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Original Research Article

Serology and molecular characterisation of *Cryptosporidium* specie detected from cattle in Sokoto State, North Western Nigeria

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ABSTRACT

Molecular characterization of *Cryptosporidium* has led to increased recognition of the diversity of species infecting animals. A *Cryptosporidium* Copro-Enzyme Linked Immuno Assay (Copro-ELISA) antigen detection manufactured by Savyon® Diagnostics Ltd., Ashdod, Israel, was used to screen faecal samples from cattle. Copro-ELISA *Cryptosporidium* antigen positive faecal samples were subjected to Nested PCR for the amplification of 830bp fragments of small subunit (SSU) rRNA gene and followed by nucleotide sequencing by targeting the 18S gene in cattle. Of the 384 bovine faeces screened, 31 (8.07%) were ELISA positive out of the 384 screened samples and 19.4% (6/31) were *Cryptosporidium* positive using PCR technique. Nucleotide sequencing of the bovine PCR products showed the presence of *C. bovis* 2 (6.5%), *C. ryanae* 2 (6.5%), *C. andersoni* 1 (3.2%) and *C. ubiquitum* 1(3.2%). *C. ubiquitum* has also been found in this study in Wurno LGA of Sokoto state, it is a new specie in the genus previously identified as *Cryptosporidium* Cervine genotype which was reported in wild and domestic ruminants, rodents, carnivores and primates including humans. In calves, there were no significant associations ($p>0.05$) in prevalence of Cryptosporidium between breeds; between sources of drinking water; between physical nature of faeces (OR=3.61:95% CI on OR: 0.36<OR<34.71); between management practices (OR=1.71: 95% CI on OR: 0.26<OR<11.41) and between sexes (OR=0.58: 95% CI on OR: 0.08<OR<3.88). Absence of the zoonotic spp (*C. parvum*) in the area suggests that the age group of calves studied are not likely to be source of human cryptosporidiosis.

Keywords: Cattle, *Cryptosporidium specie*, molecular characterisation, Nested PCR, Nigeria

INTRODUCTION

Cryptosporidium species are apicomplexan parasites that infect the microvillus border of the gastrointestinal epithelium of a wide range of vertebrate hosts, including humans (Xiao *et al*., 2004) causing diarrhea. In many animals, *Cryptosporidium* infection is not associated with clinical signs though there could be acute self-limiting illness. There are 18 valid *Cryptosporidium* specie and over 40 genotypes with new genotypes continually being discovered (Slapeta, 2016; Fayer, 2018). However most studies on the biology, morphology, biochemistry, host preferences, immunology, pathogenicity, physiology and prevalence have been conducted on one species. *Cryptosporidium parvum* because it is both of veterinary and medical importance, geographically widespread, infects many host species (this makes it more easily obtainable for studies than other species) and can be grown and tested *in vitro* and in animal models (Fayer, 2018). The genus *Cryptosporidium* is one of over 300 genera that includes 4800 named species in the phylum apicomplexa (Fayer, 2018). One major reason for the long disputes in *Cryptosporidium* taxonomy is the difficulty in fulfilling the definition of biological species. Definition of species as being groups of interbreeding populations isolated reproductively from other groups cannot be applied to *Cryptosporidium*, because it is difficult to conduct genetic crossing studies with many *Cryptosporidium* specie (Xiao *et al.*, 2004). Morphology, especially oocysts measurements, represents the cornerstone of apicomplexan taxonomy. For an adequate description of a new *Cryptosporidium* specie,

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oocysts morphometric alone cannot be used rather a combination of experimental transmission, followed by electron microscopy has proven useful. Other methods are infectivity which has been used at times, and finally host specificity and biochemical differences (Maikai *et al*., 2012; Akinkuotu *et al*., 2014). Molecular studies have uncovered an overwhelming amount of genetic diversity within the genus *Cryptosporidium* and this has become a key essential element in defining a new *Cryptosporidium* species (Da Silva *et al*., 2013). There is evidence of emergence of numerous genotypes of the parasite of animal origin that are zoonotic worldwide (Maikai *et al*., 2012). Therefore results from this study can enhance public health.

MATERIALS AND METHODS

STUDY DESIGN

This study was a prospective cross-sectional study carried out between December, 2018 and October, 2019.

STUDY AREA

Sokoto state is geographically located at the North Western part of Nigeria between longitude $11^0$30' to 13^0 50', East and latitude 4^0 to 6^0 40', North. The state has common borders with Niger Republic to the North, Kebbi State-Nigeria to the South and Zamfara State-Nigeria to the East (Bala *et al.*, 2014). It falls in the dry Sahel surrounded by sandy Sudan type Savannah (Bala *et al.*, 2014). Four Local Government Areas (LGA) were selected from the state using simple random sampling method from the four agricultural zones of Sokoto state (one LGA from each zone) as outlined by the State Ministry of Agriculture (Figure 1). They include: Yabo, Sokoto, Wurno and Gwadabawa LGA's.

