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Molecular Characterisation of *Cryptosporidium* Species from Extensively Managed Cattle Slaughtered inAbattoirs in Kaduna State, Nigeria

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ABSTRACT

Cryptosporidium species are recognised as important cause of diarrhoea in both animal and man especially children, the aged and HIV infected individuals. This study examined the prevalence, distribution, genotypes and subgenotypes of Cryptosporidium implicated in animal cryptosporidiosis in Kaduna State. A total of 300 animal faecal samples were collected and analysed. All samples were screened by microscopy after modified Ziehl-Neelsen acid-fast staining. Cryptosporidium-positive faecal samples were genotyped by nested PCR followed by RFLP. The samples were further subgenotyped by nested PCR and amplification of gp60 kDa gene and subsequent nucleotide sequencing. Prevalence of cryptosporidiosis in cattle was found to be 15.7% (47/300) as determined by microscopy. PCR-RFLP analysis of the 18S rRNA gene fragment revealed that three genotypes including Cryptosporidium bovis (57.1%), Cryptosporidium ryanae (14.3%) and Cryptosporidium parvum (28.6%) were identified in the samples. Nucleotide sequence analysis of the gp60 kDa gene yielded subtype families IIa and IIdin Cryptosporidium parvum; these subtypes had equal occurrence with subgenotypes IIaA15G2R1 (100.0%) and IIdA15G1 (100.0%) respectively. This study showed that cryptosporidiosis is prevalent in the study population and the occurrence of IIa and IId subtype families suggests that zoonotic transmission is important and likely transmission route of cryptosporidiosis in the Kaduna State.

Keyword: *Cryptosporidium*, Cryptosporidiosis, Oocyst, Cattle, gp60 kDa, Genotype, Subgenotype, Kaduna State

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INTRODUCTION

Cryptosporidium species are important intestinal parasites that infect a wide host range including humans and animals. Cattle are particularly considered to be one of the main animal reservoir of *Cryptosporidium*[1]. *Cryptosporidium* infections frequently result in morbidity, weight loss and delayed growth, and mortality of young animals. It has been reported that contamination of cattle manure has led to several foodborne and waterborne outbreaks of human cryptosporidiosis [1,2].

Extensive genetic variation exists within the genus *Cryptosporidium*. On the basis of molecular analysis technique, PCR-based, seven *Cryptosporidium* species have been identified in cattle, these include *C. parvum, C. andersoni, C. bovis, C. ryanae, C. hominis, C. felis* and *C. suis*[3]. The first-four *Cryptosporidium* species above have been implicated in most cases of bovine cryptosporidiosis though with different clinical manifestations [4].

In Nigeria, information on molecular identification and genetic characterization of *Cryptosporidium* species in cattle are limited in some States and lacking in most States. Since detection of *Cryptosporidium* in Nigeria is done based on microscopy after Modified Ziehl-Neelsen staining which could not differentiate between the species of the parasite, the goal of this work was to use molecular technique to detect the parasite as well as identify the species and genotype circulating in the study area. This research work therefore provides molecular baseline information on *Cryptosporidium* in Kaduna State. The present study therefore focused on the investigation of *Cryptosporidium* in extensively managed cattle slaughtered in abattoirs in Kaduna State. The aim of the study was to determine the distribution and species/genotypes of *Cryptosporidium* in the cattle.

MATERIALS AND METHODS

The Study Area

Kaduna State is located on the southern end of the high plain of North-western region of Nigeria bounded by latitude 9°03′N and 11°05′N and longitude 6°05′E and 8°048′E. It occupies a total land mass estimated at 46,030 sqkm, approximately 5% of the total land mass of the country. There is a marked seasonality in Kaduna State with the cool to hot season being longer than wet (rainy) season. Rainfall varies, decreasing from an average of about 1530 mm in the southeast to about 1013 mm in the northeast [5]. Three abattoirs located in Zaria, Kaduna and Kafanchan were used as the sample sites.

