/2020	3.7×10^1	1.5×10^1	4.8×10^1	Animal
ECO-52	RC	87°10′7.136″ W	14°4′58.629″ N	DC	Rainy/2020	7.4×10^1	1.4×10^1	0	N/A
ECO-93	CA	86°54′45.999″ W	14°6′12.513″ N	MO	Dry/2021	1.2×10^4	1.4×10^1	1.3×10^2	Animal
ECO-120	YE	87°2′43.977″ W	13°59′40.757″ N	TAT	Dry/2022	4.0×10^1	1.2×10^1	3.8×10^1	Animal
ECO-135	CA	87°2′27.391″ W	14°19′42.839″ N	CA	Rainy/2020	1.6×10^3	1.2×10^1	2.5×10^1	Animal
ECO-25	RC	87°11′28.989″ W	14°5′58.559″ N	DC	Rainy/2021	6	1.0×10^1	0	N/A
ECO-54	RC	87°12′30.01″ W	14°6′24.68″ N	DC	Rainy/2020	3.2×10^1	1.0×10^1	0	N/A
ECO-60	RC	87°9′29.49″ W	14°6′41.027″ N	DC	Dry/2021	4.3×10^1	1.0×10^1	0	N/A
ECO-67	RC	87°11′11.883″ W	14°6′2.284″ N	DC	Rainy/2020	1.8×10^1	1.0×10^1	0	N/A
ECO-74	YE	87°0′58.187″ W	13°55′36.551″ N	MA	Rainy/2020	1.8×10^2	1.0×10^1	6.0×10^1	Animal
ECO-81	SJ	87°20′43.432″ W	13°58′7.944″ N	DC	Rainy/2020	5.1×10^2	1.0×10^1	1.2×10^2	Animal
ECO-84	GUA	87°15′54.488″ W	14°3′2.404″ N	DC	Rainy/2020	3.6×10^1	1.0×10^1	0	N/A
ECO-88	RC	87°9′29.49″ W	14°6′41.027″ N	DC	Rainy/2020	1.1×10^1	1.0×10^1	0	N/A
ECO-90	GUA	87°21′18.164″ W	14°3′33.726″ N	DC	Rainy/2021	2.0×10^3	1.0×10^1	1.7×10^2	Animal
ECO-103	SJ	87°13′56.159″ W	13°56′30.157″ N	SA	Rainy/2021	2.0×10^3	1.0×10^1	4.5×10^2	Animal
ECO-111	GUA	87°17′14.495″ W	14°8′9.232″ N	DC	Rainy/2021	2.5×10^1	1.0×10^1	3.3×10^1	Animal
ECO-116	CA	87°9′58.941″ W	14°5′10.581″ N	DC	Rainy/2021	1.7×10^2	1.0×10^1	2.8×10^1	Animal
ECO-118	SJ	87°12′42.381″ W	13°56′39.729″ N	SA	Rainy/2021	2.3×10^1	1.0×10^1	0	N/A

