Original Research Article

Molecular Characterization of *Cryptosporidium* Oocysts in Under-five Children with Diarrhoea in Ile-Ife, Nigeria.

Abstract

Aim: Cryptosporidiosis is the fifth leading cause of diarrhoea-related death globally among under-five children. This study was conducted to identify and characterize oocysts of *Cryptosporidium* in under-five children with diarrhoea in Ile-Ife.

Study design: It was a case-control study.

Place and duration of the study: Department of Medical Microbiology and Parasitology, Obafemi Awolowo University between April, and October 2019.

Methodology: Ethical approval was obtained for this study. Consent was also obtained from the parents of the children. Stool samples from 53 children with diarrhoea (cases) and 94 children without diarrhoea (controls) under five years of age were collected. A structured proforma was used to obtain relevant information from the parents of the children. Stool samples were collected and examined macroscopically for consistency, appearance, and colour. Modified Ziehl-Neelsen staining was carried out on all the stool samples. The samples positive for *Cryptosporidium* oocysts by staining were genotyped by nested polymerase chain reaction and confirmed by sequencing of their 18S rRNA genes. Data generated were analyzed using descriptive and inferential statistics with SPSS software version 20 and STATA 15.

Results: The prevalence of cryptosporidiosis among the study participants was 23.1% with rates of 43.4% (23) and 11.7% (11) for diarrhoeic and non-diarrhoeic children respectively. Of the 34 stool samples analysed, 6 (17.6%) were amplified by nested PCR and 4 (11.8%) were identified by sequencing. The species of *Cryptosporidium* identified were *Cryptosporidium parvum* (75%) and *Cryptosporidium bovis*(25%).

Conclusion: In conclusion, Cryptosporidiosis is prevalent among under-five children with *Cryptosporidium parvum* and *Cryptosporidium bovis* as the infecting species.

Key words- Cryptosporidiosis, diarrhoea, under-five children, *Cryptosporidium*, Infection.

INTRODUCTION

The protozoal parasite*Cryptosporidium*is an important cause of diarrhoeal disease in humans and animals globally. There are presently over40 recognized species with most of these species found in humans(Innes*et al*., 2020).*Cryptosporidium*causes Cryptosporidiosis which is a significant diarrhoeal disease that affects young children especially those under the age of 5 years and individuals that are immunocompromised (Innes*et al*., 2020). Like many other important protozoan parasites such as *Giardia*, *Cryptosporidium*is spread through the faecal-oral route. Infection can be acquired through ingestion of oocyst infected water, food and or a direct contact with an infected individual (Shirley *et al*., 2012; Checkley *et al*., 2015; Johansen *et al*., 2015). It is also transmitted zoonotically as *Cryptosporidium* is known to have a wide host range. It was first recognized as a human pathogen in 1976 (Ryan *et al.,* 2021) and since then its status has sailed up from a mere curiosity to a pathogen of worldwide importance.*Cryptosporidium* has recently been established as the second most important infectious agent after rotavirus, and the single most important parasitic cause of gastroenteritis (Kotloff*et al*., 2013).

Studies have also established a link between *Cryptosporidium* infection and growth impairment, physical fitness, and cognitive function of children. (Kotloff*et al*., 2013; Korpe*et al*., 2016; Khalil *et al*., 2018; Rogawski*et al*., 2018). Approximately 7.6 million diarrhoea casesannually are attributable to *Cryptosporidium*, withabout 2.9 million in Sub-Saharan Africa (Sow *et al*., 2016 and Mutai *et al.* 2020). Recent studies have also linked cryptosporidiosis to colorectal cancer (Innes*et al*., 2020). Molecular tools have revolutionized identification of microrganisms and ofthe 44 recognised *Cryptosporidium* species and greater than 120 genotypes, 19 species, and four genotypes have been reported in humans with *Cryptosporidiumhominis*,*Cryptosporidiumparvum*, *Cryptosporidiummeleagridis*, *Cryptosporidiumcanis* and *Cryptosporidiumfelis* being the most prevalent (Ryan et al., 2021). However, there is paucity of information on the species infecting children in Ile-Ife environment. It was against this background this study sought to assess the prevalence and species of *Cryptosporidium* in under-five children.

