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Molecular characterization of *Cryptosporidium* spp from stool samples of people living with human immunodeficiency virus in Maiduguri, Nigeria

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Abstract:

Background: *Cryptosporidium* is an important cause of diarrhea in immuno-compromised individuals including children. Recent advances in molecular techniques have led to the discovery of different species that are more commonly associated with diarrhea. This study aimed at isolating and characterizing the C*ryptosporidium* spp among HIV patients with diarrhea in Maiduguri, Nigeria.
Methodology: Stool samples were collected from 269 adult HIV patients with diarrhea at the antiretroviral clinic of University of Maiduguri Teaching Hospital. The stool samples were first screened for *Cryptosporidium* using nested PCR after DNA extraction with QIAamp DNA stool mini-extraction kit following the manufacturer's protocol. The identified *Cryptospodium* parasites were further characterized into different species using resyriction fragment polymorphism (RFLP) analysis that targets the amplified small subunit (SSU) ribosomal DNA and glycoprotein 60 (gp60) genes.
Results: From the 269 HIV patients studied, 52 (19.6%) were positive for *Cryptosporidium* by the nested PCR assay. RFLP analysis characterized the *Cryptosporidium* species into *C. hominis* (40.4%, n=20) and *C. parvum* (59.6%, n=32).
Conclusion: Majority of the *Cryptosporidium* species in this study were *C. parvum*, supporting the notion that zoonotic transmission is the main route of cryptosporidiosis transmission. Nonetheless, the observation of *C. hominis* in some patients suggests that anthroponotic transmission is also an important contributor to *Cryptosporidium* pathogenesis in Maiduguri.

Keywords: *Cryptosporidium*; HIV; immuno-compromised; diarrhea; PCR-RFLP; gp60; SSU

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Caractérisation moléculaire de *Cryptosporidium* spp à partir d'échantillons de selles de personnes atteintes du virus de l'immunodéficience humaine à Maiduguri, au Nigéria

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Résumé:

Contexte: *Cryptosporidium* est une cause importante de diarrhée chez les personnes immunodéprimées, y compris les enfants. Les progrès récents des techniques moléculaires ont permis la découverte de différentes espèces plus fréquemment associées à la diarrhée. Cette étude visait à isoler et à caractériser les *Cryptosporidium* spp chez des patients atteints du VIH et souffrant de diarrhée à Maiduguri, au Nigéria.
Méthodologie: Des échantillons de selles ont été prélevés auprès de 269 patients adultes atteints du VIH et souffrant de diarrhée à la clinique antirétrovirale de l'Hôpital universitaire de Maiduguri. Les échantillons de selles ont d'abord été analysés pour *Cryptosporidium* par PCR nichée après extraction d'ADN à l'aide du kit de mini-extraction d'ADN de selles QIAamp, conformément au protocole du fabricant. Les parasites *Cryptosporidium* identifiés ont été caractérisés en différentes espèces à l'aide d'une analyse du polymorphisme des fragments de réplication (RFLP) ciblant les gènes de l'ADN ribosomique de la petite sous-unité amplifiée (SSU) et de la glycoprotéine 60 (gp60).
Résultats: Parmi les 269 patients VIH étudiés, 52 (19,6 %) étaient positifs pour *Cryptosporidium* par PCR nichée. L'analyse RFLP a caractérisé les espèces de *Cryptosporidium* en *C. hominis* (40,4%, n=20) et *C. parvum* (59,6%, n=32).
Conclusion: La majorité des espèces de *Cryptosporidium* dans cette étude étaient *C. parvum*, ce qui étaye l'idée que la transmission zoonotique est la principale voie de transmission de la cryptosporidiose. Néanmoins, l'observation de C. hominis chez certains patients suggère que la transmission anthroponotique est également un contributeur important à la pathogenèse de *Cryptosporidium* à Maiduguri.

Mots-clés: *Cryptosporidium*; VIH; immunodéprimé; diarrhée; PCR-RFLP; gp60; SSU

Introduction:

Cryptosporidium species are Apicomplexan protozoan that is recognized as one of the most important diarrheal pathogens affecting people worldwide, particularly in Africa (1). *Cryptosporidium* is second only to rotavirus in causing diarrhea and death in children in developing countries, responsible for 2.9 million cases annually in children aged <24 months in sub-Saharan Africa (2). People living with HIV/AIDS suffer from numerous and frequent opportunistic infections, many of which pose a serious threat to their health and are life-threatening. Cryptosporidiosis is one of the most common causes of gastroenteritis in immunocompromised people (3). In people with weakened immune systems (HIV-infected people, cancer patients, and organ recipients), cryptosporidiosis can be severe, long-lasting, and sometimes fatal.

