

## Molecular characterisation of *Cryptosporidium* species among Patients Presenting with Diarrhoea in Some Parts of Kaduna State, Nigeria

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### ABSTRACT

*Cryptosporidium* species have been widely reported to be responsible for significant diarrhoea in humans and animals. However, little information is available on the prevalence and molecular characterisation of *Cryptosporidium* among humans in Kaduna State. This study examined the distribution, genotypes and subgenotypes of *Cryptosporidium* implicated in cryptosporidiosis in some parts of Kaduna State, Nigeria. A total of 30 *Cryptosporidium* positive samples were collected and analysed. The samples were genotyped by nested PCR followed by restriction fragment length polymorphism using restriction enzymes *RsaI*, *SspI* and *VspI*. The samples were further subgenotyped by nested PCR and amplification of *gp60* kDa gene and subsequent nucleotide sequencing. PCR-RFLP analysis of the 18S rRNA gene fragment revealed that 54.5% (12/22) and 45.5% (10/22) of the *Cryptosporidium* were *Cryptosporidium parvum* and *Cryptosporidium hominis* respectively. Nucleotide sequence analysis of the *gp60* kDa gene yielded 3 subtype families, namely Iia (58.3%), Iic (8.3%) and Iid (33.3%), among the *Cryptosporidium parvum* obtained from the human samples. Three subtype families were also identified in *Cryptosporidium hominis* [Ia (50.0%), Id (40.0%) and Ie (10.0%)]. The zoonotic subgenotype IiaA15G2R1 (85.7%) predominated over the IiaA16G2R1 (14.3%) subgenotype. One anthroponotic *Cryptosporidium parvum* subgenotype (IicA5G3a) was detected in humans. The *Cryptosporidium hominis* subgenotypes included IaA14R6 (60.0%), IaA15R3 (40.0%), IdA10G2 (75.0%), IdA10 (25.0%) and IeA11G3T3 (100.0%). This study showed that cryptosporidiosis is prevalent in the study population. The similarity of prevalence of *Cryptosporidium parvum* (54.5%) and *Cryptosporidium hominis* (45.5%) indicates that anthroponotic transmission is as important as zoonotic transmission of cryptosporidiosis in the study population.

**Keywords:** *Cryptosporidium*, 18SrRNA, *gp60* kDa, genotype, subgenotype, Diarrhoea

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## 1. Introduction

*Cryptosporidium* is an important genus of parasitic protozoa (apicomplexa) infecting humans and other vertebrates globally. Members of this genus are faecal-orally transmitted, often through water or food [1], resulting predominantly in gastrointestinal disease (cryptosporidiosis).

Human cryptosporidiosis is known to be caused predominantly by *Cryptosporidium hominis* or *Cryptosporidium parvum* [2, 3], although infections by other species (e.g., *C. meleagridis*, *C. felis* or *C. canis*) or genotypes have been reported [4]. The transmission of human cryptosporidiosis has been inferred to occur from human-to-human (anthroponotic transmission) for *Cryptosporidium hominis* and *Cryptosporidium parvum*, or animal-to-human (zoonotic transmission) for *Cryptosporidium parvum* [5, 6]. In developed countries, human cryptosporidiosis often occurs sporadically [7] but is also frequently linked to outbreaks associated with contaminated drinking or swimming pool water, child day-care centres or petting farms [8, 9].

*Cryptosporidium* species have been estimated to infect up to 500 million people annually in developing countries. In Africa, about 20 to 35% are infected with the parasite and 32.5 to 40% harbour this organism in sub-Saharan Africa [10]. A prevalence as high as 32% was reported among children in Guatemala, with a significant variation between female (44%) and male (17%) children [11]. In Malawi, molecular epidemiologic studies of cryptosporidiosis in children showed that 41 of 43 were infected with *Cryptosporidium hominis* and only 2 with *Cryptosporidium parvum*, while in Korea a prevalence of 1% was reported among HIV patients [11].

In Tanzania a prevalence of about 17.3% was documented amongst HIV patients [12]. In Guinea Bissau, *Cryptosporidium parvum* had a prevalence of 7.7% and was the second most common parasite with a marked seasonal variation, with peak prevalence found consistently at

the beginning or before the rainy seasons. In the Republic of South Africa, a prevalence rate of 5.6 to 8.5% of *Giardia* cysts and *Cryptosporidium* oocysts were found in all types of water tested including surface water, sewage or treated effluents respectively [10].

Molecular identification of *Cryptosporidium* species is generally lacking in Africa. Species data have been collected in only six countries: Kenya, Malawi, Uganda, Equatorial Guinea, South Africa and recently Nigeria and include *Cryptosporidium hominis*, *C. parvum*, *C. canis*, *C. felis*, *C. meleagridis* and *C. muris*. However, only in Kenya have all six species been reported [13, 14, 15].

Molloy *et al.* [14] reported a *Cryptosporidium* oocysts prevalence range of 15.6% to 19.6% over one year period in four semi-urban villages in Ile-Ife, Osun State, Nigeria. Ayinmode *et al.* [15] in a study carried out in Ibadan, Oyo State reported a prevalence of 11.6%. Relatively few studies have been reported on genotype distribution data for *Cryptosporidium* in Nigeria [14], particularly in the Northern Nigeria.