MATERIAL AND METHODS

Study design- It was a case control study.

Study area - The study area was Ile-Ife, Osun State, Nigeria. Ile-Ife lies between Latitudes 7^0 31' N and 7^0 35' N and Longitudes 4^0 30' E and 4^0 35' E. The research study was conducted in the Department of Medical Microbiology and Parasitology, Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria.

Study population - The participants (under-five children) were recruited from Enuwa Primary Health Center, Oke-ogbo State Hospital and Obafemi Awolowo University Teaching HospitalsComplex(OAUTHC), Ile-Ife, Osun state.

Ethical approval and confidentiality – Approval for the study with ethical clearance certificate was obtained from the Ethics and Research committee ofOAUTHC, Ile-Ife with study protocol number (ERC/2019/04/15). The mothers of the children were informed about the research and gave their consent. Confidentiality of all patients and data was strictly maintained.

Sample size and selection of subjects-A minimum sample size of 147 was calculated for this study comprising 94 controls (children under-five without diarrhoea) and 53 cases (children under-five with acute diarrhoea). The proportion ratio used was 2:1 (controls to cases). The children were randomly selected from these centers.

Demographic information- A structured proforma was used to obtain relevant information such asage, sex, weight, height, history of diarrhoea, source of drinking water and contact with pets.

Sample collection, processing, and storage - The stool samples were collected into clean grease free universal bottles. Each sample was divided into 2 portions, a portion was preserved with 10% formalin while the other part was preserved with 2.5% Potassium dichromate. Stool samples preserved with 2.5% potassium dichromate were stored at 4ºC for molecular analysis, while those preserved in formalin were used for formol-ether concentration and microscopy as recommended (Lemos *et al.*, 2011).

The samples were processed by carrying out macroscopy, direct wet mount examination,formal-ether sedimentation technique, Modified Ziehl-Neelsen staining and

molecular analysis.The stained slides were observed using immersion oil objective lens and the size of the oocysts was confirmed using an ocular micrometer. Samples that were positive for *Cryptosporidium* oocysts were further analysed using nested polymerase chain reaction.

Molecular analysis

DNA Extraction

The positive faecal samples that were preserved with 2.5% of potassium dichromate at 4°C were diluted with nuclease-free water at 1:1 proportion. To each of the fecal sample 500µl lysis buffer [50µl Tris-HCl 100 mM (pH=8.0); 50µl ethylenediaminetetraacetic acid (EDTA) 50 mM (pH=8.0); 50 µl 10% sodium dodecyl sulfate (SDS); 3.5µl 70 mM β-mercaptoethanol; 1µl 1% polyvinylpyrolidone (PVP) and 345.5µl sterile distilled water] were added and incubated at 65°C in a water bath for 90 minutes. Twenty micro-liters of proteinase K (20 mg/ml) was applied after incubation. The samples were incubated for 15 minutes in an ice bath. The tubes were shaken to mix well, and the mixture was centrifuged at 12000 rpm for 5 minutes. After centrifugation, the aqueous phase which is the supernatant was gently removed to a new eppendorf tube, 500µl chloroform was added; homogenized and centrifuged at 12000 rpm for 15 minutes. The supernatant seen after centrifuging was put into another eppendorf tube and precipitated with 500µl ice-cold isopropyl alcohol and held for 10 minutes in an ice bath. After 10 minutes, it was centrifuged at 12000 rpm for 3 minutes. The supernatant was gently discarded, and the precipitated DNA was washed with 500µl of 70% ethanol and centrifuged at 12000 rpm for 3 minutes. The supernatant was discarded, and the DNA was dried.

18S rRNA Nested PCR Assay

The primary mix containing the extracted DNA was amplified using the PCR thermal cycler (GeneAmp® PCR System 9700). The cycling condition were selected as follows: one cycle of denaturation at 94˚C for 5 minutes; 45 cycles of 94˚C for 30 seconds (denaturation), 48˚C for 20 seconds (annealing), 72˚C for 30 seconds (extension); then a final extension of 72˚C for 7 minutes and held at 4˚C until removed from the thermo-cycler.

