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Genotypic and Phylogenetic Profile of Cryptosporidium Parvum Strains from HIV Positive Patients in Maiduguri, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author *MYI designed the study, prepare, typed, and scrutinize the manuscript. Author UMA collected and analyzed the clinical specimens. Authors MRS, SYD and HT performed the molecular analysis authors IMT, ZMK, SMP and HSM scrutinized the manuscripts, organized the data and carried out the statistical analysis. Author ABS is the general overseer who supervised the entire work and led the research team. All authors read and approved the final manuscript.*

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ABSTRACT

Background: Cryptosporidium is an etiologic agent of gastro-intestinal disorders and severe, profuse watery diarrhoea in immunocompromised patients, such as people living with HIV/AIDS.
Aim: This study was carried out to determine the genotypic and phylogenetic characteristics of *Cryptosporidium parvum* among HIV patients who usually received antiretroviral therapy (ART) in four selected hospitals in Maiduguri Metropolis.
Study Design: This is a clinical laboratory study of opportunistic pathogens in HIV/AIDS Patients.
Place and Duration of Study: This was conducted at the Department of Medical Laboratory

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Science, the University of Maiduguri in collaboration with the Biological Sciences Department, ATBU Bauchi, Nigeria, between March 2021, and February 2022.

Methods: A total of 400 faecal specimens were collected from confirmed HIV-positive patients and screened for Cryptosporidium-specific antigen by Enzyme Link Immunosorbent Assay (ELISA). Genomic DNA Extraction was done by Quick-DNA^{TM} Faecal Microbe Miniprep with PCR amplification and sequencing of 18s SSU rRNA gene using specific reference primers.

Results: Out of the 70 HIV/AIDS and Cryptosporidium co-infected patients, 20 of the samples contained 18s SSU rRNA genes, with the highest frequency (45.0%) found in patients from the University Teaching Hospital. The results of phylogenetic analyses indicated that there is significant intra-species diversity in the genus Cryptosporidium. The four human *C. parvum* isolates differ from the bovine and the two avian isolates in three regions of the 18s rRNA gene.

Conclusion: The human genotype (genotype I) found in this study is exclusively human and in a single non-human primate, in bovine genotype (genotype II) has proved to be anthroponotic and zoonotic to the livestock. The study, therefore, advocates further genotyping of *Cryptosporidium* from both HIV-infected patients and immunocompetent hosts from various regions together with surveillance of animal and environmental reservoirs is highly recommended.

Keywords: Cryptosporidium; HIV/AIDS; 18s SSU rRNA; Zoonotic; Maiduguri.

1. INTRODUCTION

Cryptosporidium parvum is one of the causative agents of human cryptosporidiosis responsible for many outbreaks [1,2]. *Cryptosporidium hominis* infection may result in more severe conditions than *C. parvum* and other species found in animals [3,4]. In healthy people, the parasite is confined to the distal ileum and proximal part of the large intestine but colonizes the entire gut, biliary and respiratory tracts in immunocompromised individuals. Low innate, humoral, or cellular immunity in a patient infected with *Cryptosporidium* can lead to chronic or prolonged illness. The life-threatening potential of Cryptosporidiosis in immunodeficient and immunosuppressed persons has increased the significance of the disease as a public health problem in the world [5].

Many studies tried to portray the genomic properties of *Cryptosporidium* prior to the advent of sequencing technology. Analysis of *Cryptosporidium* karyotypes shows that it contains eight chromosomes, with a size of 0.945 to 2.2 Mb, equivalent to a total haploid genome size of10 Mb approximately [6-8]. Furthermore, *C. parvum* was found to contain two small extra-chromosomal cytoplasmic viruses-like double-stranded RNAs (1,786 and 1,374 nucleotides, respectively) [9]. The RNAs have a single open reading frame each, which encodes a putative RNA-dependent RNA polymerase and a protein with limited homology to mammalian protein kinases, respectively [10] investigated the *C. parvum* rRNA gene organization and revealed that the small and large rRNA subunits are 1.7 and 3.6 kb, respectively, plus a 151-bp putative 5.8S rRNA. It was also found that *Cryptosporidium* harboured some mitochondrial biosynthesis genes [11] and, unlike other apicomplexans, an apicoplast is absent [12].