TARGET POPULATION

Faecal samples of cattle were collected from four agricultural zones of the state using simple random sampling criteria i.e. Wurno LGA (Isah zone), Tambuwal LGA (Tambuwal zone), Tureta LGA (Sokoto zone) and Gwadabawa LGA (Gwadabawa zone) in Sokoto State. The samples were collected from farms, herds and household within the study areas.

Inclusion criteria were (i) owners consent and approval and (ii) cattle that were day old to one year old with or without diarrhea.

QUESTIONNAIRE

A close ended questionnaire was also administered to animal owners for information on each selected animal, while samples were collected.

FECAL SAMPLE COLLECTION

Three hundred and eighty four faecal samples were collected in totality and 8-10 samples were collected on weekly basis. Convenience and simple random sampling methods were employed during collection of the faecal samples the faecal samples collection. The fecal sample-analyses were carried out at Central Research Laboratory of Usmanu Danfodiyo University, Faculty of Veterinary Medicine, City Campus Complex Sokoto, Sokoto State. While the molecular analysis of the Copro-ELISA positive samples was carried out at DNA Labs Limited, RC: 1027690, Q5 Danja Road, Off Katuru Road, Unguwar Sarki, Kaduna.

DETECTION OF *CRYPTOSPORIDIUM* COPRO-ANTIGENS BY COPRO-ELISA

Detection of *Cryptosporidium* species coproantigens in the samples was done using a commercially available Copro-ELISA kit for faecal sample (*Cryptosporidium* Copro-Enzyme Linked Immunosorbent Assay™ for Humans manufactured by Savyon® Diagnostics Ltd., Ashdod, Israel). The procedure was carried out according to the manufacturer's instruction as follows: 0.1g of each faecal sample was homogenized in 300µl of sample dilution buffer and centrifuged. 200µl of Negative and 100µl of positive controls, and 100µl of each of the sampled specimens were added in different wells of a microtitre plate coated with anti-*Cryptosporidium* species antibodies and incubated at room temperature for 1 hour. The plate was washed five times with a washing buffer (300µl), 100µl of HRP-Conjugate was added and incubated at room temperature for one hour and washed five times. 100µl of TMB-Substrate was then added and incubated for 15 minutes, a 100µl of stop solution was added to each of the wells and read using the ELISA reader (BIOTEX; Model: ELx800, Biotex Instruments, USA) at 450/620nm. Samples with Optical Density (OD) reading higher than 1.0 were read as positive, while those with OD lesser than 1.0 were reported as negative for *Cryptosporidium* coproantigens.

A Nested Polymerase Chain Reaction (SSU rRNA PCR) and DNA sequencing was used in analyzing all Copro-ELISA positive samples.

DNA EXTRACTION

Thirty one *Cryptosporidium* Copro-ELISA positive samples were frozen at -80°C with 75% ethanol (Jongwutiwes *et al*., 2002). Specimens stored in formalin were washed off thoroughly by centrifugation and rinsing with cold distilled water six times in order to remove preservative prior to DNA extraction. Pea sizes of each sample (about 200 mg) in formalin was suspended in 500µl of ASL buffer and then vortexed for 30 seconds. DNA was extracted from fecal samples by the QIAamp® DNA stool mini kit (QIAGEN Ltd., Crawley, West Sussex, UK) according to the manufacturer's instructions at DNA LABS Ltd, Ungwar Sarki Kaduna. The DNA was further purified following the manufacturer-suggested procedures included in the QIAamp DNA Stool Mini Kit and stored at -20°C until it was used for PCR assays.