Table 2. *Cont.*

Code	Sub-Basin	Coordinates		Municipality	Season/Year	TC	TtC	IE	Putative Source of Contamination
		Longitude	Latitude			CFU/100 mL	CFU/100 mL	CFU/100 mL	According to TtC/IE Ratio
ECO-122	YE	86°58′27.391″ W	13°55′5.266″ N	GA	Dry/2022	5.6×10^2	1.0×10^1	1.3×10^2	Animal
ECO-124	CA	86°55′1.54″ W	14°6′15.122″ N	MO	Dry/2022	4.6×10^1	1.0×10^1	6	Mixed
ECO-129	GUA	87°17′8.841″ W	14°3′54.693″ N	DC	Dry/2022	4.0×10^1	1.0×10^1	1.3×10^1	Mixed
ECO-21	RC	87°11′31.419″ W	14°5′13.637″ N	DC	Rainy/2020	8.4×10^1	7	0	N/A
ECO-65	CA	86°51′52.369″ W	14°16′37.795″ N	CA	Dry/2021	1.0×10^2	7	4.1×10^1	Animal
ECO-86	SJ	87°14′34.574″ W	13°57′22.331″ N	SA	Dry/2021	1.2×10^2	7	2.7×10^1	Animal
ECO-114	GUA	87°15′54.488″ W	14°3′2.404″ N	DC	Rainy/2021	2.0×10^1	6	0	N/A
ECO-132	RC	87°8′22.844″ W	14°7′21.464″ N	DC	Rainy/2020	1.4×10^1	5	4	Mixed
ECO-158	YE	87°3′43.358″ W	14°2′25.498″ N	SAO	Rainy/2020	4.8×10^1	4	1.6×10^1	Animal
ECO-64	CA	86°55′1.54″ W	14°6′15.122″ N	MO	Dry/2021	1.0×10^3	3	9	Animal
ECO-17	GUA	87°13′3.67″ W	14°4′47.649″ N	DC	Dry/2021	3	2	0	N/A
ECO-23	RC	87°9′22.173″ W	14°5′32.643″ N	DC	Rainy/2020	1.1×10^2	2	0	N/A
ECO-63	CA	87°0′13.144″ W	14°9′31.227″ N	VA	Dry/2021	6.0×10^2	2	1.9×10^1	Animal
ECO-141	SJ	87°21′9.426″ W	14°1′0.002″ N	OJ	Rainy/2020	1.2×10^2	2	0	N/A
ECO-144	CA	87°6′45.806″ W	14°6′58.371″ N	SL	Rainy/2020	3.3×10^2	2	0	N/A
ECO-56	YE	87°2′3.604″ W	14°10′27.206″ N	VA	Rainy/2021	2	1	1	Mixed
ECO-97	GUA	87°16′10.496″ W	14°7′49.125″ N	DC	Rainy/2021	1.5×10^1	1	4×10^1	Animal
ECO-152	RC	87°6′38.747″ W	14°10′50.657″ N	DC	Rainy/2020	4	1	3	Animal
ECO-168	RC	87°11′40.086″ W	14°5′9.171″ N	DC	Rainy/2020	6	1	0	N/A
ECO-6	SJ	87°12′31.039″ W	14°1′24.918″ N	DC	Rainy/2021	8	0	0	N/A
ECO-20	SJ	87°18′27.629″ W	13°55′53.561″ N	OJ	Dry/2021	6	0	0	N/A
ECO-38	CA	86°55′1.54″ W	14°6′15.122″ N	MO	Rainy/2021	2	0	0	N/A
ECO-73	CA	86°51′58.996″ W	14°7′34.236″ N	MO	Rainy/2020	4.8×10^3	0	0	N/A
ECO-140	RC	87°11′23.24″ W	14°5′3.13″ N	DC	Rainy/2020	1.0×10^1	0	0	N/A
ECO-163	RC	87°12′0.902″ W	14°5′16.38″ N	DC	Dry/2021	1.3×10^1	0	0	N/A

Notes: The samples are listed in this table from those with the highest concentration of thermotolerant coliforms to those with the lowest concentration. N/A = Not applicable. Key to the municipalities: CA: Cantarranas; DC: Distrito Central; GA: Galeras; LP: Lepaterique; MA: Maraita; MO: Morocelí; OJ: Ojojona; SA: Santa Ana; SAO: San Antonio de Oriente; SL: Santa Lucía; TAL: Talanga; TAT: Tatumbla; VA: Valle de Ángeles; VSF: Villa de San Francisco.

3.2. Risk Analysis

Categories of potential health risks associated with *E. coli* or TtC concentrations have been established by the WHO: Low (<1 CFU/100 mL), intermediate (1–10 CFU/100 mL), high (11–100 CFU/100 mL), and very high (>100 CFU/100 mL) [25]. A total of 94% (n = 93) of the samples examined here did not meet the WHO drinking water quality recommendations, and 36.3% of the samples posed a very high risk for human consumption. Likewise, 32.3% were categorized as high risk, 25.3% as intermediate risk, and only 6% were found acceptable for consumption according to WHO risk parameters.