Of the 40 currently recognized *Cryptosporidium* species, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for most human infections. However, other species have also been found in immunocompromised patients, including *C. meleagridis, C. canis,* and *C. felis* (1). Cryptosporidiosis remains a common cause of chronic diarrhea in AIDS patients in developing countries, with up to 74% of AIDS patients with diarrhea harboring this parasite in their stool (4).

The main challenge in the proper identification of *Cryptosporidium* spp is to distinguish oocysts from other small particles in fecal and environmental specimens such as yeasts, molds, algae, and plant debris (5). Of the nearly 20 *Cryptosporidium* species and genotypes reported in humans*, C. hominis* and *C. parvum* are responsible for most infections (3). Therefore characterization of *Cryptosporidium* at the species level will be useful in identifying infection and contamination sources (6).

Detection of cryptosporidiosis using PCR-based methods is more sensitive than conventional microscopical and serological methods for detecting oocysts in faeces. Molecular methods can also identify the species/ genotypes and subtypes of *Cryptosporidium,* important for epidemiology of cryptosporidiosis and predicting transmission routes (6). Since the description of the first PCR-based tool for the differentiation of *C. parvum* and *C. hominis*, molecular techniques in the diagnosis of cryptosporidiosis became popular, especially due to their genotyping capabilities (6). This study was conducted to determine the prevalence and identify the different *Cryptosporidium* species among people living with HIV in Maiduguri, Borno State, Northeastern Nigeria using the nested-PCR technique and restricted fragment length polymorphism (RFLP) analysis.

Materials and method:

Study area:

The study was carried out at the University of Maiduguri Teaching Hospital, Borno State, Nigeria. Borno State, also called Yerwa by its locals, is one of the northeast states with Maiduguri as its largest city and

the capital. Maiduguri was founded in 1907 as a military outpost by the British and is home to the Kanem-Bornu Empire. The residents are mostly Muslims, including Kanuri, Hausa, Shuwa, Bura, Marghi, and Fulani ethnic groups. There is also a considerable Christian population.

Borno State has been affected by the Boko Haram insurgency, and almost all the local government areas have relocated to the metropolitan areas, of which Maiduguri is one. The internally displaced individuals are accommodated in camps with inadequate social amenities. The University of Maiduguri Teaching Hospital is a tertiary health center located in Maiduguri with a bed capacity of 650 and 21 departments.

Study design, participants and ethical approval:

This descriptive cross-sectional study was carried out from March 2020 to April 2021 at the University of Maiduguri Teaching Hospital, a tertiary referral health institute, and a center for HIV /AIDS management under the PEPFAR. A total of 269 consenting adults living with HIV who had complained of diarrhea were consecutively enrolled into the study.

A pre-designed structural questionnaire was used to collect socio-demographic characteristics of the patients. Informed consent was obtained from all study participants. The study was approved by the ethical committee of the University of Maiduguri Teaching Hospital, Borno State, Nigeria.

Inclusion and exclusion criteria:

All consenting adult HIV-infected patients presenting with diarrhea attending the ARV clinic in UMTH or were admitted at the time of the study were recruited while all HIV-infected patients who have diarrhea and are on any of the prophylactic drugs against parasitic infection were excluded from the study.

Study instrument:

A structured questionnaire was used to collect information from all the respondents. The tool extracted information about demography, socio-economic characteristics of the respondents, history of risk factors, history of medication, clinical history, and antiretroviral therapy. The data collection tool was adapted from a similar previous study (7).

The questionnaire was pretested at a secondary health facility in the same geographical region as the study area. Before the commencement of data collection, we ensured that the questionnaire issues were identified and corrected.

Specimen collection:

Stool samples from recruited HIV-infected patients with diarrhea were collected by the patients in clinics, wards, and some at home in a wide-mouth screw cap labeled container and transported to the Medical Microbiology laboratory of UMTH Maiduguri for analysis. Each sample was allocated a laboratory number. In case of delay in sample processing, it was stored at 2-8°C for 24 hours.

Macroscopic examination:

The samples were examined macroscopically to detect the presence of any color, consistency (whether formed, semi-formed, soft, or watery), and presence of blood, mucus, and adult worms. The sample was then divided into two portions, one for molecular studies and the other portion for microscopy.