Presently, only limited or no molecular epidemiological data on *Cryptosporidium* species are available for most African countries [16]. The current knowledge is skewed toward developed countries. Considering the skewed global distribution of human cryptosporidiosis with infection being significantly more common in impoverished than in wealthy nations [17], and particularly considering the relationship between chronic and often fatal human cryptosporidiosis, poverty and HIV/AIDS [18, 19, 20], the present lack of data on this parasite for developing countries in general and Nigeria in particular [14, 15] represents a major gap in our knowledge and understanding of the epidemiology of *Cryptosporidium* species in humans [2]. Cryptosporidiosis had been studied in Kaduna State. However, these studies either used conventional microscopic method which can neither differentiate the species nor the subspecies of the parasite, or were on animal population; since humans and particularly the age group less than 5 years as well as immunocompromised individuals are the most susceptible as shown by reports from different countries [21, 22], the current research aims at exploring the prevalence rate among humans based on conventional as well as molecular techniques. This work is therefore essential for a more precise understanding of the epidemiology of *Cryptosporidium* species in the study area by determining the prevalence and species distribution of *Cryptosporidium* among diarrhoea patients in Kaduna State, Nigeria.

## 2. Materials and Methods

### 2.1 Sampling

Each sample was divided into two parts: one portion of the stool was preserved with 2.5% potassium dichromate in a proportion of 1 g of stool in 1 ml of potassium dichromate for molecular analysis and the second portion was preserved with 10% formalin in a proportion of 1g of stool in 3ml of formalin for analysis using modified Ziehl-Neelsen method [23]. A total of 30 *Cryptosporidium* positive samples were, as part of a larger study, collected from diarrhoeic patients attending three selected hospitals in Kaduna State, Nigeria.

### 2.2 Stool sample concentration using formalin-ethyl acetate sedimentation method

Samples were concentrated according to the method outlined by Centres for Disease Control and Prevention [24]. Specimens were mixed well and 5 ml of each faecal suspension was strained through wetted cheesecloth type gauze placed over a disposable paper funnel into a 15 ml conical centrifuge tube. Formalin (10%) was added through the debris on the gauze to bring the volume in the centrifuge tube to 15 ml. The sample was centrifuged at 500 x g for 10 minutes. The supernatant was decanted and 10 ml of 10% formalin was added to the sediment and mixed thoroughly with wooden applicator stick. Four milliliters (4 ml) of ethyl acetate was added and the tube was stoppered and shaken vigorously in an inverted position for 30 seconds, after which the stopper was carefully removed. Sample was then centrifuged again at 500 x g for 10 minutes. The plug of debris was freed from the top of the tube by ringing the sides with an applicator stick. The top layer supernatant was decanted. A cotton-tipped applicator was used to remove debris from sides of the centrifuge tube. The concentrated specimen was re-suspended in five drops of 10% formalin.

### 2.3 Sample staining using Modified Ziehl-Neelsen (mZN) method

Concentrated faecal samples from section 2.8.1 was thinly smeared on a microslide, air-dried, fixed with methanol for 5 minutes and stained by modified Ziehl-Neelsen (mZN) technique [25]. The slides were stained with carbol-fuchsin (0.34% fuchsin and 4% w/v phenol) for 30 minutes and washed with distilled water. The slides were differentiated in 1% hydrochloric acid-alcohol (70%) for 1 minute and counter-stained with 1% methylene blue for

another 1 minute. Finally, the stained smears were microscopically examined using 1000x magnification. Oocysts stain pink to red or deep purple against a blue background.

#### 2.4 Genomic DNA isolation/extraction

Cryptosporidial genomic DNA was extracted from faecal material using a modification of a previously described simple alkali wash and heat lysis method by Millar *et al.* [26] as described by Millar *et al.* [27]. Faecal suspensions were prepared by adding saline (0.8% w/v) in a ratio of 1:1 (v/v). Faecal material (0.1 ml) was added to 1.4 ml of alkali wash solution (0.5 M NaOH and 0.05 M sodium citrate) in a 1.5 ml Eppendorf tube and mixed for 30s at room temperature by repeated inversion. The mix was subsequently centrifuged at 13,000g for 5 min and the cell pellet containing any cryptosporidial DNA was re-suspended in 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and centrifuged at 13,000g for 5 min. This latter step was repeated and the resulting pellet was re-suspended in Tris-EDTA (0.1 ml, 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) and the DNA extracted from the oocysts by 10-14 cycles of freeze-thawing (in ice for 1 min; in water bath at 100°C for 1 min). The resulting extract was centrifuged at 13,000g for 15 min and the supernatant containing cryptosporidial DNA was transferred to a clean tube and stored frozen prior to PCR.

#### 2.5 Genotyping of *Cryptosporidium* species

**Polymerase Chain Reaction (PCR):** All mZN-positive specimens were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the COWP and SSU-rRNA genes. A fragment of the COWP gene was amplified by PCR, as described previously [28]. The PCR was performed with primers Cry-9 (5'-GGACTGAAATACAGGCATT ATCTTG-3') and Cry-15 (5'-GTAGATAATGGA AGAGATTGTG-3') which amplified a 550-bp fragment. The PCR mixture contained 10 µl of template DNA, 10x Taq buffer S, 10 mM dNTP, 20 pmol of each primer, 2.5 U of Taq DNA polymerase, and 0.5 µl of non-acetylated BSA (10 mg/ml) in a 50-µl reaction volume. PCR cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 min and final cooling at 4°C.