As a parasite of public health importance, *Cryptosporidium* was part of the genome-sequencing analysis done earlier. Two reference strains served as genome representatives: *C. parvum* Iowa and *C. hominis* TU502. The genome sequences revealed similar genome sizes of 9.11 and 9.16 Mb, respectively, with 3 to 5% sequence divergence [13,14]. In addition, the genome sequencing and assembly of *C. muris* strain RN66, a less relevant *Cryptosporidium* species from a public health perspective, are essentially complete [15]. Genome analysis showed extremely streamlined metabolic pathways and absence of many cellular structures compared to other apicomplexans [16,17]. Genome sequences showed that *Cryptosporidium* species have genes associated with apical complex organelles but showed that they lack an apicoplast and possess only a degenerate mitochondrion that has lost its genome [13,15]. The existence of a relict mitochondrion was subsequently confirmed by ultramicroscopy [18,19].

A comprehensive genome database, Crypto DB, serves as a public interface for *Cryptosporidium* genome sequences [20]. This website offers access to sophisticated tools which enable the identification of genes based on text, sequence similarity, and motif queries [21]. The sequencing of *Cryptosporidium* genomes has revealed more

information, contributing to a better knowledge of microbiology, pathogenicity, evolution, and virulence. The quest for the molecular basis of virulence has exploited these genomic data to search for genes that may ultimately unravel the regulation of virulence, host range, and transmissibility at the genetic level [22]. Several comparative genomics studies have been carried out since the completion of genome sequences from apicomplexan parasites of medical and veterinary significance. Abrahamsen et al. [14] showed that *Cryptosporidium* spp. and *Plasmodium* spp. share over 150 ancestral apicomplexan proteins, involved mainly in interactions with eukaryotic host cells and the biogenesis of the apical complex [23].

Gordon and Sibley [24] used genome sequences of *Toxoplasma gondii*, *Plasmodium* spp., *Cryptosporidium* spp., and *Theileria* spp. to show the conservation of actin-like proteins among these parasites, which rely on actin-based motility for cell invasion, while comparative genomics of *Plasmodium* spp., *Cryptosporidium* spp., and *Toxoplasma gondii* revealed that calcium-regulated proteins (plant-like pathways for calcium release channels and calcium-dependent kinases) were also conserved [25]. In addition to conserved genes, comparative genomics can identify unique, novel, and uncharacterized virulence genes. Kuo et al. [26] compared the genome sequences of three apicomplexan parasites (*Plasmodium*, *Theileria* and *Cryptosporidium*) and showed that as many as 45% of the *Cryptosporidium* genes could be considered genus-specific.

The comparative genomics study by Kuo et al. [26]; however, identified 334 putative *C. hominis*-specific genes and 178 putative *C. parvum*-specific genes by interrogations of the *Cryptosporidium* database into which the gene sequences had been placed. A similar *in Silico analysis* that compared the genome sequences of *C. hominis* and *C. parvum*, aiming to uncover genetic loci responsible for host preference, showed 93 and 211 putative genes specific for *C. hominis* and *C. parvum*, respectively [27]. However, when tested experimentally, the majority of the genes were found to be present in both species with slight interspecies and inter-subtype sequence variability.

Nevertheless, PCR results showed experimental evidence for one *C. hominis*-specific and *C. parvum*-specific genes [27]. A subsequent study by Widmer [28] which investigated the genetic basis of *Cryptosporidium* host specificity used genome-wide comparisons of *C. parvum* zoonotic, *C. parvum* anthroponotic (IIc subtype), and *C. hominis* isolates. They reported that for some genetic loci, there was actually more sequence similarity between *C. parvum* anthroponotic and *C. hominis* strains than there was between *C. parvum* anthroponotic and *C. parvum* zoonotic strains. A proteomic analysis of *C. parvum* proteins expressed during excystation showed overexpression of three apicomplexan-specific and five *Cryptosporidium*-specific proteins [29]. A recent *C. parvum* genome analysis showed that several protein kinases of *Cryptosporidium* are distinct from those of other apicomplexans (*P. falciparum* and *T. gondii*) Artz et al. [30].

Many HIV/AIDs patients suffer from profuse diarrhoea due to Cryptosporidiosis, leading to severe resulting in a severe ill-health conditions with fatal outcomes. Cryptosporidiosis has consistently been a significant opportunistic infection among HIV-positive patients in developing countries like Nigeria [31]. Although genotypic analysis of *Cryptosporidium* has been performed from clinical stool specimens of HIV-infected persons in different parts of the world [1] with little information on this area where HIV/AIDS is still endemic, hence the need for this study.