DNA extraction:

DNA extraction was done on the aliquots of the stool sample after defrosting itusing commercially available QIaAmp stool Minikit (Qiagen, Germany) as per the manufacturer's instructions, with some modifications as described by Okojokwu et al., (8). Fecal suspensions were prepared by adding saline (0.8% w/v) in a ratio of 1:1 (v/v). Next, fecal material (0.1ml) was added to 1.4 ml of alkali wash solution (0.5M NaOH and 0.05 M sodium citrate) in a 1.5ml Eppendorf tube and mixed for 30 seconds at room temperature by repeated inversion.

The mixture was subsequently centrifuged at 13,000g for 5min, and the cell pellet containing any cryptosporidial DNA was re-suspended in 0.5ml of 0.5M Tris-HCl (pH 8.0) and centrifuged at 13,000g for 5min. This latter step was repeated, and the resulting pellet was re-suspended in Tris-EDTA (0.1 ml, ten mM Tris-HCl, pH 8.0, containing one mM EDTA) and the DNA extracted from the oocysts by 10-14 cycles of freeze-thawing (in ice for 1 min; in a water bath at 100°C for 1 min). The resulting extract was centrifuged at 13,000g for 15min, and the supernatant containing cryptosporidia DNA was transferred to a clean tube and stored frozen before PCR. This procedure was performed at the University of Maiduguri Biotechnology Center.

Nested PCR amplification:

A nested PCR targeting the small subunit of the 18S rRNA gene was performed at University of Maiduguri Biotechnology center with the use of initial primers Cr18PA: (5′-TTCTAGAGCTAATACATGCG-3′) and Cr18PB: (5′-CCCATTTCCTTCGAAACAGGA-3′), which amlified a 1.3 kb fragment of 18S-rRNA gene. The inner primer Cr18NA: 5′-GGAAGGGTTGT ATTTATTAGATAAAG-3′ and Cr18NB: 5′-CTCA

TAAGGTGCTGAAGGAGTA-3' then amplified a 826-864bp fragment of initial amplified sequence.

The reaction mixture of the PCR consists of 3µL, 1xPCR buffer (50mM KCl, 20 mM Tris-HCL, 2.5 mM $Mgcl_2$, pH 8.4) and 1µL of 0.1µg/ml BSA, 2µL of the initial primers, and 1µL of 0.3mM concentration of each of dNTPs, 0.5µL of recombinant Taq polymerase and 2 µL of the purified DNA and 2µL of 1.5 mM $Mgcl_2$. The reaction mixture was prepared as described above for the second round of amplification, except for the primers and DNA template. Again, 1 µL of the first PCR product as a source of DNA and the inner primers was used.

Primary amplification was carried out in 35 cycles, each consisting of denaturation at 95ºC for 30 seconds, annealing at 45ºC for 30 seconds, and extension at 68ºC for 30 seconds, with an initial denaturation at 95ºC for 3 min and a final extension at 68ºC for 5 min. The secondary round of amplification consisted also of 35 cycles with identical temperatures and times,and annealing temperature of 48 ºC. All PCRs were run in a Thechne PCR thermocycler.

***Cryptosporidium* speciation by restriction fragment length polymorphism analysis:**

RFLP analysis of the secondary PCR products using restriction enzymes *SspI* (New England Biolab, Ipswich, USA) and *VspI* (Promega, Madison, USA) was used to characterize *Cryptosporidium* species. Six microliters (6µL) of the PCR product were digested with 6 units of each enzyme in the same reactions at 37ºC for 4 hours. Positive controls, including PCR mixture reaction with the DNA extract of known *Cryptosporidium* positive stool samples were used in both PCR assays while PCR mixture reaction without DNA was used as negative control in both PCR.

Loading and electrophoresis of the sample:

Five microliters (5µL) of amplified undigested and digested PCR products were mixed with 2.0µL of loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. A 1500 bp molecular weight marker was loaded in one well to determine the size of the amplified PCR products and 1000 bp for RFLP analysis. Electrophoresis was carried out at 100 volts for 35 minutes. The intensity and size of all amplicons were assessed by electrophoresis in 1.5% ethidium bromide-stained agarose gels using Tris Borate EDTA (65 mMTris-HCl, 27 mM boric acid, one mM Ethylene diamide tetra acetate, pH 9; Bio-Rad, USA) as the buffer and 1.5kbp molecular marker (Fermentas).

The amplified products of the study samples were visualized in a trans-illuminator, and the gel was photographed by a digital camera and transferred data to a computer for further documentation. Negative control was used for each amplification.

Data analysis:

The information obtained from the patient questionnaire and other relevant data from this study was entered into a computer program. Data were analyzed using the Statistical Package for Social Sciences (SPSS) version 23.0. The data were presented in tables/figures as frequencies and proportions for univariate analysis, while for bivariate analysis Chi-square test was used to test for association between independent variable of socio-demographic/risk factors and dependent variables.