The SSU-rRNA gene was amplified by nested PCR, as described previously [29]. The primary PCR was performed with primers SSU-F2 (5'-TTCTAGAGCTAATACATGCG-3') and SSU-R2 (5'-CCCATTCCTTCGAAACAGGA-3') resulting in an approximately 1,300-bp

fragment. The primary PCR mixtures contained 10 µl of template DNA, 10x Taq buffer S (PeqLab, Erlangen, Germany), 10 mM deoxynucleoside triphosphate mix (dNTP) (Invitrogen, Karlsruhe, Germany), 20 pmol of each primer, 2.5 U of Taq DNA polymerase (PeqLab), and 0.5 µl of non-acetylated bovine serum albumin (BSA; 10 mg/ml) (New England Biolabs, Frankfurt, Germany) in a 50-µl reaction volume. Primary PCR cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min, with a final extension of 72°C for 7 min and final 4°C cooling. The secondary PCR was performed with primers SSU-F3 (5'-GGAAGGGTTGTATTTATT AGATAAAG-3') and SSU-R4 (5'-CTCATAAGG TGCTGAAGGAGTA-3'), which resulted in an approximately 830-bp fragment. The reaction conditions were similar to those described above for the primary PCR, except that 5 µl of the primary PCR product was used as the template and no BSA was added. Cycling conditions for the secondary PCR consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 58°C for 45 sec, and 72°C for 1 min with a final extension of 72°C for 7 minutes and final cooling at 4°C. Positive (purified *Cryptosporidium* DNA) and negative controls (master mix without a DNA template) were included in each batch of PCR amplification reactions [30].

## 2.6 Restriction fragment length polymorphism (RFLP)

*Cryptosporidium parvum* and *Cryptosporidium hominis* was differentiated from any other *Cryptosporidium* species that may infect human by analysis of restriction patterns of the 18S rRNA amplicons. Ten microliters (10 µl) of amplified products was digested with 10 U of endonuclease *Ssp* I and 2 µl of 10x NE Buffer 3 in a total volume of 20 µl, and was incubated at 37°C in a water bath for 2 hrs under conditions recommended by the supplier. To distinguish between *C. parvum* and *C. hominis* the same amount of amplified product was digested with 10 U *Vsp*I (or *Ase* I) under the same conditions. The endonucleases digestion products were fractionated (electrophoresed) in 2% agarose gel and visualised by ethidium bromide staining.

## 2.7 Sixty kilodalton Glycoprotein (GP60 kDa) gene PCR amplification for *Cryptosporidium parvum* and *Cryptosporidium hominis* subgenotyping

A 60-kDa glycoprotein (GP60) gene fragment was amplified by nested PCR as previously described [31, 32, 33]. The primary PCR was performed with primers GP60-AL3531 (5'-ATAGTCTCCGCTGTATTC-3') and GP60-AL3535 (5'-GGAAGGAACGATGTATCT-3'). The primary PCR mixtures contained 10 µl of template DNA, 10x PCR buffer (Roche

Diagnosics, Mannheim, Germany), 10 mM deoxynucleoside triphosphate mix (dNTP) (Roche), 20 pmol of each primer, 2 U of Fast start Taq DNA polymerase (Roche) and 0.5 µl of BSA (10 mg/ml) (New England Biolabs) in a 50-µl reaction volume. Primary PCR cycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 45 sec and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes and final cooling at 4°C. The nested PCR was performed with primers GP60-AL3532 (5'-TCCGCTGTATTCT CAGCC-3') and GP60-AL3534 (5'-GCAGAGGAACCAG CATC-3') or GP60-LX0029 (5'-CGAACCACAT TACAAATGAAGT-3') which amplified approximately 850 and 390-bp fragment respectively. The reaction conditions was similar to those described above for the primary PCR; except that 1 µl of the primary PCR product was used as the template and no BSA was added. Cycling conditions for the secondary PCR consisted of 95°C for 3 minutes, followed by 35 cycles of 95°C for 1 minute, 50°C for 45 sec, and 72°C for 1 minute with a final extension at 72°C for 7 minute and final cooling at 4°C.

## 2.8 DNA sequencing

Secondary GP60 PCR products were purified using the procedures of a commercial kit (QIAquick<sup>®</sup> Gel Extraction kit, Qiagen, Hilden, Germany). Direct DNA sequencing of the gel purified PCR product was performed. The primers used to sequence were GP60-AL3532 (5'-TCCGCTGTATTCTCAGCC-3') and GP60-AL3534 (5'-GCAGAGGAACCAGCATC-3') [32]. DNA sequences were aligned with each other and with previously reported sequences using the BLAST program (<http://blast.ncbi.nlm.nih.gov/>) and ClustalX software ([ftp://ftp.ebi.ac.uk/pub/software/clustal\\_w2/](ftp://ftp.ebi.ac.uk/pub/software/clustal_w2/)) for identification of genotypes and subgenotypes.

## 2.9 Data Analysis

DNA sequences were analysed with the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/>), Molecular Evolutionary Genetics Analysis version 6 (MEGA 6) tool and ClustalX software ([ftp://ftp.ebi.ac.uk/pub/software/clustal\\_w2/](ftp://ftp.ebi.ac.uk/pub/software/clustal_w2/)).