NESTED PCR AMPLIFICATION AND RFLP ANALYSIS

The 18S rRNA gene was amplified using the nested PCR protocol as described by Xiao *et al*. (1999). A primary PCR step gave a PCR product that was about 1,325 bp long, followed by a secondary amplification of an internal fragment with a length of approximately 840 bp. This fragment has been shown to be very specific to *Cryptosporidium* species and for genotyping. Then, the different species were identified by applying restriction digestion of the secondary product. The primary PCR was amplified by using primers 5-TTC TAG AGC TAA TAC ATG CG-3 and 5- CCC TAA TCC TTC GAA ACA GGA-3. Each reaction mixture contained 10µl of Perkin-Elmer 10× PCR buffer, 12µl MgCl2, 8µl dNTPs, 2.5µl of each primer, 0.5µl of *Taq* DNA polymerase, 2µl of DNA template, 1µl bovine serum albumin (1%) and HPLC water to make a final volume of 50µl. The PCR reactions were carried out in a Techne Thermal cycler (Techne Ltd., Cambridge, UK) using the following PCR protocol; an initial hot start at 94°C for 3 minutes, followed by 35 cycles, each consisting of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 1 minute; and a final extension step at 72°C for 7 minutes. Positive and negative controls were included in every PCR reaction. PCR products were visualized by UV after electrophoresis in 1% agarose gel and staining with ethidium bromide. For the secondary PCR step, a PCR product that was 819 to 825bp long (depending on the species) was amplified by using 2µl of the primary PCR product and primers 5-GGA AGG GTT GTA TTT ATT AGA TAA AG-3 and 5-AAG GAG TAA GGA ACA ACC TCC A-3. The PCR mixture and cycling conditions were identical to the conditions used for the primary PCR step, except that 6µl of MgCl2 (3mM) was used in the PCR mixture (Jongwutiwes *et al*., 2002). RFLP analysis of the secondary PCR product was carried out by digesting with *SspI* for species identification and with *Vsp*I for genotyping of *Cryptosporidium* species as described previously (Jongwutiwes *et al*., 2002). Briefly, for the restriction digestion (37°C for 80 minutes), each reaction mixture contained 15µl of the secondary product, 1µl of *Ssp*I (20 U), 2.5µl of restriction buffer and 11.5µl of HPLC water to make a final volume of 30 µl for species identification. Furthermore, *Vsp*I (Boehringer Mannheim, Germany) at the same concentration described for *SspI* was used for genotyping. The digestion products were separated on a 2% agarose gel and visualized after ethidium bromide staining. Isolates were assembled according to their RFLP patterns, and a representative of each group was selected for sequence analysis.

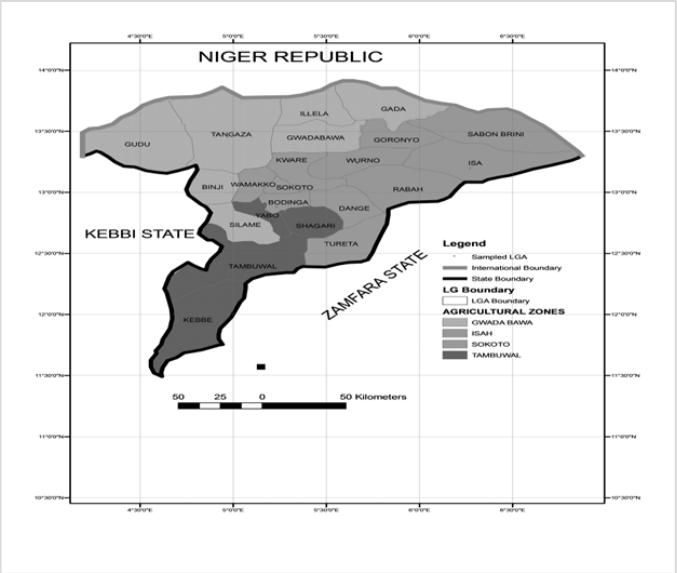

Figure 1: Map of Sokoto state-Nigeria showing the four Agricultural zones. Source: Sokoto State Ministry of Animal Health and Fishery Development.

SEQUENCING

PCR products were sequenced directly following purification using either QIAquick Gel Extraction Kit (QIAGEN, West Sussex, UK) or the MicroSpin Columns Kit (Amersham Biosciences, Buckinghamshire, UK). The ChromasPro programme version 1.3 (2003, Technelysium Pty. Ltd., Australia) was used to read all the amplicon sequences. DNASTAR version 5.06 (2003) was used for editing the consensus sequences and multiple alignments of the DNA sequences. To confirm the identity of the sequences from the GenBank, Blast Local Alignment Search Tool (BLAST) searches in National Center for Biotechnology Information (NCBI) were undertaken. The DNA distance based Neighbor Joining (NJ) analysis was performed by using the phylogenetic analysis software TREECON for windows version 1.3b. The GC/AT content was determined by using a programme on the European Bioinformatics Institute (EBI) site (www.ebi.ac.uk).

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The five sequences used in this study have been deposited in the GenBank database under the accession no DQ002918.1, DQ010950.1, DQ010951.1, JX547008.1 and JX547010.1.

STATISTICAL ANALYSIS

Chi-square test was used to test for association within the result variables using Graphpad instat Statistical software version 3.0 for 64-bit PC. Graphpad instat was used to analyze data PC from the questionnaire and determine if there is a relationship between the variables and the infection caused by *Cryptosporidium.* Values were considered to be significantly different when the P-value obtained was less than 0.05.