Results:

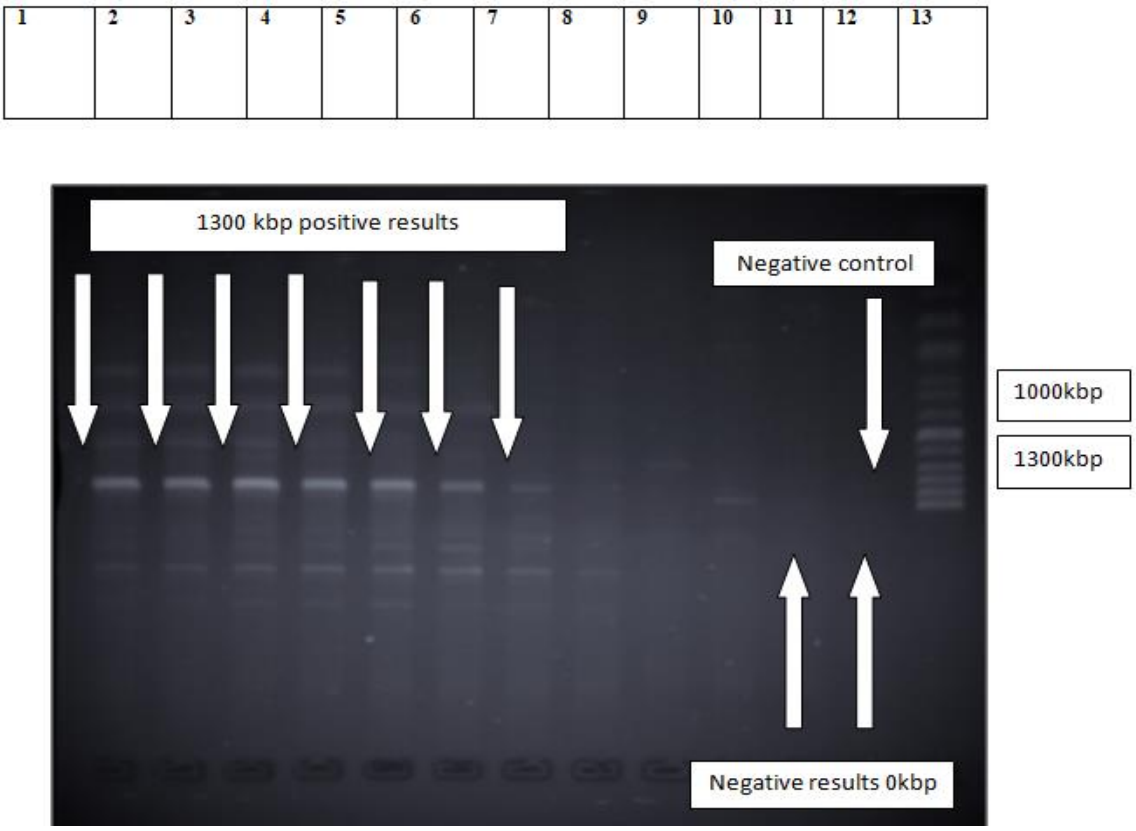
Two hundred and sixty-nine HIV patients were recruited and their samples analyzed. Majority of the patients were females (n=189, 70.3%) while 80 (29.7%) were males (Table 1). Stool samples of 52 of the 269 HIV patients were amplified by PCR, giving the prevalence of cryptospiridiosis of 19.3% in the study. The PCR SSU rRNA genes of *Cryptosporidium* produced bands with a molecular size of 1,300 bp (Fig 1). The secondary PCR products of the 18S rRNA gene yielded bands that coincided with the expected 830 bp for *Cryptosporidium* species (Fig 2).

The prevalence of *Cryptosporidium* infection was higher in males (n=20, 25.0%) compared to females (n=32, 16.9%) but the prevalence difference was not significantly different (p=0.1729) (Table 1). The prevalence was highest in the age group 18-27 years (n=7, 23.5%), while the lowest prevalence (n=5, 11.6%) was in the age group 48-57 years, but there was nosignificant difference in prevalence with respect to age group (p=0.4905) (Table 1). Patients with no or primary education had the highest prevalence (n=19, 24.1%), while patients with tertiary education had the lowest prevalence (n=7, 16.3%) but the prevalence difference was not significantly different with respect to education (p=0.4404) (Table 1). Based on occupational status, the prevalence was highest among the patients who are farmers (n=5, 31.3%) but there was no significant difference in prevalence with respect to occupation (p=0.5440) (Table 1).