The secondary mix containing the primary PCR product was amplified using the same thermo-cycler. The cycling condition were selected as followed: one cycle of denaturation at

94˚C for 5 minutes; 45 cycles of 94˚C for 30 seconds (denaturation), 50˚C for 20 seconds (annealing), 72˚C for 30 seconds (extension); then a final extension of 72˚C for 7 minutes and held at 4˚C until removed from the thermo-cycler.Sequencing of 18S rRNA PCR products was carried out using the Nimagen, BrilliantDye^{TM} Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to the manufacturer's instructions. The labelled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053). The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50 cm array, using POP7. The sequence chromatogram analysis was performed using FinchTV analysis software and was changed to FASTA format before blasting using NCBI database. Table 1 showsprimers used forthe nested PCR while Tables 2 and 3 show the primary and secondary PCR work sheets.

Table 1. Primers used for nested PCR.

Primer name	Sequence 5'-3'	Target gene	Amplicon size (bp)
Primer 1-R	CACCAGACTTGCCCTCCAAT	18S rRNA	215
Primer 1-F	CAATGACGGGTAACGGGGAA	18S rRNA	215
Primer 2-R	GCCTGCTGCCTTCCTTAGAT	18S rRNA	78
Primer 2-F	CGGGTAACGGGGAATTAGGG	18S rRNA	78

Table 2. Primary PCR work sheet

Primary PCR Mix	n = 1	n = 34
Master mix	12.5 µl	687.5 µl
Primer 1-F	0.5 µl	27.5 µl
Primer 1-R	0.5 µl	27.5 µl
Extracted DNA working solution	4 µl	220 µl
Water	7.5 µl	412.5 µl
Total	25 µl	1375 µl

Table 3. Secondary PCR work sheet

Secondary PCR Mix	n = 1	n = 6
Master mix	12.5 µl	75 µl
Primer 2-F	0.5 µl	3 µl
Primer 2-R	0.5 µl	3 µl
Primary PCR Product	3 µl	18 µl
Water	8.5 µl	51 µl
Total	25 µl	150 µl

Data Analysis – Data were analyzed usingfrequency, proportion, percentages, tables, and Pearson's chi-square was used to determine the association between Cryptosporidiosis and associated symptoms.

Results

Socio-Demographic Characteristics of the Respondents

Out of the 147 children recruited, 38.8 % were infants while 61.2.% were more than 12 months in age. Also, 51 % were male while 49.0 % were female. Diarrhoea occurred in 36.1% of the study participants while 63.9% did not have diarrhoea. Out of those with diarrhoea, 34.0 % were exposed to all kinds of domestic animals while 66.0% were not exposed. For the children without diarrhoea, 59.6% were exposed to domestic animals while 40.4% were not.Majority of the respondents were Christians 84.4%; Muslims were 15% and other religions 0.6 %. For the father's occupation, 27.9 % were artisans and 27.2 % traders while for the mother's occupation, 27.2 % were artisans and 36.1 % were traders. Majority of the guardians of the children hadtertiary education (46.3%), 43.5% had secondary education, 8.8% had primary education and 1.4 % had no education. Forty-four-point two percent of the children have not started school at all, and majority of the children (89.8%) had their mother as their informant.

Table 4 shows the prevalence of *Cryptosporidium* from the stool samples of the study participants by the Modified Ziehl-Neelsen technique.

Table4: Prevalence of *Cryptosporidium* from the stool samples of under-five children analysed by Modified Ziehl-Nelseen staining technique.

Stool Consistency	***Cryptosporidium* Positive**	***Cryptosporidium* Negative**	χ^2	***P-value***
Diarrhoeic (n= 53)	23 (43.4)	30 (56.6)	19.1479	**<0.001**
Non-diarrhoeic (n= 94)	11 (11.7)	83 (88.3)		
Total (n= 147)	**34 (23.1)**	**113 (76.9)**		

Identification of *Cryptosporidium* from the stool Samples of Under-five Children analysed by nested PCR.