## 3. Results

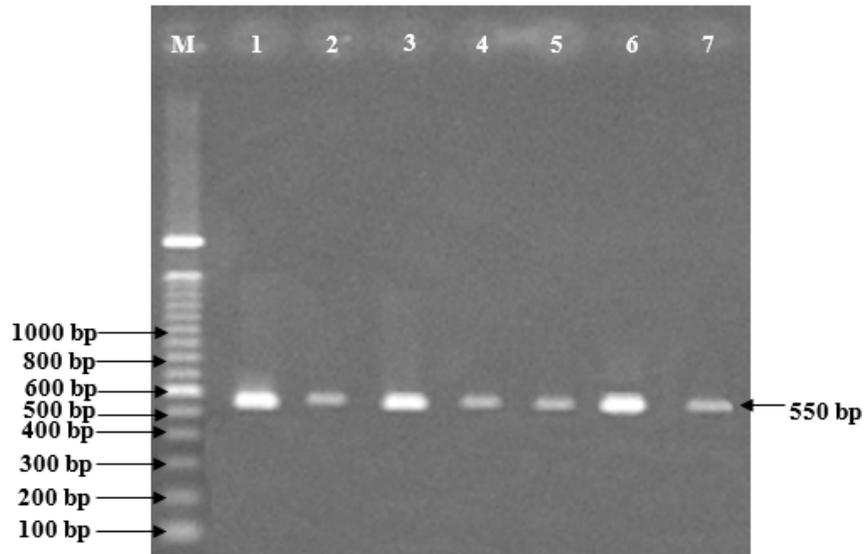
Twenty two (22) out of the thirty samples used for the molecular studies were successfully amplified, while 8 of the samples failed to amplify. The PCR products of the COWP and 18S rRNA genes produced bands with molecular size of 550bp and 1,300bp respectively (Plates I and

II). The secondary PCR products of the 18S rRNA gene yielded bands that coincided with the expected 830bp that are diagnostic of *Cryptosporidium* species (Plates III).

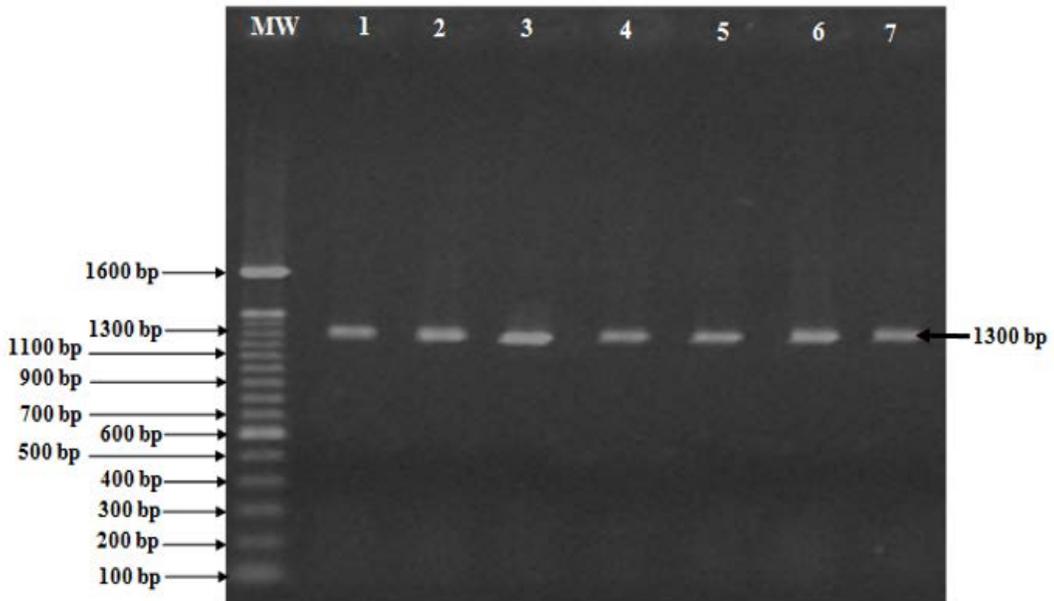
The *Rsa* I endonuclease digestion of COWP gene produced two distinct digestion patterns. The molecular size of the digestion products in lanes 1 and 2 reflected the position of the *Rsa* I cleavage sites identified in *Cryptosporidium parvum* characterised by three band patterns of 410bp, 106bp and 34bp. The other endonuclease digestion pattern (lanes 3, 4, 5, 6 and 7) comprising isolates of *Cryptosporidium hominis*, displayed four restriction fragments of 285bp, 125bp, 106bp and 34bp (Plate IV).

The restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA gene sequence using restriction enzymes *Ssp* I and *Vsp* I was done to identify the *Cryptosporidium* genotypes circulating in the study area. Representative electrophoresis profile of the digestion products are presented in the plates below. Plates V shows the *Ssp* I digestion products of 18S rRNA gene of *Cryptosporidium* species by PCR-RFLP. The bands were of molecular sizes 108bp, 111bp, 269bp and 450bp and were indicative of *Cryptosporidium hominis* and *Cryptosporidium parvum*. Restriction analysis of the *Vsp* I digestion products produced a band pattern that was diagnostic of *Cryptosporidium hominis* and *Cryptosporidium parvum*. Lanes 1, 2, 3, 5, 6 and 7 were *Cryptosporidium hominis* and lane 4 was *Cryptosporidium parvum* (Plate VI).

