GENOTYPIC CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF CRYPTOSPORIDIUM PARVUM STRAINS FROM HIV POSITIVE PATIENTS IN MAIDUGURI, NIGERIA

ABSTRACT

Background: Cryptosporidium is a common cause of gastroenteritis 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 clinico-laboratory study of opportunistic pathogens in HIV/AIDS Patients.

Place and Duration of Study: This was conducted at the University of Maiduguri Teaching Hospital in collaboration with the Biological Sciences Department, ATBU Bauchi, Nigeria, between March 2012 and February, 2022.

Methods: A total of 400 Stool samples 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™ 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 of Maiduguri Teaching Hospital (UMTH). 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 molecular analysis of *Cryptosporidium* isolates 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,

INTRODUCTION

Human cryptosporidiosis is mainly caused by *Cryptosporidium hominis* and *C. parvum*, which are responsible for most of the outbreaks of Cryptosporidiosis [1]. Other less common species are *C. meleagridis*, *C. cuniculus*, *C. viatorum*, *C. muris*, *C. canis*, *C. felis*, *C. suis*, and *C. Andersoni* [2,3]. *C. Hominis* may cause more severe infection than *C. parvum* and other zoonotic species [4]. In contrast, in a study from India, HIV-positive patients, infected with *C. Parvum* and other zoonotic species tend to have fever more regularly than those infected with *C. Hominis* [4]. Thus, this disparity in clinical manifestations might be due to the different *Cryptosporidium* species. In immunocompetent individuals, the parasite is localized in the distal small intestine and proximal colon, but occurs throughout the gut, biliary and respiratory tracts in immunocompromised hosts. Deficiencies in innate, humoral or cellular immunity in a patient infected with *Cryptosporidium* results in a severe or prolonged illness. The life intimidating

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potential of *Cryptosporidium parvum* in immunocompromised and immunosuppressed persons has increased the importance of cryptosporidiosis as a worldwide public health problem [5].

Several studies tried to reveal the characteristics of the *Cryptosporidium* genome ~~prior to~~ the sequencing era Karyotypic analyses suggested that *Cryptosporidium* contains eight chromosomes, ranging in size from 0.945 to 2.2 Mb, giving a total haploid genome size of approximately 10 Mb [6,7]. In addition, *C. parvum* was shown to have two small extra-chromosomal cytoplasmic viruses-like double-stranded RNAs (1,786 and 1,374 nucleotides, respectively) [8]. 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 reported 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 demonstrated that *Cryptosporidium* does retain some mitochondrial biosynthesis genes [11] but that, unlike other apicomplexans, it lacks an apicoplast [12].

As a pathogen of public health relevance, *Cryptosporidium* was included in early genome-sequencing projects. Two reference strains served as genome representatives: *C. parvum* Iowa and *C. hominis* TU502. The genome sequences showed 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 revealed extremely streamlined metabolic pathways and a lack of many cellular structures and metabolic pathways found in other apicomplexans [16].

Energy metabolisms largely from glycolysis and both aerobic and anaerobic metabolisms are available, thus conferring environmental flexibility [17]. Limited biosynthetic capabilities and minimal metabolism have been reported, suggesting a large dependence on nutrient acquisition from the host [18]. An extensive array of transporters was discovered, which enable the import of essential nutrients from the host [15]. Genome sequences showed that *Cryptosporidium* species have genes associated with apical complex organelles but suggested 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 ultrastructural studies [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 a vast amount of information, contributing to a better knowledge of microbial biology, 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 performed since the completion of genome sequences from apicomplexan parasites of medical and veterinary importance. 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

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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. Although the predicted gene counts were not identical, the papers reporting the genomes of *C. parvum* (3,952 genes) and *C. hominis* (3,994 genes) found no evidence for unique genes between the species, noting that what variation appeared to be present reflected primarily gaps in one genome or the other and speculating that the phenotypic differences likely arose from polymorphisms in coding regions and differences in gene regulation [26].

The comparative genomics study by Kuo *et al*. [26]; however, identified 334putative *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 albeit 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 also aimed to investigate the genetic basis of *Cryptosporidium* host specificity used genome-wide comparisons of *C. parvum* zoonotic, *C. parvum* anthroponotic (IIc subtype), and *C. hominis* isolates. Those authors 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-infected patients suffer from profuse diarrhoea due to *Cryptosporidium* infections, resulting in a significant decrease in quality of life or death. Cryptosporidiosis has consistently been an important opportunistic infection among HIV-infected 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 mainly been studied in patients with HIV infection. HIV infection diagnosis is usually carried out by applying the ELISA method to detect antibody and, and western blot analysis recording the patient's status during chronic incubation is done through counting CD4 in peripheral blood samples and the rate of CD4/CD8 number. 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.