Tables5 and 6 shows the analysis done by PCR for further identification of *Cryptosporidium* targeting 18S rRNA gene. Of the 34 stool samples analysed by nested PCR, 6 (17.6%) were amplified and recorded as positive for *Cryptosporidium* infection by possession of 18S rRNA gene. Plates 1 and 2 are amplicons of primary and secondary PCR.

Table 5: Identification of *Cryptosporidium* from the stool Samples of Under-five Children analysed by PCR.

Variables	**Frequency**	**Percentage (%)**
PCR		
Negative	**28**	**82.4**
Positive	**6**	**17.6**
Total	**34**	**100**

Table 6: Prevalence of *Cryptosporidium* from the stool Samples of Under-Five Children analysed by PCR.

Variables	***Cryptosporidium* positive children**		χ^2	***P-value***
PCR	Diarrhoea n=23	Non-Diarrhoea n=11	0.8191	0.365
Negative	18(78.26)	10(90.91)		
Positive	5(21.74)	1(9.09)		
Total	**23(100.00)**	**11(100.00)**		

L 1 2 3 4 5 6 L

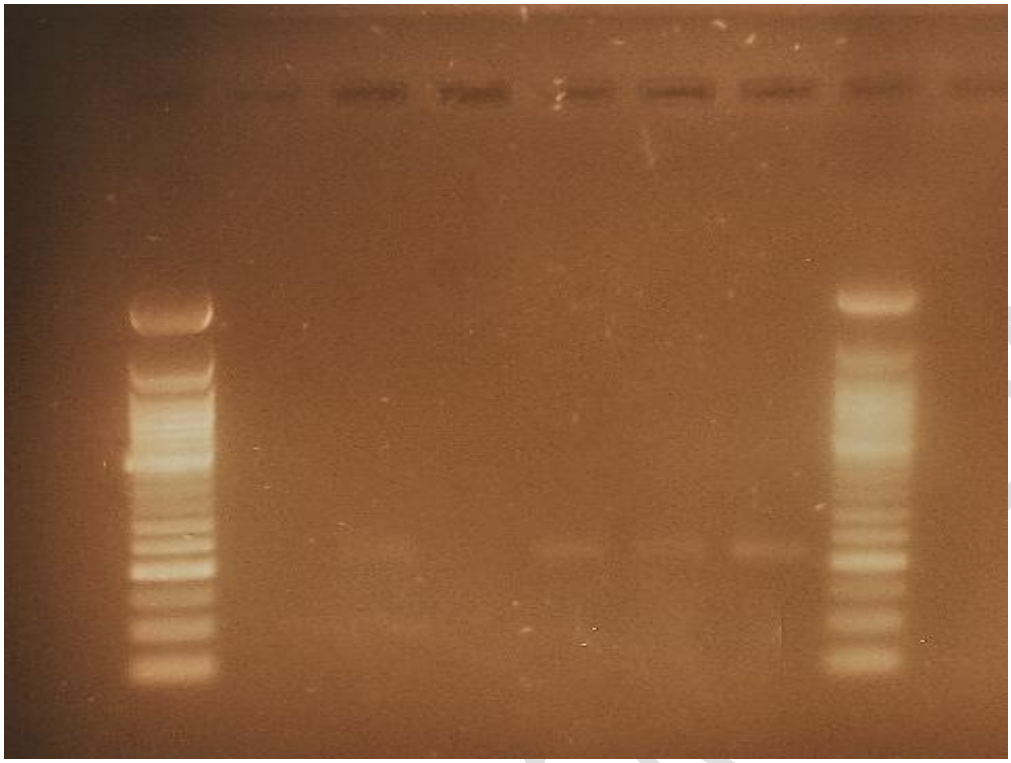

Plate 1: Amplicons of primary PCR of 18S rRNA gene.