RESULTS

PREVALENCE OF *CRYPTOSPORIDIUM* SPECIE IDENTIFIED IN CALVES USING SSU RRNA PCR FROM COPRO-ELISA POSITIVE SAMPLES IN RELATION TO EACH LGA SAMPLED

6 (19.4%) out of the 31 (100%) Copro-ELISA positive samples analyzed by Nested PCR produced the expected SSU rRNA gene products of 830 bp region from samples of different locations. The *Cryptosporidium* species isolated from different locations are as follows; Yabo Local Government Area (LGA) had the highest prevalence of 3 (33.3%) species, followed by Wurno LGA with 2 (25%), Sokoto LGA had 1 (14.3%) and Gwadabawa LGA had zero (Table Ia).

C. bovis and *C. ryanae* had the highest prevalence of 6.5% each and both were isolated from Yabo/Wurno and Sokoto/Gwadabawa respectively. *C. andersoni* was isolated from Yabo (3.2%) only while *C. ubiquitum* was detected from Wurno (3.2%) only (Table Ib).

Table Ia: Prevalence of *Cryptosporidium* in calves in Local Government Areas of Sokoto State, Nigeria

LGAs	No of samples Examined (%)	No Positive (Prevalence)
Yabo	9 (29)	3 (50)
Sokoto	7 (22.6)	1 (16.7)
Wurno	8 (25.8)	2 (33.3)
Gwadabawa	7 (22.6)	0 (0)
Total	**31 (100.0)**	**6 (19.4)**

*x^2= 2.22; *p*-value= 0.53 *df = 3

PREVALENCE OF *CRYPTOSPORIDIUM* SPECIE IDENTIFIED IN BREEDS OF CATTLE IN SOKOTO STATE, NIGERIA

Sokoto Gudali were found to be of the highest prevalence (33.3%) followed by White Fulani with a prevalence of 16% (Table III). There was no significant difference in distribution of the infection between the two breeds of cattle (x^2= 0.04; *p*= 0.82).

Table 1b: Prevalence of *Cryptosporidium* specie in calves in LGA's of Sokoto State, Nigeria using SSU rRNA PCR on Copro-ELISA positive samples.

LGAs	C. bovis	C. ryanae	C. andersoni	C. ubiquitum	Total
Yabo	1	0	1	0	2
Sokoto	0	1	0	0	1
Wurno	1	0	0	1	2
Gwadabawa	0	1	0	0	1
Total	2 (6.5%)	2 (6.5%)	1 (3.2%)	1 (3.2%)	6 (9.4%)

PREVALENCE OF *CRYPTOSPORIDIUM* SPECIE AMONG CALVES OF DIFFERENT SEXES, UNDER DIFFERENT MANAGEMENT AND AMONG THOSE WITH DIFFERENT NATURE OF FAECES IN SOKOTO STATE NIGERIA

Though there was a higher prevalence of *Cryptosporidium spp* among male cattle (28.6%) than in females (16.7%), the difference was not statistically significant (OR=0.58: 95% CI on OR: 0.08 < OR < 3.88) (Table IV).

A higher prevalence (27.8%) of *Cryptosporidium* was seen in diarrheic cattle compared to non-diarrheic cattle (7.7%). However, the association was not statistically significant (OR=3.61: 95% CI on OR: 0.36 < OR < 34.71) (Table IV).

Two (28.6%) out of the 7 (22.6%) faecal samples from cattle raised under non-extensive system of management were positive for *Cryptosporidium*, while out of the 24 (77.4%) fecal samples from cattle raised under extensive system of management, 4 (12.5%) were positive for *Cryptosporidium*. There was no statistical significance (Table IV).

PREVALENCE OF *CRYPTOSPORIDIUM* SPECIE IN CALVES IN SOKOTO STATE NIGERIA WITH RELATION TO SOURCE OF WATER

Faecal samples collected from calves that were not drinking water yet, but fed only on their dam's milk had a higher prevalence (25%) of *Cryptosporidium* than those (18.5%) that drank from various sources of water (Table V) though the association was not statistically significant (x^2= 0.61; *p*=0.81) between prevalence of *Cryptosporidium* and source of water.

PREVALENCE OF *CRYPTOSPORIDIUM* IN CALVES THAT COHABITED WITH OTHER ANIMAL-SPECIES IN SOKOTO STATE, NIGERIA

There was a higher prevalence *Cryptosporidium spp* (66.7%) among calves that close contact with dogs and sheep, followed by those that had contact with dog, sheep and chickens (16.7%) and those that had no contact with any other species (14.3%). While dog, sheep, and goat (13.3%) were the least in the prevalence (Table VI). However, the association was not statistically significant (x^2= 2.43; *p*= 0.49).