MATERIALS AND METHODS

Study design

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

Inclusion and exclusion Criteria

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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 were disconsent to be part of the study were excluded.

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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, and the samples were transported to the laboratory. On arrival, safety precautions were observed throughout the period of processing the specimens as described by Asmita *et al.* [1].

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Antigenic Detection of Cryptosporidium

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

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 ~~process~~ [27].

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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 Zymo-Spin™ III-HRC Filter in a clean 1.5 ml micro-centrifuge tube and was centrifuged at 16,000 x g for 3 minutes. The filtered DNA was then used for PCR and sequencing.

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).

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,

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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]

Agarose 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 [33].

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].

RESULTS AND DISCUSSION

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).

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)

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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.

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 prevalence of *Cryptosporidium* spp 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.

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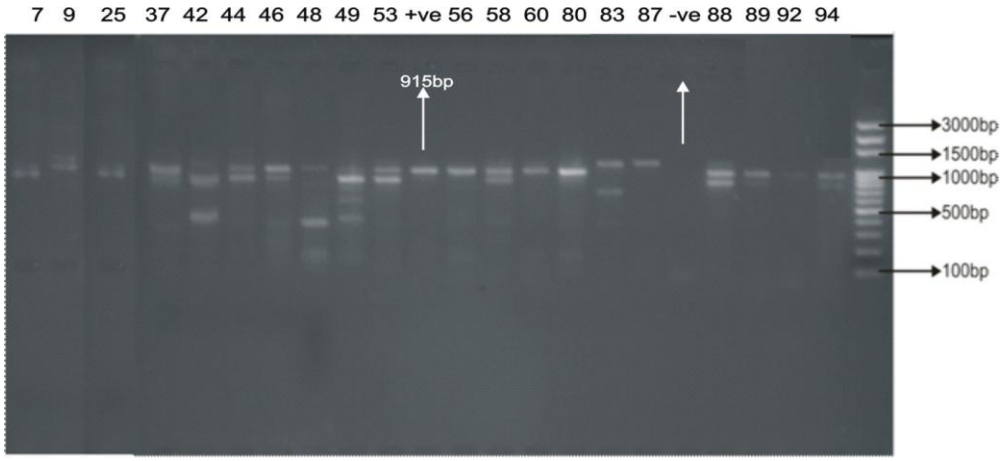

Figure 1: Gel image of positive amplicons lane 7-94 show a band that amplified the 18s rRNA gene with a100bp ladder. Positive control (genomic DNA from ATCC sample) with 915bp and negative control (containing all PCR reagents but no target DNA added).

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

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Identification of *Cryptosporidium* species 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].

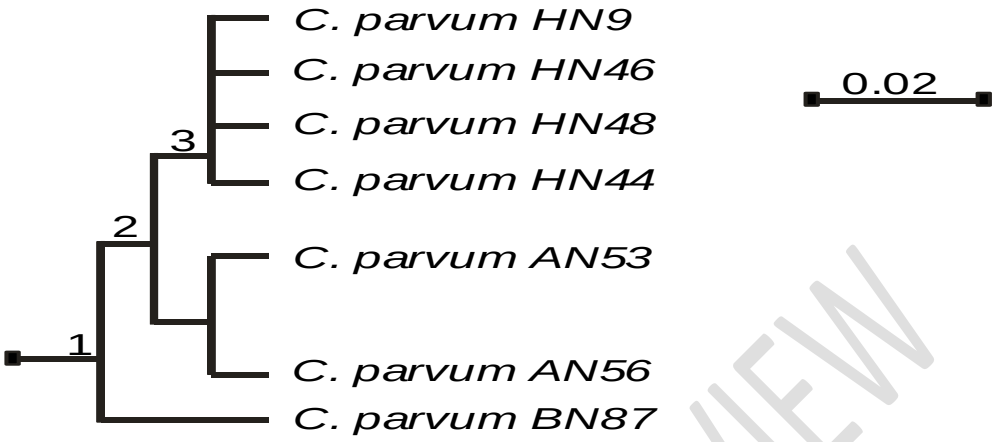

Figure 2: Phylogenetic tree of the amplicons of Cryptosporidium parvum
A book trap of 0.02 with 3 clades representing sub-genotypes at a node 1,2, and 3 coming from their common ancestor showing 2 genotypes