(Lane L: DNA ladder (50 bp), Lane 2, 4, 5, and 6 = 215 bp)

L 1 2 3 4 5 6 L

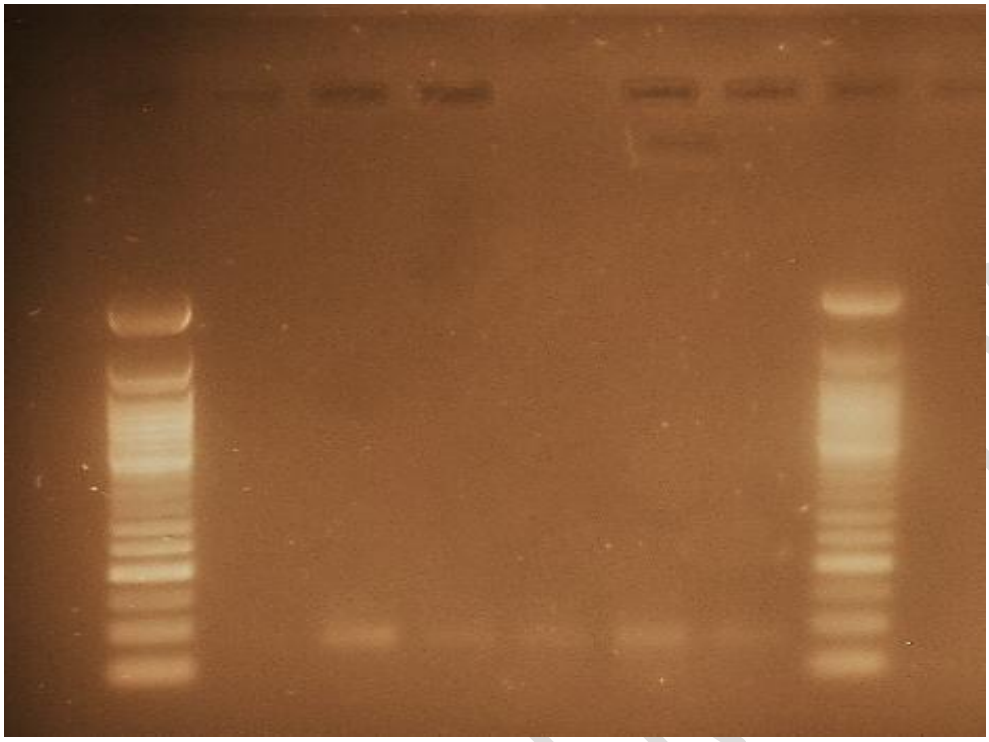

Plate 2: Amplicons of secondary PCR of 18S rRNA gene.

(Lane L: DNA ladder (50 bp), Lane 2, 3, 4, 5 and 6 = 75 bp)

Characterization of *Cryptosporidium* species from the stool Samples of Under-five Children analysed by sequencing of 18S rRNA PCR products.

Table 7shows the identified *Cryptosporidium* species analysed by sequencing of the 18S rRNA gene. Of the 6 *Cryptosporidium* isolates that were characterised by sequencing analysis,3 were identified as *Cryptosporidiumparvum* and 1 was identified as *Cryptosporidiumbovis*. The 4 isolates were from children with diarrhoea while the other 2 were not identified.

Table 7: *Cryptosporidium* species identified by sequencing analysis of 18S rRNA PCR products.

Species Identified	Number of Species Identified (%)	Accession Number	Partial Sequence
Cryptosporidiumparvum	3(75%)	FJ039877.1	GGAGCCTGAGAACGGCTACCACATCTAAGGAAGGCAGCAGGC
Cryptosporidiumbovis	1(25%)	KY809006.1	GCAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGC

Discussion

The prevalence of *Cryptosporidium* among the under-five children investigated in this study by Modified Ziehl-Neelsen (MZN) staining techniqueis 23.1%. This rate is high, and this finding substantiates *Cryptosporidium* as an important cause of diarrhoea in children. Wellington *et al*. (2008) and Musa *et al.* (2014) reported prevalence rates of 17.1% and 17.8% in Lagos and Zaria respectively which are lower than the observed prevalence in this study. However, other studies such as Nassar *et al*. 2017, Shinkafi and Muhammed, 2017 reported higher prevalence rates of 38.3% in Osun and 62.5% in Sokoto states respectively. The varying prevalence rates could be due to differences in geographical location and possibly levels of hygiene.This study also revealed thatdiarrhoea was significantly associated with presence of *Cryptosporidium* in the stool samples of the children which is similar to the reportsof Inyang-Etoh*et al*. 2007, Dabas *et al*. 2017 andTamomh *et al*. 2021.