PREVALENCE OF *CRYPTOSPORIDIUM* SPECIE IN CATTLE RELATION TO STOCK SIZE IN SOKOTO STATE, NIGERIA.

The smaller the number of cattle in each farm, the more the prevalence of *Cryptosporidium* Herds of 201-300

cattle had the highest (21.4%) prevalence, followed by the 0-100 group (20%) and the least prevalence was 100-200 group (16.7%) (Table VII). However, association between stock size and the prevalence was not statistically significant (x^2= 3.71; p= 0.16).

The relationship between *Cryptosporidium* and total number of cattle in each farm or herd is shown in Table VII. The smaller the number of cattle in each farm, the more the prevalence of *Cryptosporidium* as seen in the 201-300 group that has the highest (21.4%) prevalence, followed by the 0-100 group (20%) and the least prevalence was 100-200 group (16.7%). The association was not statistically significant (x^2= 3.71; p= 0.16).

Table III: Prevalence of Cryptosporidium in calves of different Breeds of Cattle in Sokoto State, Nigeria.

Breed	No. examined (%)	Positive samples (%)	Chi-square (x^2)	*p*-value
Sokoto Gudali	6 (19.4)	2 (33.3)	0.04	0.83
White Fulani	25 (80.6)	4 (16)	Df=1	
Total	31 (100)	6 (19.4)		

Table IV: Odds ratio and 95% Confidence interval on influence of sex, management practices and nature of faeces on prevalence of *Cryptosporidium* species in calves in Sokoto state, Nigeria.

Factors	No. examined	Positive samples	Specific rate (%)	Odds ratio (OR)	95% Confidence Interval on OR
Sex					
Male	7	2	28.6	0.58	0.08-3.88
Female	24	4	16.7	1.00	
Faeces					
Non-Diarrheic	13	1	7.7	3.61	0.36-34.71
Diarrheic[ref]	18	5	27.8	1.00	
Management					
Extensive	24	4	12.5	1.71	0.26-11.41
Non-extensive	7	2	28.6	1.00	

Ref: Reference Category

Table IIa: Prevalence of *Cryptosporidium* in calves in Local Government Areas of Sokoto State, Nigeria

Age	No of samples Examined (%)	No Positive
0-90	9 (29)	3 (33.3)
91-180	15 (48.4)	2 (13.3)
181-270	6 (19.4)	1 (16.7)
271-360	1 (3.2)	0 (0)
Total	31 (100)	6 (19.4)

*x2= 1.14; p-value= 0.77; *df = 3

Table IIb: Prevalence of *Cryptosporidium* spp. in young and old calves in Sokoto State, Nigeria

Age (days)	No. examined (%)	Positive samples (%)	*C. bovis* (%)	*C. ryanae* (%)	*C. andersoni* (%)	*C. ubiquitum* (%)
0-180	22 (77.4)	5 (20.8)	2 (8.3)	2 (8.3)	1 (4.2)	0 (0)
181-360	7 (22.6)	1 (4.3)	0 (0)	0 (0)	0 (0)	1 (4.3)
Total	31 (100)	6 (19.4)	2 (6.5)	2 (6.5)	1 (3.23)	1 (3.23)

*x^2= 0.10; p-value= 0.035; df = 1

Table V: Prevalence of Cryptosporidium in calves on their dams` milk and those already drinking from water sources in Sokoto State, Nigeria.

Type of water	No. of water samples examined (%)	Positive samples (%)	Chi-Square	*p*-value
Water Sources	27 (87.1)	5 (18.5)	0.061	0.81
None	4 (12.9)	1 (25)	df=1	
Total	31(100.0)	31 (8.07)		

p > 0.05

**Water Sources: Dam, Pond, River, Stream, Tap, Well water, Borehole*

DISUCUSSION

Lower prevalence of 19.4% using PCR was also observed in the study, probably because the *Cryptosporidium* oocysts may have degraded due some unfavorable environmental factors like temperature as oocysts are affected by both temperatures lower than 5^0C and higher than 15^0C (Guven *et al*., 2013) and also the methodology employed in the study can contribute a lot in the lower prevalence recorded, especially the ELISA kit used. High rates of *Cryptosporidium* infection has been reported in calves 1 to 3 weeks of age (Santin *et al.*, 2004a: Guven *et al.*, 2013: Akinkuotu and Fagbemi, 2014). In this study, most of the calves were older

than 3 weeks of age. Detection of *C. bovis, C. ryanae* and *C. andersoni* in this study is supported by the fact that levels of *C. bovis, C. ryanae* and *C. andersoni* increase with increasing age and in post-weaned calves (2 to 11 months of age) with *C. bovis* and *C. ryanae* being predominant species

Table VI: Prevalence of Cryptosporidium in calves that cohabited with other animal-species in Sokoto State, Nigeria.

