FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 8 No. 1, February, 2024, pp 190 - 194
DOI: https://doi.org/10.33003/fjs-2024-0801-2262

MOLECULAR DETECTION OF WATER PROTOZOAN PARASITES FROM SELECTED RIVERS IN RIVERS STATE, NIGERIA

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ABSTRACT

Drinking water or potable water also known as improved drinking water is said to be any water that is free from physical, chemical, biological or radiological form of contamination. Water samples were collected from 5 rivers in Omuchi Aluu, Choba, Chokocho, Oyigbo, and Aleto communities, in Rivers State, Nigeria, used for activities such as washing, bathing, fishing, and dredging by community indigenes. Water samples were collected from various sampling stations and, transported to the laboratory, African Biosciences, Ibadan for analysis to characterize and identify the protozoan parasites using molecular techniques. This study aimed at characterizing the genotype of protozoan parasites of water from selected rivers in River State, Nigeria, using molecular methods to check for portability and purity of water. DNA Primers of five protozoan parasites, *Giardia* species, *Entamoeba* species, *Cryptosporidium* species, *Cyclospora* species, and *Toxoplasma* species were used to analyze and detect the presence of the genes of these protozoans. The study showed the presence of *Giardia duodenalis* and *Cryptosporidium parvuum*, and the absence of *Entamoeba histolytica*, *Cyclospora cayetanensis*, and *Toxoplasma gondii* from the water samples collected from Omuchi Aluu, Choba, Chokocho, Oyigbo, and Aleto rivers. It was concluded that water samples collected from rivers contaminated with parasites, is unsuitable for drinking, hence community locals should be sensitized to embark on good water sanitary and hygiene (WASH) practices, for a healthy life and sustainable environment.

Keywords: Molecular techniques, Nigeria, Protozoan parasites, Water, WHO

INTRODUCTION

Safe water is important for public health, whether it is used for drinking, domestic use, food production or recreational purposes. Improved water supply and sanitation, and better management of water resources, can boost a country's economic growth and can contribute greatly to poverty reduction (Feng *et al*., 2011). Drinking water or potable water also known as improved drinking water is said to be any water that is free from physical, chemical, biological or radiological form of contamination (Gyang *et al*., 2017). Biological contaminants, including bacteria, viruses, fungi, protozoa, and helminths, are the primary causes of food-borne and water-borne diseases. These contaminants can lead to a spectrum of health issues, ranging from mild discomfort to severe or even life-threatening illnesses (Ani and Itiba, 2015). Different sources of water such as wells, bore holes, ponds and streams need be protected from pollution and contamination by potential parasites, micro-organisms and harmful chemical substances (Ayaz *et al.*, 2011). These contaminants gain access into water bodies through human and animal faecal matter from patients and healthy carriers of parasitic diseases. The most dangerous form of water pollution occurs when faecal contaminants enter water sources (Kalpana *et al.*, 2011).Contaminated water and poor sanitation are linked to the transmission of diseases such as: cholera, diarrhea, hepatitis A, typhoid and polio and is the major cause of illness, death, and childhood mortality (Rahmanian *et al*., 2015).

Pathogenic protozoans have been known to cause public health problems and remain the major cause of death worldwide. Many countries have identified waterborne protozoan pathogens such as *Giardia lamblia*, *Naegleria fowleri*, *Acanthamoeba* species, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Isospora belli*, *Toxoplasma gondii*, *Blastocystis hominis*, *Balamuthia mandrillaris* and *Microsporidia* due to lack of water hygiene. These parasites cause infectious diseases which are transmitted by contaminated foods, recreational water, surface water, ground water for drinking, and are dangerous to the health of community locals (Angelici and Karanis, 2019).

Molecular biological techniques offer a better alternative in the study of protozoan parasites because their sensitivity and specificity allow the discrimination of microorganisms at the species and strain levels. This technique provides information that may be relevant in evaluating the sources of infection in humans, and the health implications or risks posed by these protozoan parasites (Sánchez *et al*., 2018). Molecular methods and procedures are usually very precise for specific species and they provide historical information about pathogens (Gilbride, 2014). These methods easily reveal the host source and are better equipped for health risk assessment. This technique has great potential in parasitic protozoan detection due to its high sensitivity of microorganism at low concentrations (Kumar *et al*., 2016). Some examples of detection methods are Nucleic Acid Amplification procedure, analytical tools for detecting varieties of pathogens, real-time analysis (like real-time PCR (Polymerase Chain Reaction)). Other methods are: Polymerase Chain Reaction (PCR), multiplex PCR, quantitative PCR, microarrays, pyrosequencing, biosensing, fluorescence *in situ* hybridization (FISH) and immunology – based methods (Srivastava *et al.*, 2020).This study aimed at using molecular methods to detect protozoan parasites of water from selected rivers in River State, Nigeria, to check for portability and purity of water.