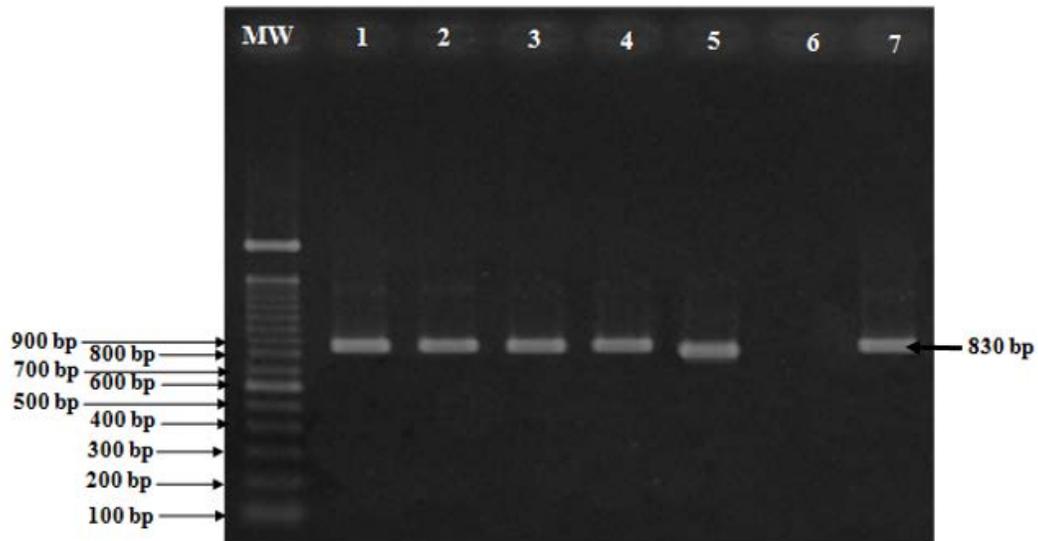
The isolates that amplified were genotyped into *Cryptosporidium parvum* 54.5% (12/22) and *Cryptosporidium hominis* 45.5% (10/22) as shown in Table 1. The distribution of *Cryptosporidium* genotypes in the study populations based on sample locations showed that 70.0% (7/10) of the genotyped samples from Zaria were *Cryptosporidium parvum* while 30.0% (3/10) were *Cryptosporidium hominis*. Out of the 10 samples from Kaduna that were subjected to genotyping, only 60.0% (6/10) were successfully amplified; 33.3% (2/6) of these were genotyped to be *Cryptosporidium parvum* and 66.7% (4/6) were *Cryptosporidium hominis*. Six (6) out of the 7 samples (85.7%) from Kafanchan were successfully amplified and genotyped. Of these, 50.0% (3/6) were *Cryptosporidium parvum* while the remaining 50.0% (3/6) were *Cryptosporidium hominis* (Table 1).