Genotyping of *Cryptosporidium* has been mainly studied in HIV patients as one of the AIDS-defining diseases/opportunisticinfectionsn. The disease diagnosis is usually performed by using the Rapid immunochromatographic assay/RDT in addition to stool microscopy. Therefore, the present study aimed to perform the molecular characterization and phylogenetic studies of C*ryptosporidium parvum* among HIV/AIDS patients attending some Hospitals in Maiduguri, Borno State, Nigeria.

2. MATERIALS AND METHODS

2.1 Study Design

The study is a cross-sectional hospital-based study involving HIV patients in some Hospitals within the Maiduguri metropolis.

2.2 Inclusion and Exclusion Criteria

All HIV Seropositive in and outpatients attending the selected hospitals' antiretroviral therapy clinics, during the period of this study are

included while all HIV patients that refuse to be part of the study were excluded.

2.3 Sample Collection

A total of 400 Stool samples were collected from confirmed HIV-positive patients attending Mamman Shuwa Memorial Hospital, State Specialist Hospital, Umaru Shehu Ultra-modern Hospital, and University of Maiduguri Teaching Hospital. The participants were given a consent form attached to a questionnaire, the specimens were labeled appropriately and registered with the patient's study number. On arrival to the laboratory, safety precautions were observed throughout the period of processing the specimens as described by Asmita et al. [1].

2.4 Antigenic Detection of Cryptosporidium

Cryptosporidium-specific antigen present in the stool specimens was detected by Enzyme Link Immunosorbent Assay (ELISA) [27].

2.5 Genomic DNA Extraction by Quick-DNA™ Faecal Microbe Miniprep

For optimal performance, beta-mercaptoethanol was added to the Genomic Lysis Buffer to a final dilution of 0.5% (v/v) 2.5 ml per 500 ml. 2.5 grams (5 g max.) of the positive faecal samples was added to the bead/filter chamber of a ZR BashingBead™ Lysis/Filtration Tube. A 6 ml BashingBead™ Buffer was added to the sample and processed [27].

The Filtration Tube was centrifuged at ≥3,000 x g (5,000 x g max.) for 5 minutes using an Eppendorf high-speed centrifuge. The bead/filter chamber was removed from the top of the tube and transferred supernatant from the bottom to a clean 50 ml tube. An 18 ml Genomic Lysis Buffer was added to the supernatant (~3:1) and vortex mix.

The entire mixture was filtered from the previous step using a Zymo-Spin™ V-E Column/Zymo-Midi Filter™ assembly mounted on a vacuum manifold with a vacuum source set at ≥600 mm Hg. the Zymo-Spin™ V-E Column/Zymo-Midi Filter™ assembly was disconnected. Zymo-Spin™ V-E Column was transferred to a Collection Tube and Spined at 10,000 x g for 1 minute in a microcentrifuge, 300 µl DNA Pre-Wash Buffer was added to the column and spun at 10,000 x g for 1 minute, the flow-through was discarded, 400 µl gDNA Wash Buffer was added to the column and centrifuged at 10,000 x g for 1 minute. The flow-through was discarded and the wash step was repeated [27].

Zymo-Spin™ V-E Column was transferred to a 1.5 ml microcentrifuge tube and 150 µl DNA Elution Buffer was added directly to the column matrix5. It was kept for 1 minute and then centrifuged at 10,000 xg for 1 minute to elute the DNA. A Zymo-Spin™ III-HRC Filter was placed in a clean Collection Tube and a 600 µl Prep Solution was added. It was centrifuged at 8,000 x g for 3 minutes. The eluted DNA was transferred to a prepared micro-centrifuge tube and was centrifuged at 16,000 x g for 3 minutes. The filtered DNA was then used for PCR and sequencing.

2.6 Quantification/Purification of DNA

Extracted DNA was quantified using a NanoDrop™ (2000C, Thermo scientific spectrophotometer, CA, USA) where 10µl of the extracted DNA was placed on the tip of the NanoDrop machine and the lid was closed to read and the quantity of the DNA in the extracted samples was measured, where all the samples fall within the normal range (1.8-2.0ng).