Representative electrophoresis profiles of the restriction enzymes digests of the PCR amplified products are presented in Fig 3 and Fig 4. The bands were of molecular sizes 450 bp, 260 bp, 111 bp, and 108 bp and were indicative of *C. hominis* and *C. parvum* (Fig 3). Of the 52 positive samples, 40.4% were *C. hominis,*and 59.6% were *C. parvum* (Fig 5).

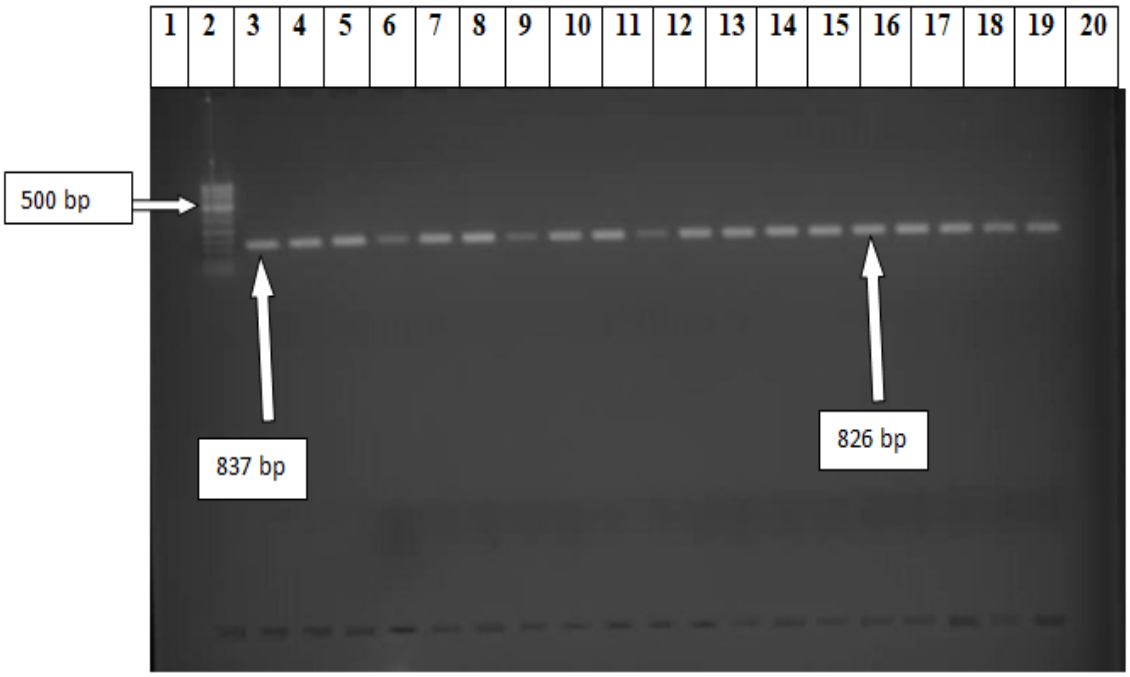
Table 1: Socio-demographic characteristics and prevalence of cryptosporidiosis in the study participants

Variable	Frequency (%) (n=269)	Prevalence by PCR (%) (n=52)	x^2	*p* value
Age group (years)				
18-27	28 (10.4)	7 (25.0)	3.417	0.4905
28-37	85 (31.6)	20 (23.5)		
38-47	100 (37.2)	8 (18.0)		
48-57	43 (15.9)	5 (11.6)		
≥ 58	13 (4.8)	2 (15.4)		
Sex				
Male	80 (29.7)	20 (25.0)	1.858	0.1729
Female	189 (70.3)	32 (16.9)		
Level of education				
None/Primary	79 (29.4)	19 (24.1)	1.640	0.4404
Secondary	147 (54.6)	26 (17.7)		
Tertiary	43 (16.0)	7 (16.3)		
Occupation				
Business	82 (30.5)	12 (14.6)	0.3083	0.5440
Civil service	68 (25.3)	13 (19.1)		
Farming	16 (5.9)	5 (31.3)		
Housewife	91 (33.8)	20 (22.0)		
Student	12 (4.7)	2 (16.7)		