**Plate I: Amplicons of COWP gene.**  
(MW= 100bp Molecular weight marker; lanes 1 to 7 = 550 bp)

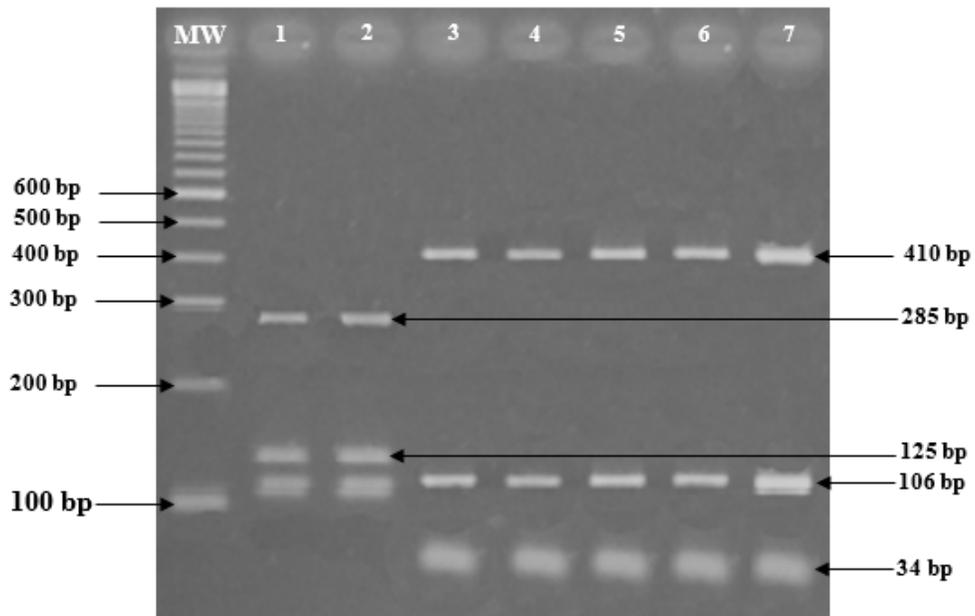


**Plate II: Amplicons of primary PCR of 18S rRNA gene.**  
(MW= 100bp Molecular weight marker; lanes 1 to 7 = 1,300 bp)



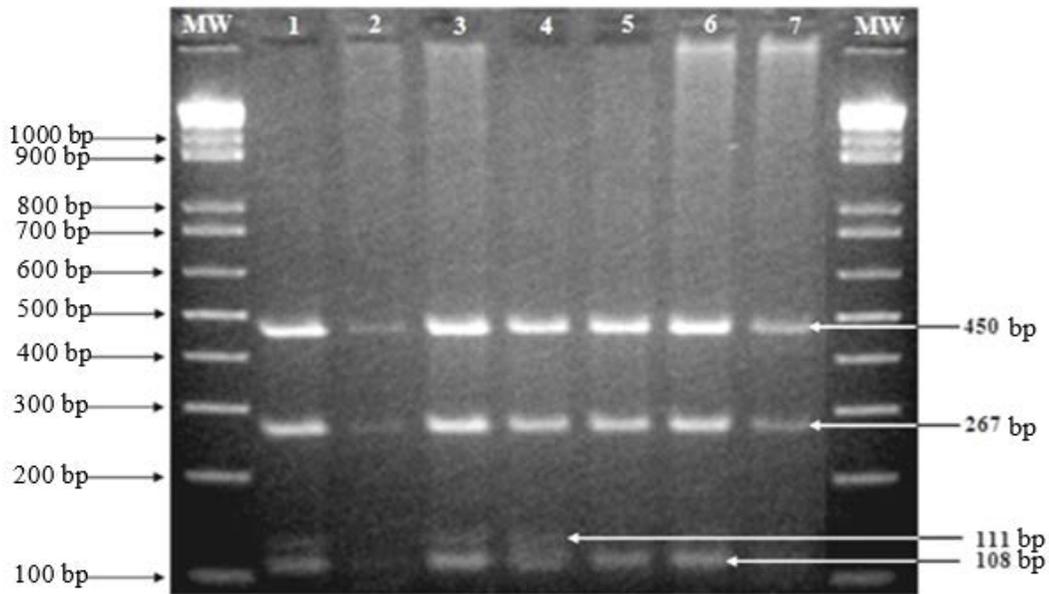
**Plate III: Amplicons of secondary PCR of 18S rRNA gene.**

(MW= 100bp Molecular weight marker; lanes 1, 2, 3, 4, 5 and 7 = 830 bp)

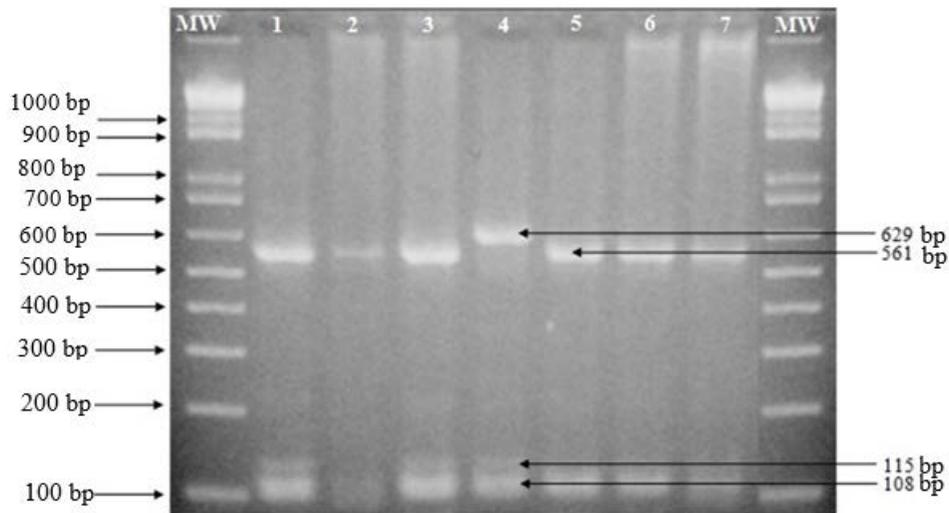


**Plate IV: *Rsa* I digestion products of the COWP gene.**

(MW=100bp molecular marker; Lanes 1 and 2 = *C. hominis*; 3, 4, 5, 6 and 7 = *C. parvum*)



**Plate V: *Ssp I* digestion products of 18S rRNA gene of *Cryptosporidium* species by PCR-RFLP.**



**Plate VI: Genotyping of the *Cryptosporidium* species by a nested PCR-RFLP procedure based on *VspI* digestion of the 18S rRNA gene.**

(MW = 100 bp molecular weight marker; Lanes 1, 2, 3, 5, 6 and 7 were *C. hominis*, lane 4 was *C. parvum*.)

**Table 1: Distribution of *Cryptosporidium* genotypes among humans based on sampling locations**

Location	No. of sample	No. of sample amplified (%)	No. positive (%)		No. failed to amplify (%)
			<i>C. parvum</i>	<i>C. hominis</i>	
Zaria	13	10 (76.9)	7(70.0)	3(30.0)	3(23.1)
Kaduna	10	6 (60.0)	2(33.3)	4(66.7)	4(40.0)
Kafanchan	7	6 (85.7)	3(50.0)	3(50.0)	1(14.3)
Total	30	22 (73.3)	12(54.5)	10(45.5)	8(26.7)

Table 2 presents the subtype families or alleles of *Cryptosporidium* subtypes. Analysis of the gp60 kDa gene sequence of *Cryptosporidium parvum* yielded 3 subtype families namely; Ila, Ilc and IId. The Ila subgenotype accounted for 58.3% (7/12) of the *Cryptosporidium parvum*, Ilc was 8.3% (1/12) of the *C. parvum* in humans and 33.3% (4/12) of the *Cryptosporidium parvum* belong to subgenotype IId. Also, 3 *Cryptosporidium* subtypes were identified in *Cryptosporidium hominis* with the following subtype families: 50.0% (5/10) were in subgenotype Ia, 40.0% (4/10) belong to subgenotype Id and subgenotype Ie accounted for 10.0% (1/10).