Sample Processing

A total of 300 stool samples were collected from cattle slaughtered at the abattoirs with 100 samples collected from each abattoir. 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 [6].

Stool sample concentration using formalin-ethyl acetate sedimentation method

Samples were concentrated according to the method outlined by Centres for Disease Control and Prevention [7]. 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.

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 [8]. 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.

Genomic DNA 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*. [9] as described by Millar *et al.*[10. 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 mMTris-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.

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 [11]. The PCR was performed with primers Cry-9 (5´-GGACTGAAATACAGGCATTATCTTG-3´) and Cry-15 (5´-GTAGATAATGGAAGA GATTGTG-3´) which amplified a 550-bp fragment. The PCR mixture contained 10 µl of template DNA, 10x Taq buffer S, 10 mMdNTP, 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 1minute, 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 [12]. The primary PCR was performed with primers SSU-F2 (5´-TTCTAGAGCTAATACATGCG-3´) and SSU-R2 (5´-CCCATTTCCTTCG AAACAGGA-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´- CTCATAAGGTGCTGAAGGAGTA-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 [13].

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.

Sixty kilodalton Glycoprotein (GP60 kDa) gene PCR amplification and subgenotyping

A 60-kDa glycoprotein (GP60) gene fragment was amplified by nested PCR as previously described [14, 15, 16]. The primary PCR was performed with primers GP60-AL3531 (5´-ATAGTCTCCGCTGTA TTC-3´) and GP60-AL3535 (5´-GGAAGGAACGATGTATCT-3´). The primary PCR mixtures contained 10 µl of template DNA, 10x PCR buffer (Roche Diagnostics, 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´- TCCGCTGTATTCTCAGCC-3´) and GP60-AL3534 (5´-G CAGAGGAACCAGCATC-3´) or GP60-LX0029 (5`-CGAACCACATTACAAATGAAGT-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.

DNA sequencing

Secondary GP60 PCR products were purified using the procedures of a commercial kit (QIAquick® Gel Extraction kit, Qiagen, Hilden, Germany). Direct DNA sequencing of the gel purified PCR product was performed in an ABI model 3720 sequencer (Applied Biosystems, Forster City, CA) by using Big-Dye version 3.1 chemistry and automated capillary DNA sequencer. The primers used to sequence were GP60-AL3532 (5´-TCCGCTGTATTCTCAGCC-3´) and GP60-AL3534 (5´-GCAGAGGAACCAGCATC-3´) [15]. DNA sequences were used to search the GenBank database for similarities using the Blastn tool program (http://blast.ncbi.nlm.nih.gov/).ClustalWprogramme was used to compare sequences and phylogenetic tree was constructed using MEGA 6 software.

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 ClustalW software (ftp://ftp.ebi.ac.uk/pub/software/clustal w2/).

RESULTS

Cryptosporidium oocysts were detected in 47 out of the 300 stool samples giving a prevalence of 15.7%. Within Kaduna State, Zaria had the highest prevalence of 21.0% followed by Kafanchan (15.0%) while Kaduna had the least prevalence (12.0%). Results of genotyping of the *Cryptosporidium* species from animal population are presented in Table 1. Thirty percent (30.0%) of samples that were *Cryptosporidium* positive by microscopy were employed for the genotyping procedure. Three *Cryptosporidium* genotypes were identified. Table 1 shows the distribution of the various genotypes based on the sample location. Fifty percent (3/6) of the samples from Zaria were *Cryptosporidium bovis*, 16.7% (1/6) were *Cryptosporidium ryanae* and 33.3% (2/6) were *Cryptosporidium parvum*. The samples from Kaduna, on the other hand, were genotyped to be 50.0% (2/4) *Cryptosporidium bovis*, 25.0% (1/4) as *Cryptosporidium ryanae* and 25.0% (1/4) were *Cryptosporidium parvum*. There was a slight difference in the genotype distribution in Kafanchan; only two genotypes were identified. Out of the 4 samples genotyped in Kafanchan, 75.0% (3/4) were *Cryptosporidium bovis* while 25.0% (1/4) were *Cryptosporidium parvum*. *Cryptosporidium ryanae* was not identified in cattle population in Kafanchan. On the whole, genotyping of *Cryptosporidium* isolates from the cattle population revealed that 57.1% (8/14) of the *Cryptosporidium* species were *Cryptosporidium bovis*, 14.3% (2/4) were *Cryptosporidium ryanae* and 28.6% (4/14) were *Cryptosporidium parvum*.