MATERIAL AND METHODS

Description of the Study Areas

The study areas comprise of freshwater rivers surrounded by forest vegetation in these communities; Omuchi Aluu, Choba, Chokocho, Oyigbo, and Aleto in the following respective local government areas; Ikwerre, Obio-Akpor, Etche, Oyigbo and Eleme in Rivers State, Nigeria.

Station 1: Omuchi Aluu river is located in Omuchi Aluu community, Ikwerre local government area. Water samples were collected from the river, between latitudes 4.91260 and 4.91264 N and longitudes 6.89691 and 6.89695 E. The river is polluted due to oil bunkering and nearby farming activities, people who live by the river, mainly locals from the community have their bath in the river, while others defecate by the side of the river which is washed into the river by runoff.

Station 2: Choba river, in Choba community is located in Obio/Akpor local government area, water samples were collected from the center of the river, between latitudes 4.88859 and 4.88863 N and longitudes 6.89816 and 6.89820 E. The river is polluted and the water collected is unsafe for consumption due to the evidence of oil bunkering activities, slaughtering of herds and rearing of cows, and recreational activities such as swimming by the locals who live by the river side.

Station 3: Chokocho river in Chokocho community in Etche local government area, between latitudes 4.99582 and 4.99586 N and longitudes 7.06175 and 7.06179 E. Locals living by the river side carry out activities such as dredging and domestic activities such as laundering, washing of dishes, and having their baths in the river.

Station 4: Oyigbo river in Oyigbo local government area is between the boundary of Rivers state and Abia state, between latitudes 4.8859 and 4.8880 N and longitudes 7.1417 and 7.1452 E. Water is highly polluted by dredging activities, washing of slaughtered animals, and recreational activities such as swimming, and fish farming.

Station 5: Aleto river is in Aleto community, Eleme local government area. Water samples were collected between the boundary of Rivers state and Abia state, between latitudes 4.81971 and 4.81975 N and longitudes 7.14131 and 7.14135 E, and river flows beneath a bridge, and its contaminated by oil spillage due to pipeline vandalization and breakage, recreational and domestic activities.