The trinucleotide analysis showed that 6 (85.7%) of the 7 *Cryptosporidium parvum* that belong to the subtype family Ila had the subgenotype IlaA15G2R1 while 14.3% (1/7) belong to IlaA16G2R1 subgenotype. Only 1 (100.0%) belong to the *Cryptosporidium parvum* subtype family Ilc and its genotype was IlcA5G3a. There were 4 specimens that belong to the subtype family IId and all of them were subgenotyped as IIdA15G1R1 (100.0%). The subtype families identified in *Cryptosporidium hominis* on the other hand showed that subtype family Ia had two subgenotypes viz: IaA14R6, 3 (60.0%) and IaA15R3, 2 (40.0%). There were 75.0% (3/4) of IdA10G2 and 25.0% (1/4) of IdA10 subgenotypes in the Id subtype family while the subtype family Ie had only one member identified to have subgenotype IeA11G3T3, 1(100.0%) (Table 3).

Based on the nucleotide sequences obtained from the various samples subgenotyped, phylogenetic trees were drawn. In Figure 1, the phylogenetic relationship between the different *Cryptosporidium* species is depicted by 7 clades of subgenotype families.

**Table 2: Distribution of *Cryptosporidium* subtype alleles based on sampling locations**

Location	<i>Cryptosporidium parvum</i>				<i>Cryptosporidium hominis</i>			
	No. positive	IIa (%)	IIc (%)	IId (%)	No. positive	Ia (%)	Id (%)	Ie (%)
Zaria	7	4(57.1)	1(14.3)	2(28.6)	3	2(66.7)	1(33.3)	0(0.0)
Kaduna	2	1(50.0)	0(0.0)	1(50.0)	4	3(75.0)	1(25.0)	0(0.0)
Kafanchan	3	2(66.7)	0(0.0)	1(33.3)	3	0(0.0)	2(66.7)	1(33.3)
Total	12	7(58.3)	1(8.3)	4(33.3)	10	5(50.0)	4(40.0)	1(10.0)