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* isolates from HIV-infected patients in Nigeria comprise heterogeneous species. The human genotype (genotype I) which has so far been found in this study is exclusively human and in a single non-human primate, in bovine genotype (genotype II) which has also been 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 coding for the small subunit ribosomal RNA (SSU-rRNA), commonly used for taxonomic classification. Recently, it has been shown that one of the sequences used in this analysis [40] was erroneously identified as a *C. parvum* sequence, while in fact, it was *C. muris*. More recent work [10] and GenBank entry AF040725) has shown that the SSU-rRNA region of the *C. parvum* zoonotic (bovine) genotype does not show heterogeneity and is practically identical to the sequence submitted to GenBank in 1993 (accession number L16996,

Pieniazek *et al*. [44] use DNA sequencing and phylogenetic analysis to identify four distinct *Cryptosporidium* genotypes in HIV-infected patients: genotype 1 (human), genotype 2 (bovine) *Cryptosporidium parvum*, a genotype identical to *C. felis*, and one identical to a *Cryptosporidium* sp. isolate from a dog. This was the first identification of human infection with the latter two genotypes [40].

Cryptosporidium isolates from diarrhoeal stools of the human immunodeficiency virus (HIV)-infected patients in Thailand were genetically 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 a limited number of isolates analyzed, only *C. meleagridis* and *C. muris* were found in HIV-infected children, whereas the genotype 1 of *C. parvum* predominated in HIV-infected adults [34].

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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 characterized, and the phylogenetic tree was established from *Cryptosporidium* isolates and compared to those generated from 18S rRNA. The results revealed that the genus *Cryptosporidium* contained the phylogenetically distinct species *C. parvum*, *C. hominis*, *C. suis* and *C. baileyi*, which were consistent with the biological characterization and host specificity reported earlier [32]. *Cryptosporidium* species formed two clades: one included *C. hominis*, *C. suis*, *C. parvum* cattle genotypes and *C. parvum* mouse genotype; and the other comprised *C. meleagridis* and *C. baileyi* isolates. These results suggest that the AOX gene is not only equally suitable for the phylogenetic analysis of *Cryptosporidium* but also provides an outstanding and new approach to determining *Cryptosporidium* genetic origin [46].

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 molecular analysis of *Cryptosporidium* isolates from both HIV-infected patients and immunocompetent hosts from diverse geographical origins together with surveillance of animal and environmental reservoirs is highly recommended.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

REFERENCES

1. Asmita D, Ujjala G, Vikas A, Uday G, (2016). Genotyping of *Cryptosporidium* Species and Their Clinical Manifestations in Patients with Renal Transplantation and Human Immunodeficiency Virus Infection, J. Pathog. Article ID 2623602, 9 pages.
2. Cama VA, Bern C, Roberts J, Cabrera L, Sterling CR, Ortega Y. (2008) *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru. Emerg. Infec. Dis., 14:1567.
3. Elwin K, Hadfield SJ, Robinson G, Chalmers RM. (2012) The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000-2008. Epidem. Infec.140; 673– 683.
4. Ajjampur SS. Sankaran P, Kang G (2008). *Cryptosporidium* species in HIV-infected individuals in India. Nat. Med. J. India, 21(4): 178 – 84.
5. Moghaddam AA. (2007). Symptomatic and asymptomatic cryptosporidiosis in young children in Iran, Pakistan J. Bio. Sci. 10(7):1108 – 1112.
6. Blunt D, Khramtsov NV, Upton SJ, Montelone BA. (1997) Molecular karyotype analysis of *Cryptosporidium parvum*: evidence for eight chromosomes and a low-molecular-size molecule. Clin. Diag. Lab. Immunol. 4:11–13.