Table 1: Distribution of *Cryptosporidium* genotypes among cattle

Location	No. of sample	No. Positive (%)	No. of representative sample (%)	No. Positive (%)		
				Cryptosporidium bovis	*Cryptosporidium ryanae*	*Cryptosporidium parvum*
Zaria	100	21(21.0)	6(30.0)	3(50.0)	1(16.7)	2(33.3)
Kaduna	100	12(12.0)	4(30.0)	2(50.0)	1(25.0)	1(25.0)
Kafanchan	100	1(14.0)	4(30.0)	3(75.0)	0(0.0)	1(25.0)
Total	300	47(15.7)	14(30.0)	8(57.1)	2(14.3)	4(28.6)

The trinucleotide analysis showed that 6 (85.7%) of the 7 *Cryptosporidium parvum* that belong to the subtype family IIa had the subgenotype IIaA15G2R1 while 14.3% (1/7) belong to IIaA16G2R1 subgenotype. Only 1 (100.0%) belong to the *Cryptosporidium parvum* subtype family IIc and its genotype was IIcA5G3a. 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 2).

Out of the 14 samples subgenotyped from the cattle samples, only 4 were *Cryptosporidium parvum*. The subgenotype results revealed two subtype families namely IIa and IId with each subtype family having two members each. All the two members of the IIa subtype family were subgenotyped as IIaA15G2R1 while the two members of the IId subtype family had IIdA15G1 subgenotype (Table 2).

Based on the nucleotide sequences obtained from the various samples subgenotyped, phylogenetic trees were drawn. The evolutionary history was inferred by using the Maximum Likelihood method.

In the phylogenetic tree drawn using sequences obtained from cattle sample, three distinct groups were evident. These represent three different *Cryptosporidium* genotypes or species, namely: *Cryptosporidium parvum*, *Cryptosporidium ryanae* and *Cryptosporidium bovis* (Figure 1).

Table 2: *Cryptosporidium* subgenotypes identified based on *gp*60 kDa gene sequence

Source of sample	Species	Subtype family	No. in subtype family	Subgenotype: No. in subgenotype (%)
Cattle	*C. parvum*	IIa	2	IIaA15G2R1: 2(100.0)
		IId	2	IIdA15G1: 2(100.0)