2.7 PCR Amplification of 18s SSU rRNA Gene

The primer was reconstituted with a master mix and the Extracted DNA was pooled and amplified in a thermocycler (Eppendorf-Master Cycler Nexus X_2, Hamburg, Germany) to determine the optimum temperature which was denaturation at 94^0C for 3minute, 95^0C for 30sec, annealing at 47^0C for 45sec, then extension at 68^0C for 45sec, the cycle was continued for 35 cycles, at 68^0C to obtain a base pair corresponding to the primers for the Small Sub-Unit Ribosomal RNA (SSU rRNA) gene in cryptosporidium. The annealing temperature for the Nucleic Acid Test was determined and it was run at Denaturation 94^0C for 3minute, 95^0C 30sec, annealing at 47^0C 45sec, then extension 68^0C for 45sec, for 35 circles, 68^0C for 5minute. And the product of the PCR was subjected to electrophoresis in 0.8% agarose gel and visualized by a trans-illuminator.

Chart 1. Primers used in the amplification of 18s rRNA gene in this study

Primer	Sequence	Size (bp)	Reference
AL3531	5'ATAGTCTCCGCTGTATTC 3'	915	Okojokwu et al. [32]
AL3535	5' GGAAGGAACGATGTATCT 3'	915	Okojokwu et al. [32]
AL3532	5'TCCGCTGTATTCTCAGCC 3'	850	Okojokwu et al. [32]
AL3534	5´-GCAGAGGAACCAG CATC-3	850	Okojokwu et al. [32]

2.8Agarose Gel Electrophoresis

A 0.8% agar rose gel was prepared in which ethidium bromide was added in the preparation, it was gently poured inside the electrophoresis tank and solidify, a comb was used to make wells equal to the number of amplicons, and a Tris buffer was flooded inside the tank containing the gel, the amplicons were then gently dropped inside the wells created in the gel respectively, the lid of the electrophoresis machine was closed tightly and it was allowed to run for 30 minutes [32].

2.9 Determination of Cryptosporidium 18s SSU rRNA Gene Sequence

"The amplicon was sequenced using the procedures of a commercial kit (QIAquick® Gel Extraction kit, Qiagen, Hilden, Germany). Direct DNA sequencing of the gel-purified PCR product was sequenced and aligned with each other and with previously reported sequences in the NCBI database" [33].

3. RESULTS AND DISCUSSION

3.1 HIV/AIDS and Cryptosporidium Co-infection in the Hospitals

This study was carried out in four selected hospitals where HIV patients usually received antiretroviral (ART) drugs. Out of the 70 HIV/AIDS and Cryptosporidium co-infected patients, 20 of the samples harboured the 18s SSU rRNA genes, with the highest frequency (45.0%) found in samples from the University of Maiduguri Teaching Hospital (UMTH).

The distribution of patients infected with the parasites in the four selected hospitals is statistically significant (χ^2 = 0.045) USUHM lead the group with 37.1%, followed by UMTH at 31.4%, SSHM at 18.6 and MSMHM at 12.9%. This could be due to the location of the hospital close the vicinity of IDP camps where so many low-income earners live and visit the hospital for their routine antiretroviral drug collection and health care delivery. Cryptosporidium is an important protozoan parasite affecting HIV/AIDS patients. Although cryptosporidiosis occurs in both immunocompetent hosts and immunocompromised patients, the infections are more prevalent and clinically severe in the latter [34]. In this study, out of the seventy HIV and Cryptosporidiosis co-infected patients 20(28.6%) of the samples harboured the 18s SSU rDNA genes 7,9,25,37,42,44,46,48,49,53,56,58,60, 80, 83,87,88, 89,92,94 correspond with the reference amplicon size of 915bp. The prevalence rate in this study is less than the findings of studies carried out in Kano by Yunusa and Olusanyi [31] with an infection rate of 31.9% and higher than the study in south-south Nigeria with 2.9% [35] and higher in North West Nigeria with 25% respectively [36].

In another study in Jos, a report by Pam et al. [37] revealed a high prevalence rate of 83.3% in contrast with the present study, and higher than the report of Vyas et al. [38]. However, this variation may be due to the fact that the prevalence of *C. parvum* varies remarkably among regions of the world as well as among communities depending on the level of contamination of piped and drinking water with human and animal excreta (Okojokwu et al. [32]. The frequency of Cryptosporidiosis in a report by Nwokediuko et al. [39] in Enugu, North-Eastern Nigeria was 5.4% (n = 13). All 13 cases of *Cryptosporidium* spp had the stage of HIV/AIDS infection.