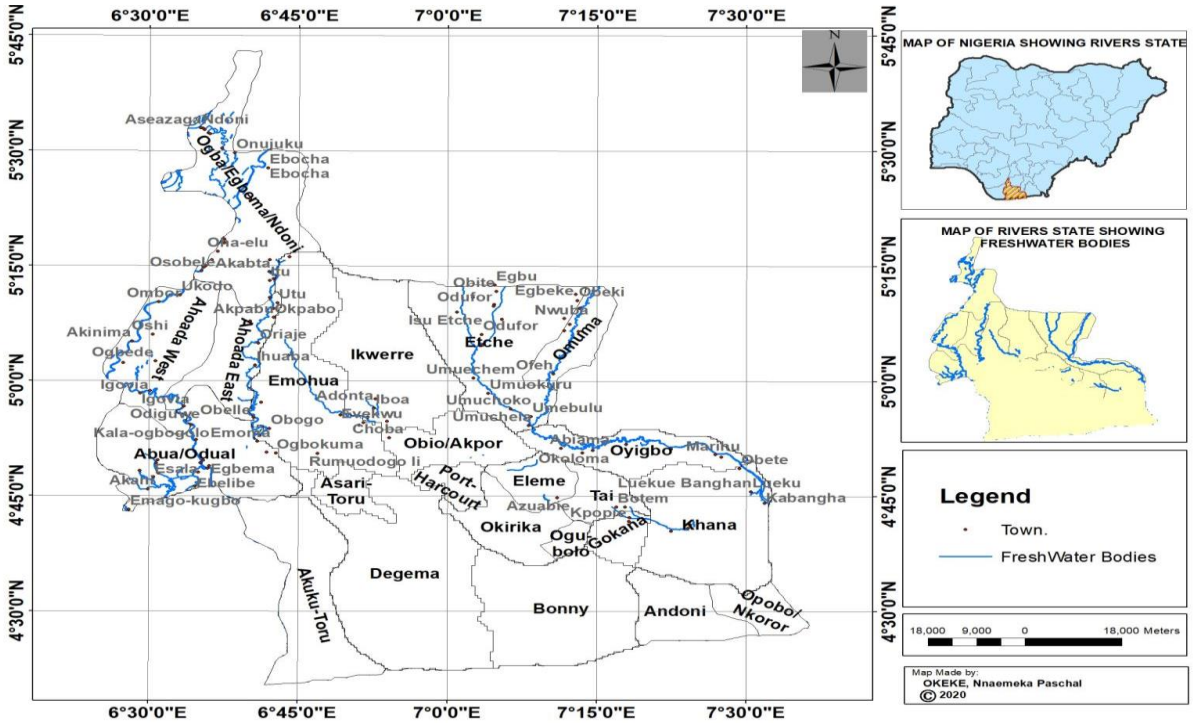

Figure 1: Map of the study areas showing sampling stations in Rivers state, Nigeria.

Sample Collection and Analysis

Water Sample Collection

Four liters of river water samples were collected from downstream of each sampling station; Omuchi Aluu, Choba, Chokocho, Oyigbo, and Aleto rivers, respectively, and inserted into in well labelled sterile bottles, which were preserved in a cooler with ice packs and transported to the laboratory, African Biosciences, Ibadan, Oyo State for molecular analysis and protozoan identification. All sample bottles were properly labeled with sampled date, sampled location and time of collection.

Molecular Analysis and Characterization

Water samples were filtered using filtration process to remove debris and solid particles, the filtrate collected were decanted into sterile bottles for further laboratory investigations.

DNA Extraction

i. Sample Lysis: 200 µl of river water sample was transferred to a bead beating tube. SL1 Buffer (750 µl) was added, followed by brief vortexing. An SL1 Buffer is a *lysis buffer solution used for the purpose of breaking open cells for* use in molecular biology experiments. The bead beating tubes were attached horizontally to a vortex and vortexed at maximum speed for 10 minutes at room temperature. After vortexing, the tubes were centrifuged at 8,000 x g for 2 minutes at room temperature to remove foam caused by detergents in the SL1 Buffer. Elution Buffer (100 µl per sample) was preheated to 60ºC for DNA elution, using Presto TM Soil DNA Extraction Kit Protocol, according to the manufacturer's instruction.

ii. PCR Inhibitor Removal: 150 µl of SL2 Buffer was added to the bead beating tube and vortexed for 5 seconds. The mixture was then incubated at 0-4ºC for 5 minutes. After

incubation, the tube was centrifuged at 8,000 x g for 1 minute at room temperature to precipitate insoluble particles and PCR inhibitors. An inhibitor removal column (purple ring) was placed in a 2 ml centrifuge tube, and 500-600 µl of clear supernatant from the bead beating tube was transferred to the column. After centrifugation at 16,000 x g for 1 minute at room temperature, the flow-through was saved for DNA binding. If a pellet was present in the flow-through, the clear supernatant was transferred to a new 1.5 ml microcentrifuge tube, using Presto TM Soil DNA Extraction Kit Protocol, according to the manufacturer's instruction.

iii. DNA Binding: 900 µl of SL3 Buffer was added to the flow-through and mixed immediately by shaking vigorously for 5 seconds. A GD (*Genomic DNA)* column (green ring) was placed in a 2 ml collection tube, and 750 µl of the sample mixture was transferred to the GD column. After centrifugation at 16,000 x g for 1 minute at room temperature, the flow-through was discarded. The remaining sample mixture was transferred to the GD column, and the column was centrifuged again at 16,000 x g for 1 minute at room temperature. The flow-through was discarded, and the GD column was placed back in the 2 ml collection tube, using Presto TM Soil DNA Extraction Kit Protocol, according to the manufacturer's instruction.