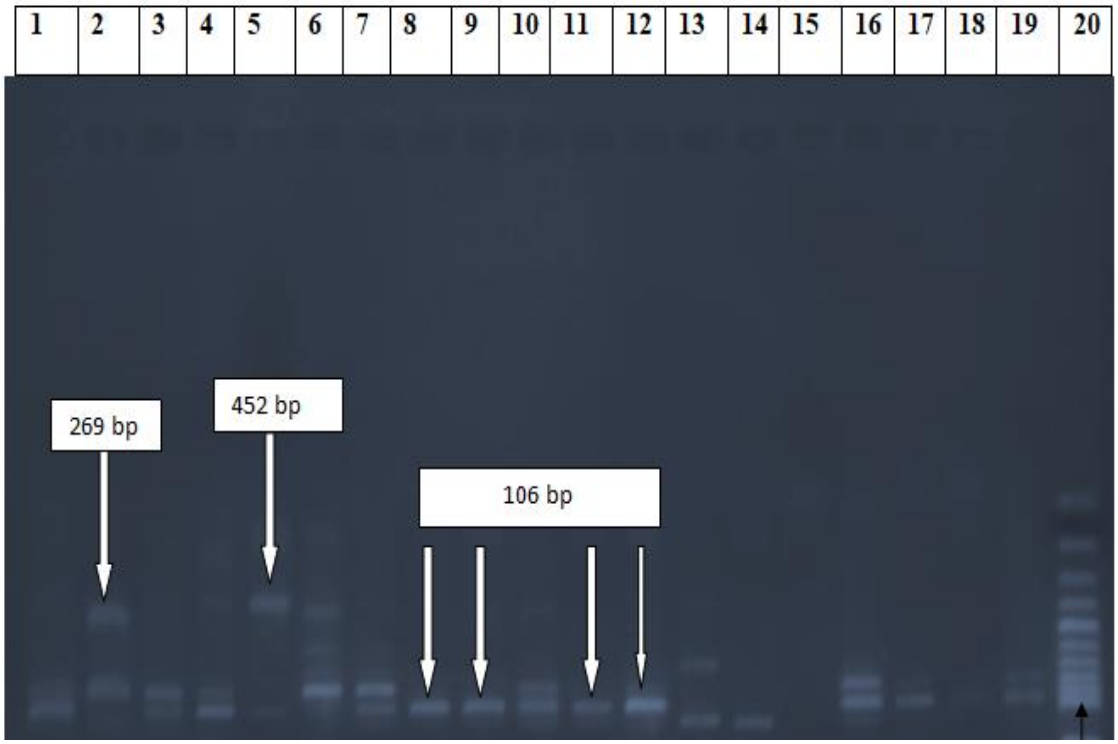
Lane 13 - 1.5 kbp molecular maker; Lane 12 - Negative control for *Cryptosporidium* spp; Lane 1-7 - Clinical samples positive for *Cryptosporidium* spp; Lane 8-11 - Clinical samples negative for *Cryptosporidium* spp

Fig 1: Gel electrophoresis picture of PCR amplification products of SSU rRNA gene



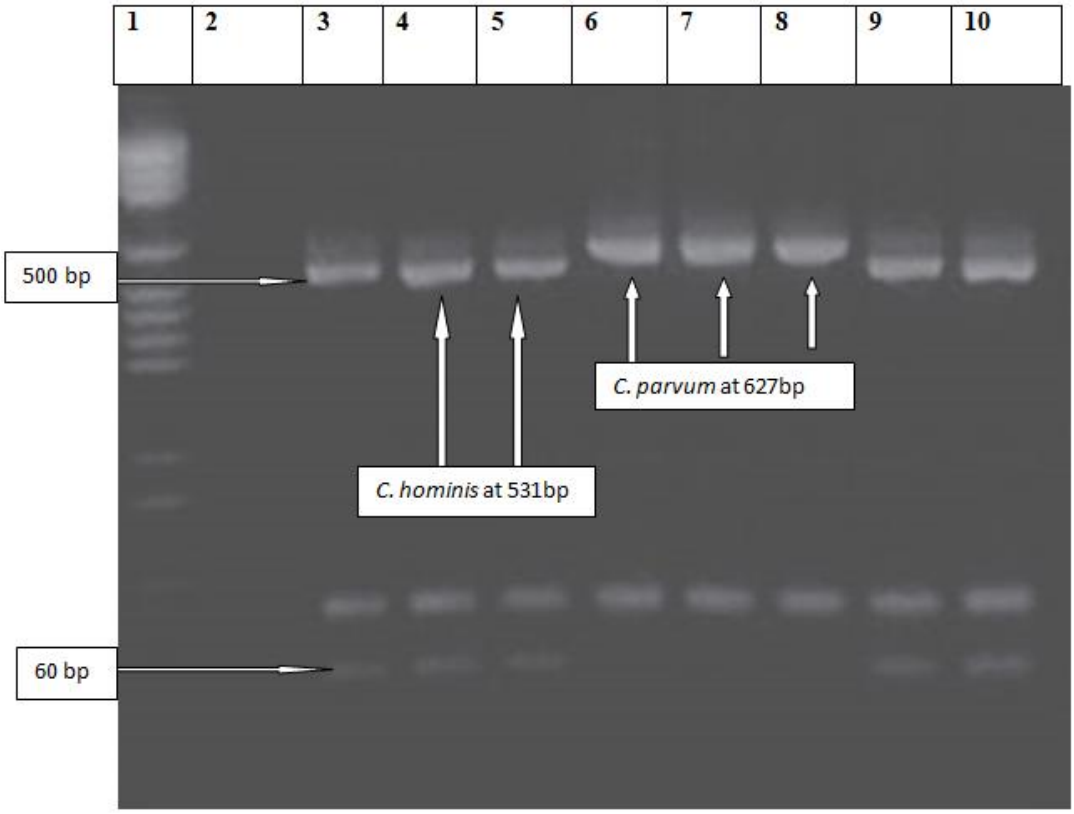
Lane 1 - molecular marker; Lane 2-20 - Positive samples between 826 – 837 bp.