The *Cryptosporidium*oocysts detected by MZN staining were further characterized by nested PCR. Out of the oocysts detected in diarrhoeicand non-diarrhoeic stools, 21.7% and 9.1% were positive for *Cryptosporidium*respectively in 6 samples due to the presence of 18S rRNA gene.Three were further identified as *Cryptosporidiumparvum*(75%)and 1(25%) was identified as *Cryptosporidiumbovis* by sequence analysis. *Cryptosporidium*species that arecommonly implicated in human cryptosporidiosis are *Cryptosporidium hominis*and *Cryptosporidium parvum. Cryptosporidiumfelis. Cryptosporidiumcanis*and *Cryptosporidiummeleagridis*also cause infections in humans especially those that are immunocompromised (Ryan *et al*.2021).Of thesespecies of *Cryptosporidium,*the two that commonly cause human infection are*Cryptosporidiumparvum*and*Cryptosporidiumhominis*,but in addition,C*ryptosporidium parvum* infects domestic or wild ruminants. (Anejo-okopi*et al*., 2016).

The finding of *Cryptosporidium parvum*in this study is much higher than the reports of Anejo-okopi*et al*., 2016 and Okojokwu[a]*et al*., (2016) where prevalencerates of 25% and 54% for werefound respectively.

*Cryptosporidiumbovis*was the other species detected in this study and it is known to commonly infect cattle in addition to three other species of *Cryptosporidium (*Thomson *et al.* 2017). In Colombia, Higuera *et al.,* 2020 reported *Cryptosporididiumbovis*as a cause of human infection. The zoonotic potential of *Cryptosporidium* was also investigated among

children less than 10 years and livestock in Egypt and *Cryptosporidiumbovis* was found in 4.1% of ruminants; Combination of *Cryptosporidium parvum* plus *Cryptosporidiumbovis* (5.3%) and combination of *Cryptosporidium parvum* plus *Cryptosporidiumbovis* 1.2% in the children. (Helmy *et al*.2013).Some other studies have also reported *Cryptosporidium bovis* as a cause of human infection. (Ng *et al*., 2012 and Khan *et al*., 2010). Cattleis the major host for *Cryptosporidium bovis* and a known reservoir of zoonotic infections (Ryan *et al*.2014).

Human infection with *Cryptosporidium bovis* has not been previously reported in Nigeria and its finding in this study suggests that zoonotic transmission of *Cryptosporidium* in Nigeria is not limited to *Cryptosporidium parvum*. However, there have been reports of *Cryptosporidium bovis*infection among cattle in Nigeria (Okojokwu[b] *et al*. 2016 and Chukwu et al., 2019)). The presence of the species identified in this study further elucidates the dynamics of transmission of *Cryptosporidium*which could be both anthroponotic and zoonotic.

Conclusion: Cryptosporidiosis is highly prevalent in under five childrenwith *Cryptosporidiumparvum*and *Cryptosporidiumbovis* as the infecting species. This study also highlights that the paediatric population are at risk for zoonotic transmission of *Cryptosporidium*.This study alsocorroborates anthroponotic and zoonotic modes of transmission in the study environment and further highlights the need for one health approach to the management of Cryptosporidiosis. Routine screening of stool samples from children for *Cryptosporidium* in addition to other intestinal pathogens should be encouraged in laboratories. Further studies are needed on children and domestic animals in Nigeria to better understand the sources of infection, transmission dynamics and genetic diversity of *Cryptosporidium*.

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