3.3. Phylogenetic Analysis of E. coli Strains

Most of the 99 *E. coli* isolates under study (33.3%) belonged to the phylogenetic group A, followed by B1 (30.3%), D (19.1%), B2 (7%), and clade I (4%). Similarly, 2% of the isolates were classified in phylogroup G, while 1% of the isolates belonged to phylogroups C, F, and clades I or II. One isolate was identified as an unknown phylogroup. Most *E. coli* isolates can be phylogenetically classified into eight phylogroups (A, B1, B2, C, D, E, F, y, and G) and five cryptic clades (I–V) [31,32]. Phylogroups B2 and D are associated with human pathogenic strains, whereas groups A and B1 are associated with commensal and antibiotic-resistant strains [31,32]. On the other hand, B2 has been recognized as the predominant phylogroup in human feces. Similarly, it has been noted that the B1 phylogroup dominates the animal microbiota, but intestinal isolates of *E. coli* that are thought to have "naturalized" (gradually adapted to natural environments), have been found to be primarily grouped in the cryptic clades [26]. In this regard, the finding of the main *E. coli* phylogroups (A, B1, B2, and D) in the water samples and the low prevalence of cryptic clades suggest fecal contamination of warm-blooded animals as the origin of these isolates.

Yet, although it is a useful tool for comprehending the populations of *E. coli* isolates, phylogroup determination has a few drawbacks, including a lack of long-term consistency in the approaches employed. Several studies are based solely on the methodology proposed by Clermont et al., 2000 [44], while other studies use the methodology proposed in 2013 [31]. Later, Clermont et al., 2019, proposed the existence of phylogroup G [32], for which the presence of some of the phylogroups could appear overestimated in the previous literature. To fully comprehend the population dynamics of *E. coli*, it is necessary to unify the methodology utilized to characterize phylogroups.

3.4. Analysis of the Groundwater Quality Situation in the Choluteca River Basin of Honduras

The unplanned and disorderly growth of the communities, which is also accompanied by a lack of effective water management, inadequate sewage treatment, and poor hygiene, are key factors contributing to the high levels of fecal contamination indicators in the sampling sites. The absence of drinking water also makes it difficult to treat organic waste properly through septic systems. Moreover, among the primary occupations in the vicinity of the sampling regions are agriculture and cattle. The high percentage of animal contamination found in this study may also be explained by the outdoor rearing of domestic animals such as chickens, cows, horses, and pigs.

Several studies have shown that the presence of fecal bacteria in water sources increases during the rainy season [28,42,45,46]. Many meteorological events occurred in Honduras during the time when the sampling for this study was conducted. Hurricanes Eta and Iota, which occurred between 3 November and 17 November 2020, stand out among these events since they flooded a significant portion of the country. These and other storms increased river flow, which may have enhanced the flow of pollutants and favored their dispersal, contributing to the high rates of TC, TtC, and IE found in groundwater samples.

3.5. Limitations

The TC, TtC, and IE counts were not compared across years or between the dry and rainy seasons. Neither the turbidity of the water sources nor the physical and chemical

parameters were evaluated. Another limitation of this study is the lack of sociodemographic data, economic activities, or hygienic conditions among the local inhabitants.

4. Conclusions

This study assessed the microbiological quality of water in the upper Choluteca River basin. Our findings show high rates of fecal contamination, as well as the predominance of *E. coli* strains from phylogroups associated with fecal contamination, which suggests that these waters are unsafe for human consumption. It is necessary to take action to increase public knowledge of sanitation issues and best practices for the management of household waste as well as waste produced by livestock and the agro-industry. In addition, water in the Choluteca River basin of Honduras must be treated with adequate methods before consumption since it could affect the health of consumers. Likewise, we suggest prompt intervention by decision-makers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15112116/s1, Table S1: Primer sequences for classifying *E. coli* strains into phylogroups, PCR conditions, and amplicon sizes. References [47,48] are cited in Supplementary Materials.

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