Detection of *Cryptosporidium* precisely has been a controversial issue as different isolates within the same species may possess overlapping features of host range, oocyst morphology and predilection site of infection [40]. "Genetic analysis of the 18S rDNA sequences by some workers has revealed the multispecies nature of the genus *Cryptosporidium"* [33]. "At least 10 species of *Cryptosporidium* (*C. parvum*, *C. muris*, *C. wrairi*, *C. felis*, *C. meleagridis*, *C. baileyi*, *C. serpentis*, *C. andersoni*, *C. saurophilum* and *C. nasorum*) have been considered to be valid based on morphological, biological and molecular evidence" [41].

Table 1. Distribution of 18s SSU rRNA genes in HIV/AIDS and Cryptosporidiosis co-infected patients

Hospitals	No. (%) of HIV patients with Cryptosporidiosis (n=70)	No. (%) of Stool samples with 18s SSU rRNA genes(n=20)
MSMHM	09 (12.9)	06 (30.0)
SSHM	13 (18.6)	04 (20.0)
UMTH	22 (31.4)	09 (45.0)
USUHM	26 (37.1)	01 (5.0)

Keys: MSMHM: Mamman Shuwa Memorial Hospital Maiduguri, SSHM: State Specialist Hospital Maiduguri, UMTH: University of Maiduguri Teaching Hospital, USUHM: Umaru Shehu Ultramodern Hospital Maiduguri

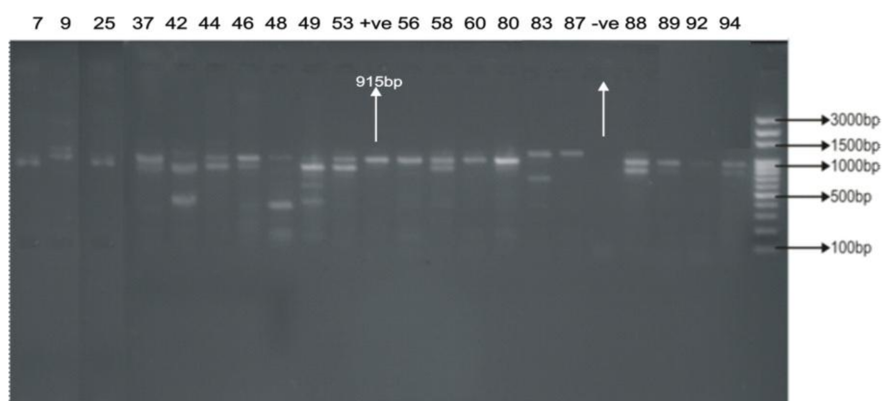

Fig. 1. Gel image of positive amplicons lane 7-94 show a band that amplified the 18s rRNA gene with a100bp ladder. Positive control (genomic DNA from ATCC sample) with 915bp and negative control (containing all PCR reagents but no target DNA added)

Table 2. Gene Sequence characteristics of the isolates of *Cryptosporidium parvum* in this study

Isolates	Species	Host	Location	Length of SSU rDNA (bp)
HN9	*C. parvum*	Human	Nigeria	915
HN46	*C. parvum*	Human	Nigeria	915
HN48	*C. parvum*	Human	Nigeria	915
HN44	*C. parvum*	Human	Nigeria	915
BN53	*C. parvum*	Bovine	Nigeria	915
AN56	*C. parvum*	Avian	Nigeria	915
AN87	*C. parvum*	Avian	Nigeria	915

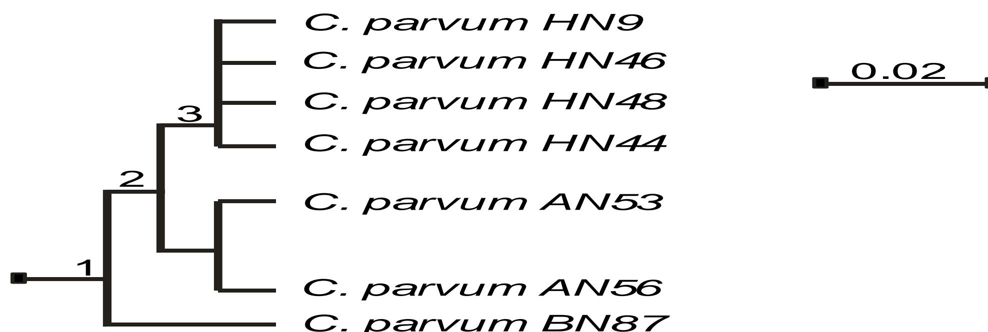

Fig. 2. Phylogenetic tree of the amplicons of Cryptosporidium parvum A book trap of 0.02 with 3 clades representing sub-genotypes at a node 1,2, and 3 coming from their common ancestor showing 2 genotypes