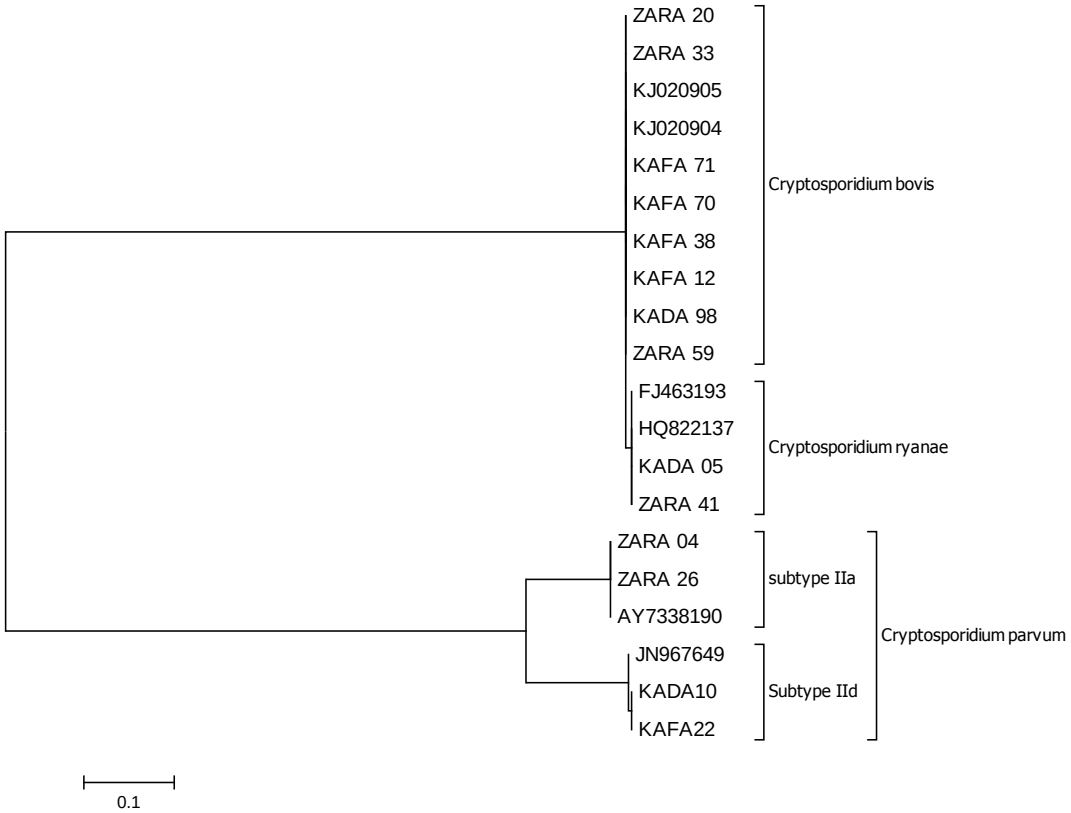

Figure 1: Phylogenetic relationships between genotypes/subgenotypes of *Cryptosporidium* defined in animal samples, together with selected reference sequences (GenBank accession numbers: KJ020905, KJ020904, FJ463193, HQ822137, AY7338190, JN967649) for comparison, inferred from gp60 kDa and 18S rRNA genes sequence data following analysis by Maximum Likelihood method

DISCUSSION

The use of molecular analysis in genotyping has become an important tool in the detection of *Cryptosporidium* species. *Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium ryanae* and *Cryptosporidium andersoni* have been identified worldwide as the four major species in cattle [17, 18]. Three of these species were identified in Kaduna State. *Cryptosporidium bovis* (57.1%) was the most common species among cattle in this study, also present in high frequencies were *Cryptosporidium parvum* (28.6%) and *Cryptosporidium ryanae* (14.3%). These findings compare with the reports of Amer *et al.* [19] where they reported 11.8% of *Cryptosporidium parvum* and 11.2% *Cryptosporidium ryanae* in cattle.

Though in this study both subtypes IIa and IId occurred in equal proportions in cattle, Xiao [20] said subtype IIa was noted to be the most prevailing allele in calves worldwide with subtypes IIaA15G2R1 and IIaAG1R1 being especially common. Therefore, it could be adduced that this occurrence could be because only adult cattle were involved in this study. In many developed countries in North America [21] and Europe [22], IIaA15G2R1 prevails [20, 23]. Several other subtypes are more regionally distributed; IIaA16G1R1 is the predominant subgenotype in the Balkan countries [20]. This study confirms the study carried out in Egypt by Amer *et al.* [24] where they hypothesize that IId subtype family may be due to an accidental infection in cattle from sheep and/or goats, with subsequent adaptation and cycling in cattle and eventual transmission to humans.

In conclusion, *Cryptosporidium bovis*, *Cryptosporidium ryanae* and *Cryptosporidium parvum* were identified in cattle using the PCR-RFLP method. The *C. parvum* showed two subtypes; IIa and IId which occurred in equal proportions. This was determined by DNA sequence analysis of gp60 kDa and 18S rRNA genes. Though these subtypes are zoonotic in nature, further epidemiologic investigations need to be carried out to establish link with environmental transmission routes of public health significance.

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