iv. Wash: 400 µl of SL3 Buffer was added to the GD Column and centrifuged at 16,000 x g for 30 seconds at room temperature. The flow-through was discarded, and the GD Column was placed back in the 2 ml Collection Tube. Wash Buffer (600 µl) was added to the GD Column, and centrifugation was done at 16,000 x g for 30 seconds at room temperature. The flow-through was discarded, and the GD Column was placed back in the 2 ml Collection Tube. Wash buffer (600 µl) was added to the GD Column again, followed by centrifugation at 16,000 x g for 30 seconds at room temperature. The flow-through was discarded, and the GD Column was placed back in the 2 ml collection tube. Centrifugation was done at 16,000 x g for 3 minutes at room temperature to dry the column matrix. Drying was completed at room temperature (15-25ºC), using Presto TM Soil DNA Extraction Kit Protocol, according to the manufacturer's instruction.

v. Elution: The dry GD Column was transferred to a new 1.5 ml microcentrifuge tube. Preheated elution buffer (30-100 µl) was added to the center of the column matrix. The column was allowed to stand for at least 2 minutes to ensure complete absorption of elution buffer, TE, or water. Centrifugation was done at 16,000 x g for 2 minutes at room temperature to elute the purified DNA, using Presto TM Soil DNA Extraction Kit Protocol, according to the manufacturer's instruction.

PCR Mixture Set up

PCR (Polymerase Chain Reaction) mixtures containing primers specific to target protozoan parasites were prepared. The components of the PCR also called the PCR cocktail was made up of the PCR Premix/ Master mix, primer (forward), primer (reverse), nuclease-free water and template DNA (extracted). Primers used in Table 1 were as previously described (Higuera *et al*., 2020).

Table 1: Primer Sequence of the Protozoan Parasites

Protozoan Parasites	DNA Sequence (5'→3'), (3'← 5')
Cryptosporidium spp.	F: 5'-AGTGACAAGAAATAACAATACAGG-3' R: 5'-CCTGCTTTAAGCACTCTAATTTTC-3'
Giardia duodenalis	F: 5'-CATGCATGCCCGCTCA-3' R: 5'-AGCGGTGTCCGGCTAGC-3'
Entamoeba histolytica	F: 5'-GTTTGTATTAGTACAAAATGGCCAATTC-3' R: 5'-TCGTGGCATCCTAACTCACTTAGA-3'
Cyclospora cayetanensis	F: 5'-TAGTAACCGAACGGATCGCATT-3' R: 5'-AATGCCACGGTAGGCCAATA-3'
Toxoplasma gondii	F: 5'-TCCCCTCTGCTGGCGAAAAGT-3' R: 5'-AGCGTTCGTGGTCAACTATCGATTG-3'

PCR Amplification

The PCR amplification was conducted with the following parameters: an initial denaturation at 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 45 seconds. Subsequently, a final extension step was performed at 72°C for 7 minutes (Toze, 1999).

Gel Electrophoresis

The PCR products were separated through gel electrophoresis to visualize and confirm the presence of the amplified DNA. 1.5% agarose gel was prepared by placing 0.75g of agarose powder in a conical flask, 50ml of Tris Boris EDTA (TBE) buffer was added to the agarose powder and allowed to dissolve using the microwave for 2 to 3 minutes. The gel was allowed to cool for a few minutes, then 5 µl of EZ vision gel (blue light) was added before pouring the gel into the tray and inserting the comb (s), which formed wells after 20 minutes of solidifying. Samples were prepared by mixing 3µl of the extracted DNA sample with 3µl of loading dye (for visualization), hence, a total of 6 µl of each sample which were loaded into different wells (one sample per well), and run at 100 volts for 20 minutes. Finally, the gel was placed in the UV transilluminator to visualize the result of protozoans' present using molecular markers (Zolgharnein *et al*., 2007).

RESULTS AND DISCUSSION

Detection of Protozoan Parasites in Water Samples

In total, 1500 ml of water samples were collected from each of the following sampling stations: Aleto river, Oyigbo river, Chokocho river, Omuchi river, and Choba river. Analysis of the water samples for various protozoa revealed differing presence levels among the samples. Specifically, amplification for *Cryptosporidium sp.* using species-specific primers was observed in 60% of the samples (3 out of 5), while *Giardia duodenalis* was amplified in 80% of the samples (4 out of 5). However, *T. gondii, E. histolytica*, and *C. cayetanensi* were not detected in any of the samples. Among the 5 rivers sampled, at least one of the tested protozoa was detected in all sites except in Choba river. Most notably, Aleto, Omuchi, and Oyigbo rivers exhibited the presence of two protozoa, whereas Chokocho river had only one protozoan detected, as shown in Table 2.