Fig 2: Gel electrophoresis of *Cryptosporidium* 18 NA amplified products with the inner primer Cry18NA.



Lane 20 - Molecular marker at 1000 bp; Lanes 2 and 5 - *Cryptosporidium hominis* at 269 bp and 452 bp respectively; Lane 6 - *Cryptosporidium hominis* positive control; Lane 1 - *Cryptosporidium hominis* negative control; Lanes 3,7,8,9,10,11,12,16,17 - *Cryptosporidium parvum* positive (106 bp); Lane 19 - *Cryptosporidium parvum* positive control; Lane 18 - *Cryptosporidium parvum* negative control

Fig 3 PCR-RFLP analysis using *Sspl* and *Vspl* restriction enzymes for differentiating *Cryptosporidium* species.



Lane 1; MW, Lane 2; negative control, Lane 3; positive control for *C. hominis*(531 bp),Lane 6; positive control for *C. parvum* (627 bp), Lanes 4,5,9,10; *C. hominis* positive isolates, Lanes 7 & 8; *C. parvum* positive isolates.

Fig 4: Species identification of *Cryptosporidium* by RFLP analyses of 18S rRNA gene with *Vspl* restriction enzyme

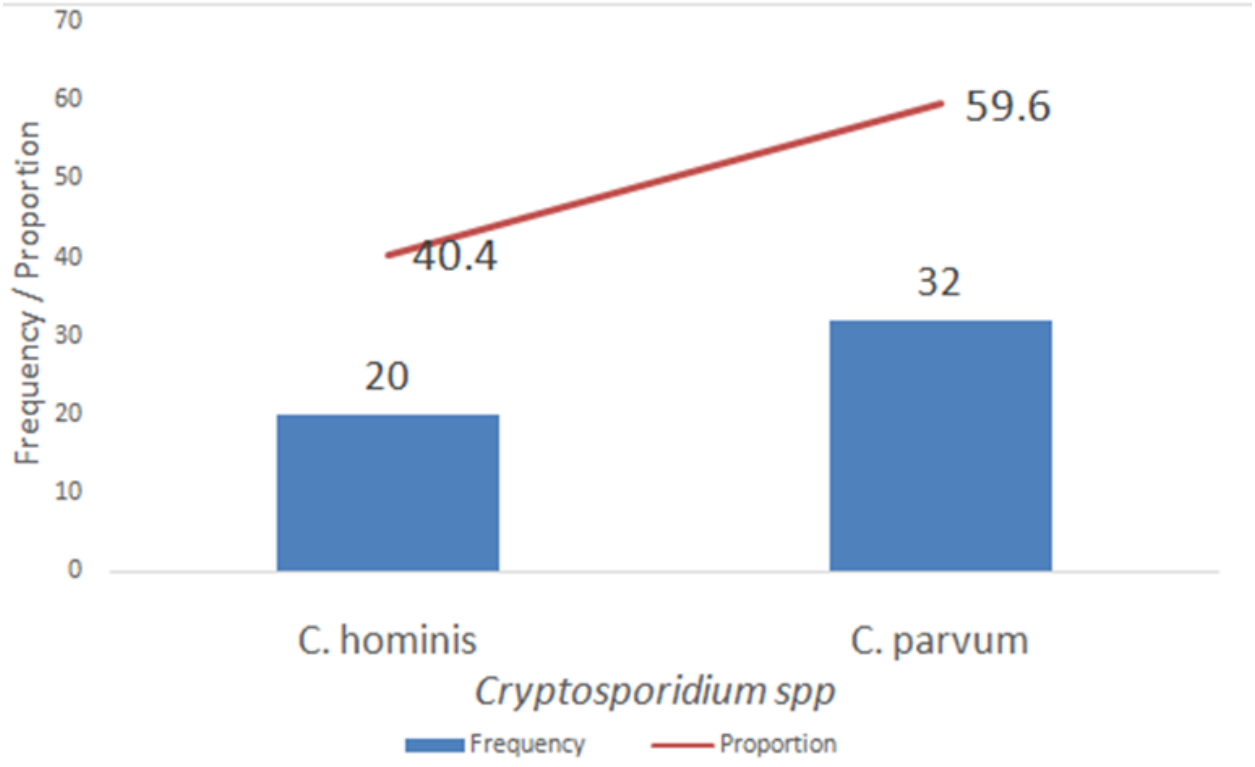


Fig 5 : Frequency of *Cryptosporidium* species identified among HIV-infected patients with diarrhea in Maiduguri

Discussion:

This study reported a prevalence of 19.6% for cryptosporidiosis amongst HIV-positive patients in Maiduguri. This prevalence is similar to earlier reported findings among HIV-infected patients in Michika, northeast Nigeria (9), andin a study done in Jos North central Nigeria, where the prevalence of cryptosporidiosis was 27% in HIV-infected patients (10). This study reported a higher prevalence compared to a study by Olopade et al., (11). This disparity could be a result of the nature of the stool sample used, as it has been shown that diarrhoeic samples yield more *Cryptosporidium* oocyst than non-diarrhoeic stool (12). Many of the participants in our study were residents of internally displaced persons (IDP) camps with poor water and sanitation hygiene, poor access to affordable and quality education, and inadequate healthcare services, among others. This setting promotes the feaco-oral transmission of infection, and may partly explain the high prevalence of cryptosporidium infection observed in the study. Immuno-suppressed individuals show a two-fold increase in prevalence rate when compared with immune-competent individuals (13).