Animal in Cohabitation with	**No. faecal samples examined (%)**	**Positive samples (%)**	**Chi-square (x^2)**	***p*-value**
Dog, Sheep, Chicken	6 (19.4)	1 (16.7)	2.43	0.49
Dog, Sheep, Goat	15 (48.4)	2 (13.3)	Df=3	
Dog and Sheep	3 (9.6)	2 (66.7)		
No animals	7 (22.6)	1 (14.3)		
Total	**31 (100)**	**6 (19.4)**		

P>0.05)

*Water Sources: Dam, Pond, River, Stream, Tap, Well water, Borehole

Table VII: Prevalence of Cryptosporidium in calves according to stock size in Sokoto State, Nigeria.

No. Cattle found in herds	**No. faecal samples examined (%)**	**Positive samples (%)**	**Chi-square (x^2)**	***p*-value**
0-100	5 (16.1)	1 (20)	3.71	0.16
101-200	12 (38.7)	2 (16.7)	Df=2	
201-300	14 (45.2)	3 (21.4)		
Total	**31 (100)**	**6 (19.4)**		

P>0.05

in this age group and a drastic drop of infections caused by *C. parvum. C. ubiquitum* observed by this study in Wurno Local Government Area (LGA) of Sokoto state, it is a new specie in the genus previously identified as *Cryptosporidium* cervine genotype (Fayer *et al.*, 2010). The specie has been reported in wild and domestic ruminants, rodents, carnivores and primates including humans (Fayer *et al*., 2010). *C. ubiquitum* has been found in humans nearly all within the past four years and has also been found in all water-sources (Fayer *et al*., 2010). Studies conducted at Erzurum Province, Turkey showed that all infections in calves of 4 weeks of age and younger were caused by *C. parvum*, while low levels of *C. bovis, C. andersoni* and *E. tenella* infections were found in animals older than 12 months with *C. andersoni* as the most prevalent specie (Guven *et al.*, 2013). Also in studies conducted at Intensive Farms at Srilanka by Abeywardena *et al*. (2014), *C. bovis* and *C. ryanae* 5.6% and 7.4% respectively, were detected in calves from the same farm. *C. bovis* and *C. ryanae* appear to be geographically widespread, having been reported in cattle from India, Denmark, China and various regions of the United States of America (Santin *et al.*, 2014a: Fayer *et al.*, 2016, 2017: Feng *et al.*, 2017; Langkjaer *et al*., 2017: Starkey *et al*., 2016). Approximately half the infections in adult cattle in the United States were found to be *C. andersoni* (Fayer *et al*., 2016, 2017). In Denmark, The Czech Republic and Japan, a higher prevalence of *C. andersoni* in older animals have been reported as well (Kvac *et al.*, 2006). Interestingly, Langkjaer *et al.* (2017) in Denmark did not detect *C. andersoni* in animals of any age, so also Helmy *et al*. (2014) in Ismaila Province of Egypt. In Northwestern Nigeria, Kaduna state, Maikai *et al*. (2011) reported a prevalence of 7.2% of *C. bovis, C. ryanae* (4.1%) and *C. andersoni* (2.5%) in native breeds of calves. Although there was concurrent occurrence of *C. bovis* and *C. ryanae* in the same calf and *C. bovis* and *C. andersoni* in another calf (Maikai *et al*., 2011). The overall prevalence of *Cryptosporidium* declines with increasing age (Santin *et al*., 2014a; Fayer *et al*., 2016, 2017; Kvac *et al*., 2006; Langkjaer *et al*., 2017). The absence of *C. parvum* in calves in this study may be due to the fact that age groups of calves studied might not be infected with the *C. parvum*.

The higher rate of *Cryptosporidium* in Yabo and Wurno LGA of Sokoto state may be attributed to the communal nature in which the cattle within the area are subjected to, as Fulani herdsmen usually allow different animals from different herds to graze together and also the presence of

Achida International Livestock Market that is few kilometers away from Wurno LGA, in which different breeds of cattle pass over before they reach the market. This practice was more within these areas than other areas samples. If cattle from one farm or herd is infected, the grazing ground may be contaminated which may serve as a means of infection for others. So also there may be a chance of zoonotic transmission between humans and cattle in Wurno LGA,

because both parties drink and bath from the same river especially during raining season. In 2011, there was an outbreak of *Schistosomiasis* in the same LGA within the people drinking and bathing in the river.