7. Hays M, Mosier DA, Oberst RD. (1995) Enhanced karyotype resolution of *Cryptosporidium parvum* by contour-clamped homogeneous electric fields. Vet. Parasitol. 58:273–280.
8. Khramtsov NV, Tilley M, Blunt DS, Montelone BA, Upton SJ. (1995) Cloning and analysis of a *Cryptosporidium parvum* gene encoding a protein with homology to cytoplasmic form Hsp70 J. Eukaryotic Microbiol. 42:416–422.
9. Clark D (1999) new insights into human cryptosporidiosis. Clin Microbiol. Rev. 12:554 –563.
10. Le Blancq SM, Khramtsov NV, Zamani F, Upton SJ, Wu TW. (1997) Ribosomal RNA gene organization in *Cryptosporidium parvum.* Mol Biochem Parasitol. 90:463–78.
11. LaGier M, Tachezy J, Stejskal F, Kutisova K, Keithly JS (2003) Mitochondrial- type iron-sulfur cluster biosynthesis genes (IscS and IscU) inthe apicomplexan *Cryptosporidium parvum*. Microbiology 149:3519–3530.
12. Zhu G, LaGier MJ. Stejskal F, Millership JJ, Cai X, Keithly JS. (2002). *Cryptosporidium parvum*: the first protist known to encode a putative polyketide synthase. Gene 298:79–89.
13. Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM (2004). The genome of *Cryptosporidium hominis*. Nature 431:1107–1112.
14. Abrahamsen M, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA. *et al* (2004). Complete genome sequence of the apicomplexan, *Cryptosporidium parvum.* Science 304:441– 445.
15. Widmer G, Tzipori S, Fichtenbaum CJ, Griffiths JK. (2007) Genotypic and phenotypic characterization of *Cryptosporidium parvum* isolates from people with AIDS, J. Infec. Dis, 178, 834-40.
16. Wanyiri J, Ward H, (2006) Molecular basis of Cryptosporidium-host cell interactions: recent advances and future prospects. Future Microbiol. 1:201–208.
17. Barta JR, Thompson RC (2006) what is Cryptosporidium? Reappraising its biology and phylogenetic affinities, Trends Parasitol. 22:463– 468.
18. Dwivedi KK, Prasad G, Saini S, Mahajan S, Lal S. and Baveja UK. Enteric opportunistic parasites among HIV infected individuals: associated risk factors and immune status. Japan J. Infec. Dis. 2007, 60(2-3): 76-81.
19. Keithly J, Langreth SG, Buttle KF, Mannella CA (2005) Electron tomographic and ultrastructural analysis of the Cryptosporidium parvum relict mitochondrion, its associated membranes, and organelles. J. Eukaryotic Microbiol. 52:132–140.
20. Puiu D, Enomoto S, Buck GA, Abrahamsen MS, Kissinger JC (2004) CryptoDB: the Cryptosporidium genome resource. Nucleic Acids Res. 32: D329–D331.
21. Casadevall A, Pirofski L. (2001). Host-pathogen interactions: the attributes of virulence. J. Infect. Dis. 184:337–344.
22. Hunter PR, Nichols G. Epidemiology and clinical features of Cryptosporidium infection in immunocompromised patients. Clin. Microb. Rev. 2002 15, 145-54.
23. Hunter PR, Nichols G. Epidemiology and clinical features of Cryptosporidium infection in immunocompromised patients. Clin. Microb. Rev. 2002 15, 145-54.
24. Gordon JL Sibley LD (2005) Comparative genome analysis reveals a conserved family of actin-like proteins in apicomplexan parasites. BMC Genomics 6:179.
25. Nagamune K, Sibley LD. (2006) Comparative genomic and phylogenetic analyses of calcium ATPases and calcium-regulated proteins in the apicomplexa Mol. Biol. Evol. 23:1613–1627.
26. Kuo C, Wares JP, Kissinger JC. (2008) the apicomplexan whole-genome phylogeny: an analysis of incongruence among gene trees. Mol. Biol. Evol. 25:2689 –2698.