The molecular assays revealed that *C. parvum* is the most common species found in HIV-infected patients in Maiduguri. This may be due to the fact that participants have a history of contact with animals and reside in overcrowded areas. This will explain both zoonotic and anthropogenic transmission. *Cryptosporidium parvum* was slightly more prevalent than *C. hominis* as the leading cause of human cryptosporidiosis in this study. Another reason could be the increased likelihood of animal-human contact among residents of the study area. Both domesticated animals are allowed to roam freely on the streets and animal dung is used as fertilizer during irrigation farming in the study area. This finding is in keeping with what was found in other parts of the world (14). Our study is in concordance with the results by Xin Yang et al., (15), and also with studies from other parts of Nigeria (7,16). The prevalence and distribution of this coccidian disease tend to vary from one locality to another and from one country to another based on the contamination rate of food, water, and contact with animals.

Furthermore, variations in sample size and sampling techniques play a pivotal role in the differences in results (7). Differences in the distribution of *Cryptosporidium* species are considered an indication of differences in the source of infection. The predominance of *C. parvum* in a population could result from both zoonotic and anthroponotic transmission, while *C. hominis* is considered to be a result of anthroponotic routes (17). Our study is limited by the fact that only two species of *Cryptosporidium* could be identified in the study because the restriction enzymes used could only detect *C. parvum* and *C. hominis.*

Conclusion:

Our study found a prevalence of 19.6% of cryptosporidiosis among HIV patients in Maiduguri with a predominance of *C. parvum.* There is need for health education of people at risk of cryptosporidiosis to interrupt transmission of this opportunistic parasite. Potable drinking water should be provided to the populace with particular emphasis on people with compromised immune status. Routine use of PCR for identification of *Cryptosporidium* in stool samples of HIV patients should be adopted.

Contributions of authors:

MUK, YMY, GBG and KBA contributed to writing the concept notes and formulating the study design, JA and ASB, MY, AUM and ZBS worked on data collection, data analysis and interpretation, laboratory analysis and interpretation. BBG, ABS, MD, ZG and all the authors contributed significantly during the manuscript drafting, revision and accepts responsibility for the integrity of the data and accuracy of analyses.

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No funding was received for the study

Conflict of interest:

No conflict of interest is declared.

Availability of data and material:

The datasets are available on reasonable request from the corresponding author

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