An age-related distribution of commonly occurring *C. parvum, C. bovis, C. ryanae* and *C. andersoni* have been confirmed in numerous studies in various countries (Maikai *et al*., 2011; Guven *et al*., 2013; Helmy *et al*., 2014). The higher prevalence in the younger calves may reflect their susceptibility to infections with low number of oocysts. The higher rate (33.3%) of *Cryptosporidium* in the younger age groups (0-90 days) in this study is in agreement with the reports of Guven *et al*. (2013), Maikai *et al*. (2011) and Helmy *et al*. (2014) who reported a higher prevalence of *Cryptosporidium* infection in younger calves than older ones in Turkey, Nigeria and Egypt respectively.

In the present study, there was a higher prevalence of *C. ubiquitum* (14.3%) than *C. bovis* (8.3%) and *C. ryanae* (8.3%) followed by *C. andersoni* (4.2%). The *C. ubiquitum* in this study may be due to the higher specific rate of older calves sampled. In cattle, only pre-weaned calves are known to be major sources of *C. parvum* (Xiao, 2008). In addition to *C. parvum*, cattle are known to be commonly infected with *C. andersoni, C. bovis* and *C. ryanae*, but rarely *C. ubiquitum* (Fayer *et al*., 2016). The majority of *C. parvum* infections appear to be limited to calves under 8 weeks of age (Santin *et al*., 2004a; Fayer *et al.*, 2006, 2007; Langkjaer *et al*., 2007). Some other studies have demonstrated that *C. bovis* can be detected in calves as from 3 weeks of age (Santin *et al*., 2014a; Fayer *et al*., 2016). Both *C. bovis* and *C. ryanae* are genetically related, but are distinctly related to some other species such as *C. pestis* that infects very young calves up to 2 weeks of age (Slapeta, 2006). Feng *et al*. (2007) reported both *C. bovis* and *C. ryanae* in all age groups of cattle in various locations worldwide. *C. andersoni* on the other hand has been mostly reported in calves older than 4 weeks of age (Santin *et al*., 2014a; Kvac *et al*., 2006). *C. ubiquitum* infects the greatest number of host species of any species of *Cryptosporidium* that has been substantiated by molecular testing (Fayer *et al*., 2010). The lack of host specificity of *C. ubiquitum* and habitat-sharing of its hosts probably contribute to its widespread distribution, because it is the most common *Cryptosporidium* found in pristine water, it is likely some other wild mammals are also hosts (Fayer *et al*., 2010).

Though there was no statistical significance between *Cryptosporidium* isolation and sex of animals, a higher prevalence was seen in males than females, which is similar to the reports of Guven *et al*. (2013), Maikai *et al*. (2009, 2011), Abeywardena *et al*. (2014) and Ibrahim *et al*. (2007). The higher rate among males may be a reflection of increased exposure to source of contamination (Nichols, 2008). It may also be some genetic predisposition of males than females to the oocysts. This needs to be investigated.

The higher rate of *Cryptosporidium* infection in White Fulani than Sokoto Gudali may be due to their susceptibility to *Cryptosporidium* infection, even though the White Fulani breeds are more resistant to other diseases than the Gudalis (Tawah and Rege, 1996). In a studies conducted by Maikai *et al*. (2011) on the native breeds of cattle in Kaduna state, Nigeria, 17.6% and 13.0% prevalence was found in White Fulani and Sokoto Gudali respectively.

There was no significant difference observed in prevalence of *Cryptosporidium* in diarrheic and non-diarrheic calves. The higher prevalence in diarrheic calves than non-diarrheic may be because the calves are infected with species of *Cryptosporidium* that causes diarrhea, although not all *Cryptosporidium* species are associated with diarrhea (Santin *et al*., 2014a, 2008) and so also some infections may go undetected until resolved by the body. Guven *et al*. (2013) reported a similar studies at Erzurum, Turkey in which 12 out the 16 *C. parvum* specie identified were from diarrheic calves and the remaining 4 from non-diarrheic. While Maikai *et al*. (2011) reported a contrast report with higher prevalence in non-diarrheic and lower prevalence in diarrheic calves.

The higher prevalence of *Cryptosporidium* in semi-intensive than extensive system of management may be attributed to the fact that the more the animals are restricted in movement, the more the contaminated environment will serve as a source of infection and the oocysts will continuously be maintained within the environment. Similar studies was reported by Maikai *et al*. (2011) and also there was no statistical significance between the two systems of management and the *Cryptosporidium* species identified.