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27. Bouzid M, Tyler KM, Christen R, Chalmers RM, Elwin K, Hunter PR. (2010) Multi-locus analysis of human infective *Cryptosporidium* species and subtypes using ten novel genetic loci. BMC Microbiol. 10:213.
28. Widmer G. (1998) Genetic heterogeneity and PCR detection of *Cryptosporidium parvum*. Adv Parasitol. 40:223–239.
29. Snelling WJ, Lin Q, Moore JE, Millar BC, Tosini F, Pozio E., (2007) Proteomics analysis and protein expression during sporozoite excystation of *Cryptosporidium parvum* (Coccidia, Apicomplexa). Mol. Cellular Proteomics 6:346 –355.
30. Artz JD, Wernimont AK, Allali-Hassani A, Zhao Y, Amani M, Lin YH, and Senisterra G. (2011) The *Cryptosporidium parvum* kinome. BMC Genomics 12:478.
31. Yunusa T, KoladeY, Oluseyi H. (2015). Prevalence of Cryptosporidiosis among HIV Seropositive Patients in a Tertiary Health Institution, Nig. J. Dent. Med. Sci. 14(5) Ver. IV, 16-20.
32. Okojokwu OJ, Inabo HI, Yakubu SE, Okubanjo OO, Akpakpan EE, Kolawole T. (2016). Molecular characterization of *Cryptosporidium* species among patients presenting with diarrhoea in some parts of Kaduna State, Nigeria. Am. J. Res. Com., 4(3), 87-106.
33. Meamar AR, Rezaian M, Rezaie S, Mohraz M, Mohebali M, Mohammad K, Golestan B, Guyot K, Dei-Cas E. (2006) ER SSU- rRNA Gene Analysis of *Cryptosporidium* spp. in HIV Positive and Negative Patients. Iranian J Pub. Health, 35 (4),1-7.
34. Tiangtip R, Jongwutiwes S. (2002) Molecular analysis of *Cryptosporidium* species isolated from HIV-infected patients in Thailand. Trop. Medicine Int. Health, 7(4), 357–364.
35. Erhabor O, Obunge O, Awah I. (2011). Cryptosporidiosis among HIV infected persons in the Niger Delta of Nigeria. Nig. J. Med., 20 (3):372-375.
36. Kumurya H. Gwarzo Y. (2013) Prevalence of Cryptosporidiosis among patient with Human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS) in Northwestern Nigeria. J. AIDS and HIV Res. 5(8):301- 305.
37. Pam VA, Dakul DA, Karshima NS, Igeh CP. (2013). Cryptosporidiosis in relation to CD4+ T-Lymphocyte counts of people living with HIV/AIDS in Jos, Plateau State, North- Central Nigeria. Asian J. Biomed. Pharm. Sci. 3(17):50-54.
38. Vyas N, Pathan N, Aziz A. (2012). Enteric pathogens in HIV-positive patients with diarrhoea and their correlation with the CD4+ T-lymphocyte counts. Trop. Parasitol. 2:29-34.
39. Nwokediuko SC, Bojuwoye BJ. Onyenekwe B. (2002) Apparent rarity of cryptosporidiosis in human immunodeficiency virus (HIV)-related diarrhea in Enugu, south-Eastern, Nigeria. Nig Postgrad Med J., 9: 70-73.
40. Cai J, Collins MD, McDonald V, Thompson DE. (1992) PCR cloning and nucleotide sequence determination of the 18S rRNA genes and internal transcribed spacer 1 of the protozoan parasites *Cryptosporidium parvum* and *Cryptosporidium muris*. Biochim Biophys Acta. 1131:317–320.
41. Bednarska M, Jankowska I, Pawelas A, Piwczyńska K, Bajer B, Wolska-Kuśnierz B, Wielopolska M, Welc-Falęciak R. (2018) Prevalence of Cryptosporidium, Blastocystis, and other opportunistic infections in patients with primary and acquired immunodeficiency. Parasitol. Res. 117: 2869–2879.
42. Wumba RD, Zanga J, Mbanzulu KM. Madone N, Mandina MN Kahindo AK, Aloni MN, Ekila MB (2015) Cryptosporidium identification in HIV-Infected Humans. Experience from Kinshasa, the Democratic Republic of Congo. Acta. Parasitol., 60(4):638-644.
43. Kilani RT, Wenman WM. (1994) Geographical variation in 18S rRNA gene sequence of *Cryptosporidium parvum*. Int J Parasitol., 24:303–306.

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44. Pieniazek NJ, Bornay-Llinares FJ, Slemenda SB, da Silva AJ, Moura NS, Arrowood MJ, Ditrich O, Addiss DG. (1999) New *Cryptosporidium* Genotypes in HIV-Infected Persons. Centers for Disease Control and Prevention, Atlanta, Georgia, USA.
45. Vanathy K, Parija SC, Mandal J, Hamide A, Krishnamurthy S. (2007) Detection of Cryptosporidium in stool samples of immunocompromised patients. Trop Parasitol., 7:4146.
46. Jin-Chan W, Wei OW, Xian ZL, Shen NC, Fu-Chun J, Feng ZJ, Ming W. (2008) Phylogenetic Analysis of Cryptosporidium isolates in Henan. Chinese J. Agric. Biotech., 4(3), 247-252.

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