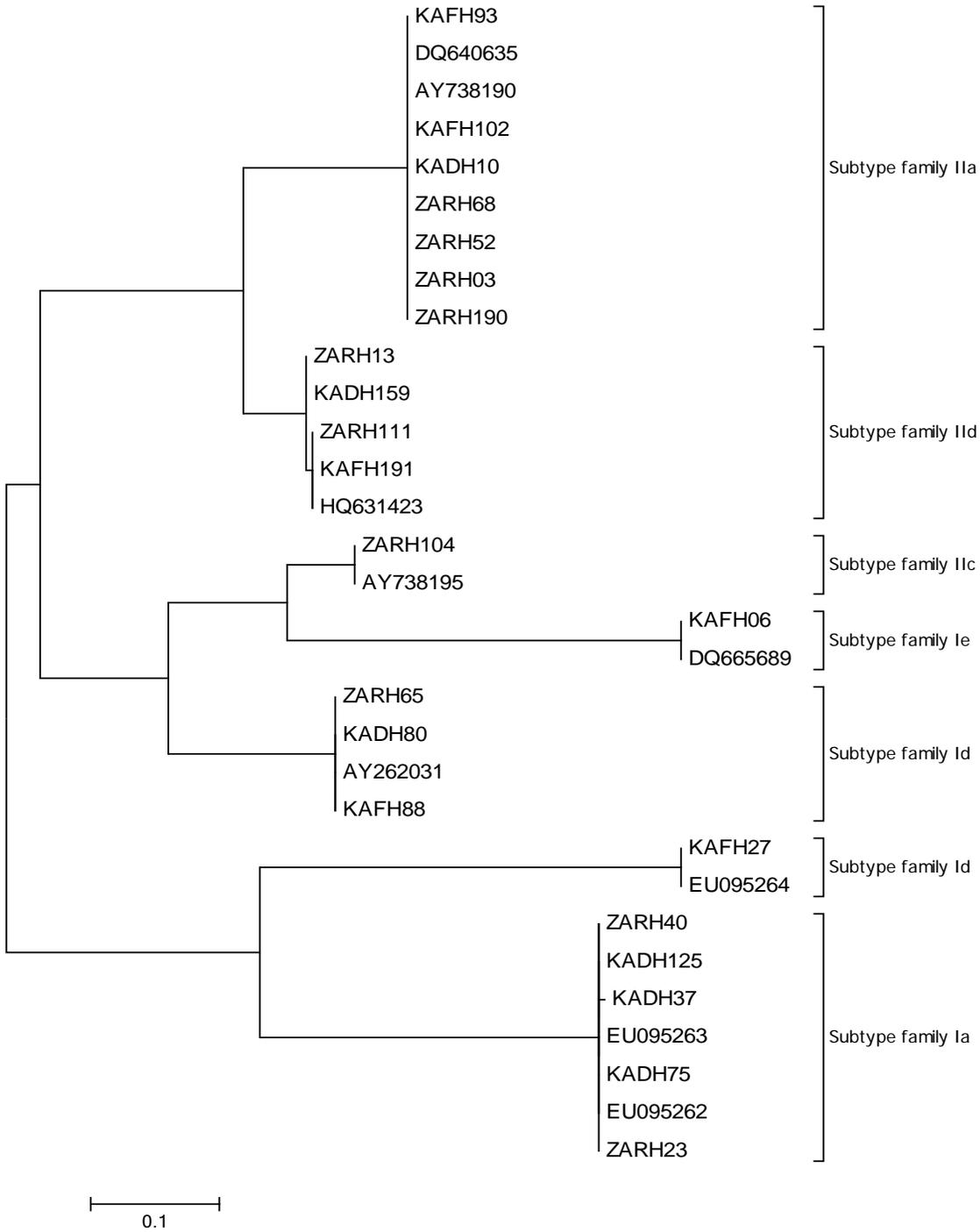
#### 4. Discussion

The application of molecular characterisation of *Cryptosporidium* isolates in field epidemiological studies is the most reliable approach for proper diagnosis and establishing of the source of cryptosporidiosis in human infections. This is because definitive identification of the species of *Cryptosporidium* parasite has not been possible by using the routine diagnostic methods [33]. Thus, the molecular characterisation of *Cryptosporidium* species used in this study has enabled determination of the origin and distribution of *Cryptosporidium* genotypes and subgenotypes in human cryptosporidiosis in Kaduna State.

However, the fact that molecular characterisation of the *Cryptosporidium* parasite by polymerase chain reaction was not successful in all the faecal samples could be explained by the inappropriate storage temperature as a result of epileptic or erratic power supply. This may have led to oocyst damage which resulted in the loss of DNA [34] and the subsequent failure of PCR amplification. In addition, although the DNA isolation kit used was especially designed for DNA preparations from faeces, the presence of inhibitors in the faecal samples may have been an additional reason for the failure in some of the PCR amplifications.

**Table 3: *Cryptosporidium* subgenotypes identified based on gp60 kDa gene sequence**

Species	Subtype family	No. in subtype family	Subgenotype: No. in subgenotype (%)
<i>C. parvum</i>	IIa	7	IIaA15G2R1: 6(85.7) IIaA16G2R1: 1(14.3)
	IIc	1	IIcA5G3a: 1(100.0)
	IId	4	IIdA15G1R1: 4(100.0)
<i>C. hominis</i>	Ia	5	IaA14R6: 3(60.0) IaA15R3 : 2(40.0)
	Id	4	IdA10G2 : 3(75.0) IdA10: 1(25.0)
	Ie	1	IeA11G3T3: 1(100.0)



**Figure 1: Phylogenetic relationships between subgenotypes of *Cryptosporidium* identified in human samples, together with selected reference sequences (GenBank accession numbers: DQ640635, AY738190, HQ631423, AY738195, DQ665689, AY262031, EU095264, EU095263 and EU095262).**

Two genotypes of *Cryptosporidium*, *Cryptosporidium parvum* (54.5%) and *Cryptosporidium hominis* (45.5%), were detected in the study subjects. *Cryptosporidium parvum* was more prevalent than *Cryptosporidium hominis* as the leading cause of human cryptosporidiosis in the study area. This is in agreement with the findings based on molecular genotyping of isolates from many regions of the world [35], as well as the prevalence of *Cryptosporidium parvum* in parts of some European countries namely France, England and Northern Ireland is higher than *Cryptosporidium hominis* [36, 37, 38]. These findings contrast with those from Kenya, Malawi, Brazil, Vietnam, and United Kingdom [21], Dijon Hospital in France [39], Iran [40] and Haiti [41]. However, since human cryptosporidiosis is not limited to the two species and others such as *Cryptosporidium felis*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*, and *Cryptosporidium canis* are also known to occasionally cause human infections [42], their relevance in causing human cryptosporidiosis cannot be ruled out or undermined. This suggests that person-to-person and probably environmental transmission is more common than zoonotic transmissions among these individuals.

Differences in the distribution of *Cryptosporidium hominis* and *Cryptosporidium parvum* are considered an indication of differences in sources of infection. The predominance of *Cryptosporidium hominis* in a population is considered to be the result of anthroponotic (person-person) transmission, whereas both anthroponotic and zoonotic transmissions are possible routes for *Cryptosporidium parvum* infections [43].

*Cryptosporidium parvum* and *Cryptosporidium hominis* in this study were grouped into subtypes based on the sequences of the 60-kDa glycoprotein gene. Three subtype families/alleles (IIa, IIc and IId) were detected; subtypes IIa and IId were reported to be zoonotic. Three (3) out of the 5 identified *Cryptosporidium parvum* subgenotypes in this study belonged to the zoonotic subtype families IIa and IId. The existence of the anthroponotic *Cryptosporidium* allele (IIc) in the studied population suggests that person-to-person transmission is an important route of transmission and therefore plays a major role in the transmission of cryptosporidiosis in Kaduna State. This is evident in the results of our investigation as nearly half of the *Cryptosporidium* species detected in humans were *Cryptosporidium hominis*. Three subtype alleles (Ia, Id and Ie) were detected.

In conclusion, the study demonstrated that *Cryptosporidium* species is prevalent and widespread in humans in Kaduna State. Humans were more parasitized by *Cryptosporidium parvum* than *Cryptosporidium hominis*. Results of our study showed that both zoonotic and anthroponotic transmission are important in the epidemiology of cryptosporidiosis in Kaduna State, Nigeria. This conclusion is based on our finding of *Cryptosporidium parvum* subtype families IIa and IIc in 11 out of 12 *Cryptosporidium parvum* subtypes in humans. The anthroponotic *Cryptosporidium* species detected were *Cryptosporidium parvum* subtype IIc and *Cryptosporidium hominis* subtypes Ia, Id and Ie.

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