The lack of association between *Cryptosporidium* and sources of water for the calves in this study may be that the herdsmen did not give accurate information pertaining to sources of water for the cattle, as calves that were not given water to drink were more infected with the oocysts. This in contrast with some studies as varying prevalence of *Cryptosporidium* has been detected in different water sources (Maikai *et al.*, 2012). There was no association between the sources of water and the *Cryptosporidium* specie identified.

There was no association between contact with other animals and *Cryptosporidium* infection in calves. Though other animals like dog, sheep, goat and chicken were found in the different farms/herds, *Cryptosporidium* infection to some degree is host specific (Fayer, 2008) and finding of the oocysts more in calves with contact with other animals may be by coincidence and it may mean that, the calves got infected through other sources not necessarily by close contact with other animals. The organism being studied has shown cross reactivity among different animals (Ayinmode

et al., 2011) and thus can be a reason for the higher prevalence here.

The prevalence of *Cryptosporidium* was shown to be more prevalent in farms/herds with higher specific rate of cattle in the farms/herds than those with less number. This may be due to the difficulties in maintaining a good sanitation in those farms/herds, because of the higher population of cattle as animals faeces were seen littering the environment. Statistically, the association was not significant between *Cryptosporidium* species identified and the farms/herds sampled.

Samples with mixed infections only showed sequencing patterns of the species that dominate the other and in instances were high rates of two species in a sample, the sequencing patterns were distorted with none of the species being able to adequately sequence. The 100% identities of the generated sequences with those already deposited in GenBank implies wide geographical distribution of the isolated species. The arrangement of *C. bovis* and *C. ryanae* with other related isolates like Bovis, Bovis-goat and Deer shows that they are more genetically related to each other than with *C. andersoni.*

CONCLUSION

Findings of this study indicate that *Cryptosporidium* copro-antigens is prevalent in calves in Sokoto state, Nigeria. They also reveal that *C. bovis, C, ryanae, C. andersoni* and *C. ubiquitum* are the most prevalent species in calves. Zoonotic *C. parvum* was not detected in the age groups of calves studied and its absence suggests that bovids in Sokoto and possibly in the whole Nigeria have limited significance as reservoirs for human infections. The study has contributed to a deeper understanding of the species of *Cryptosporidium* in Sokoto state, Nigeria. Epidemiologic data collected in this study could not identify risk factors associated with *Cryptosporidium* infections. This may be due information demanded through the questionnaire.

However, the results have thrown more light on anthroponotic and zoonotic transmission of *Cryptosporidiosis* in calves in Sokoto state, Nigeria as different anthropologic and zoonotic species and the study also suggests that epidemiology of bovine *Cryptosporidiosis* in Sokoto state is distinct from other studies even in Nigeria as it is the first study to identify *Cryptosporidium ubiquitum* in calves. The findings may help in mounting control and prevention strategies against *Cryptosporidiosis* in calves. Results of this study further suggest that the traditional screening or detection methods (Microscopy, Ziehl-Nelson staining etc.) might lead to low detection of *Cryptosporidium* species with low oocysts shedding intensity compared to immunoassays and molecular techniques that are more specific and more sensitive.

Results of our study has thrown more light on anthroponotic and zoonotic transmission of *Cryptosporidiosis* in calves in Sokoto state as different anthroponotic and zoonotic species from the areas were identified and also suggests that the epidemiology of bovine *Cryptosporidiosis* in Sokoto state is distinct with other studies reported in Nigeria in which it is the first study that identify *Cryptosporidium ubiquitum* in calves in the region based on the reviewed literature available to us. The findings in this study may help us in mounting a control and prevention strategies against Cryptosporidiosis in calves. Results of this study further suggest that the traditional screening or detection methods (Microscopy, Ziehl-Nelson staining etc.) might lead to low detection of *Cryptosporidium* species with low oocysts shedding intensity compared to immunoassays and molecular techniques that are highly specific and sensitive to the parasite.

Of the methods used for this study, Copro-ELISA technique, although reported to be highly sensitive in the detection of *Cryptosporidium* specie have the risk of producing false positive as seen with some samples in this study. Therefore molecular technique may be the best method to detect and characterize the *Cryptosporidium* species in animals as it identifies the parasites up to specie level.

Preventive measures that diminish transmission of *Cryptosporidium spp*. among animals, especially in livestock, should be emphasized on a primary prevention which reduce or eliminate causative risk factors by limiting the amount of animals density in the farms or stocks, minimizing a contraction between personnel, calves, and other herds, keeping young animals or susceptible hosts that have high risk of infection separated from adult animals, and keeping a short calving period of animals which may decrease the opportunities for *Cryptosporidium spp*. to spread within animal herds (Pumipuntu & Piratae, 2018).

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CONFLICT OF INTEREST

There is no conflict of interest whatsoever.

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