Table 2: Number and percentage of PCR positive samples for protozoan parasites for each sampled station

Sampled Stations (River)	*Giardia duodenalis*	*Cryptosporidium* sp.,	*T. gondii*	*E. histolytica*	*C. cayetanensi*
	No. of positive samples (%)	No. of positive samples (%)	No. of positive samples (%)	No. of positive samples (%)	No. of positive samples (%)
Aleto	4/5 (80)	3/5 (60)	-	-	-
Choba	-	-	-	-	-
Chokocho	4/5 (80)	-	-	-	-
Omuchi	4/5 (80)	3/5 (60)	-	-	-
Oyigbo	4/5 (80)	3/5 (60)	-	-	-

Discussion

Pathogenic protozoans have been known to cause public health problems and remain the major cause of death worldwide. Molecular methods and procedures are usually very precise for specific species and they provide historical information about pathogens (Gilbride, 2014).

The presence of *Giardia duodenalis* and *Cryptosporidium* species and absence of *Toxoplasma gondii*, *Entamoeba histolytica* or *Cyclospora cayetanensis* from this study is similar to the research carried out by Sánchez *et al*., (2018) on the Molecular detection and genotyping of pathogenic protozoan parasites in raw and treated water samples from Southwest Colombia. During their study, the water samples were analyzed, and they reported the presence of *Giardia duodenalis* and *Cryptosporidium* species in both raw and treated water samples, however, none of the water samples were positive to *Toxoplasma gondii*, *Entamoeba histolytica* or *Cyclospora cayetanensis* (Sánchez *et al*., 2018).

This study agrees with the result of the research conducted by Mahmoudi *et al.* (2015) on Genotyping of *Giardia lamblia* and *Entamoeba spp* from river waters in Iran, which also recorded the presence of *Giardia lamblia* from the water samples collected from the investigated river. In their study, they stated that the river was contaminated by human pathogenic parasites, and the high prevalence of waterborne parasites in different water sources was as a result of the significance of the transmission routes and zoonotic potential of Giardia isolates from humans and animals (Mahmoudi *et al.*, 2015).

In addition, this study recorded the infestation of Cryptosporidium species, which was also recorded in the study by Falohun *et al*., (2021) on Molecular characterization of Cryptosporidium isolates from rivers, water treatment plants and abattoirs in Ibadan, Nigeria. These researchers provided evidences that rivers and surface water in the studied area could be sources of Cryptosporidium infection for human and animal population around the studied sites and stated that there finding was of public health importance. The results of their study showed that rivers with household refuse and wastes were found to be more contaminated with Cryptosporidium oocyst, and most of the studied river's flow through residential areas, markets and farms where wastes and sewage are usually discharged into these rivers.

The result from this study showed that animal and human waste are usually sources of contamination for water bodies as described by previous authors (Barudin*et al*., 2017). Waterborne cryptosporidiosis in river water was reported by Mahmoudi *et al*., (2013) and in recreational water and other contaminated water bodies used for drinking purpose by Ehsan *et al*., (2015).

In a study conducted by Ifi *et al.* (2019) investigating the bacteriological qualities of the Okokpon River in Edo State, Nigeria, the analysis of water samples revealed high counts of heterotrophic bacteria and fungi. These elevated counts were attributed to significant anthropogenic activities, including indiscriminate laundry, defecation, car washing, bathing, as well as reduced dilution effects and surface runoff downstream prior to sampling.

Cryptosporidium species isolated from water sources in Nigeria provides the knowledge that water from waterbodies like rivers, often used for domestic and agricultural purposes may be reservoirs and sources of Cryptosporidium species that infect humans and livestock.

CONCLUSION

Giardia duodenalis and *Cryptosporidium* species were present in water samples collected from these rivers (Aleto river, Oyigbo river and Omuchi river), due to contamination of the river from human activities of community indigenes, such as swimming in the river, fishing, washing of slaughtered animals and open defecation along the river side. These identified protozoan parasites contaminate and pollutes the rivers and pose a serious health risk to community locals, hence recommendations should be made and water treatment facilities developed by the water quality management systems to reduce the health risks associated with using the water for domestic use. However, indigenes should be sensitized to embark on good water sanitary and hygiene (WASH) practices, for a healthy life and sustainable environment.

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