In the present study, the results of phylogenetic analyses also indicated that there is significant intra-species diversity in the genus Cryptosporidium. The four human *C. parvum* isolates differ from the bovine and the two avian isolates in three regions of the 18s rRNA gene. With the accession number MT08474 and the query number 36347. This differentiates between the two genotypes of *C. parvum* as reported by [42]. "The analysis in our study revealed that *Cryptosporidium* detected from HIV/AIDs patients in Nigeria comprise heterogeneous species. The human genotype (genotype I) which was observed in this study is exclusively human and in a single non-human primate, in bovine genotype (genotype II) which was also found in this study has proved to be anthroponotic and zoonotic to the livestock".

"Early studies of the polymorphism of isolates classified as *C. parvum* found significant geographic variation among isolates [43] in the region encoding for the SSU-rRNA, commonly used for taxonomic classification". "It was found that one of the sequences used in this study [40] was wrongly identified as a *C. parvum* sequence, but it was actually *C. muris"*. More recent work [10] and GenBank entry AF040725) has shown that the SSU-rRNA region of the *C. parvum* zoonotic (bovine) genotype shows no heterogeneity and is practically similar to the sequence submitted to GenBank in 1993 (accession number L16996.

Pieniazek et al. [44] use "DNA sequencing and phylogenetic studies to identify four distinct *Cryptosporidium* genotypes in HIV-positive patients: genotype 1 (human), genotype 2 (bovine) *Cryptosporidium purvum*, a genotype identical to *C. felis*, and one identical to a *Cryptosporidium species* from a dog. This was the first identification of human infected cases involving the last two genotypes" [45].

"Cryptosporidium isolates from diarrhoeal stool specimens of HIV/AIDS patients in Thailand were analyzed by sequencing the variable region in the 18S rRNA gene. Four *Cryptosporidium* species were identified, i.e. *C. parvum* (genotype 1)*, C. meleagridis, C. muris* and *C. felis* occurring. Based on small number of isolates screened, only *C. meleagridis* and *C. muris* were found in HIV-positive children, but the genotype 1 of *C. parvum* predominated in adult HIV patients" [34].

In a study by Jin-Chan et al. [46] in Henan, "the functional mitochondrial protein alternative oxidase (AOX) gene was used as a marker to analyze the phylogenetic relationship between *Cryptosporidium* isolates". This gene was profiled, and the phylogenetic tree was constructed and compared to those generated from 18S rRNA. The results revealed that the genus *Cryptosporidium* contained the phylogenetically different species *C. parvum*, *C. hominis*, *C. suis* and *C. baileyi*, which were similar to the molecular characterization and host specificity reported previously [32]. "*Cryptosporidium* species formed two clades: one included *C. hominis*, *C. suis*, *C. parvum* cattle genotypes, and *C. parvum* mouse genotype" [47,48]; and "the other comprised *C. meleagridis* and *C. baileyi* isolates [48, 49]. These results suggest that the AOX gene is not only equally suitable for the phylogenetic studies of *Cryptosporidium* but also clarifies and revealed new approach to elucidate *Cryptosporidium's* genetic origin" [46, 50, 51].

4. CONCLUSION

This study found that using the sequence of a diagnostic fragment of SSU-rDNA, a well-established genotypes of *C. parvum* was detected as human, bovine, and avian host *Cryptosporidium* genotypes among HIV/AIDS patients in Maiduguri, Nigeria. The stool samples 20 (28.6%) of HIV and Cryptosporidiosis co-infected harboured the genes, which are of zoonotic origins. Therefore, further genotyping of *Cryptosporidium* from both HIV-infected patients and immunocompetent hosts from diverse geographical origins together with surveillance of animal and environmental reservoirs is highly recommended.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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