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Cross-Reactivity of Some *Cryptosporidium* Species with *Cryptosporidium parvum* Coproantigen in a Commercial ELISA Kit

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Summary

To obtain information about the prevalence of *Cryptosporidium parvum* in cattle in Oyo State, Nigeria, a commercially available enzyme-linked immunosorbent assay (ELISA) kit made from Cryptosporidium-specific antibodies raised against antigens of Cryptosporidium parvum was used to screen 406 randomly collected fecal samples from cattle in four White Fulani herds. The overall prevalence was 32.3% (131/406) with 44 % (37/84), 36.5% (35/96) and 26.1% (59/226) prevalence in ages < 6 months, 6 – 12 months and > 12 months respectively. Further analysis of some of the ELISA-positive samples using nested polymerase chain reaction-restriction fragment length polymorphism (PCR– RFLP) and DNA sequencing of the small subunit (18S) rRNA gene identified the isolates as *Cryptosporidium bovis* and *C. ryanae*.

The use of molecular tools showed that there is cross-reactivity between *C. bovis* and *C. ryanae* with the Cryptosporidium-specific antibodies raised against antigens of *Cryptosporidium parvum*, thereby eliminating the false alarm about the possible risk of zoonotic transmission in the study area. This work showed that unlike some other livestock diseases where commercially available ELISA kits are often relied on as diagnostic tools, ELISA kits obtained from Cryptosporidium-specific antibodies are not reliable for assessing risk of zoonosis in epidemiological studies.

KEYWORDS: Cryptosporidium, ELISA, PCR-RFLP, Antibodies, Cattle, Nigeria.

INTRODUCTION

Cryptosporidium parvum is an apicomplexan protozoan parasite that is increasingly being recognized to be of public health concern in that it causes diarrhoea and gastroenteritis in immunocompromised humans such as those with Acquired Immune Deficiency Syndrome (AIDS) (Current and Garcia, 1991). Cattle have been implicated as the source of the infectious, environmentally resistant oocysts that can be transmitted either by direct contact or indirectly through the consumption of contaminated food or water (Fayer *et al.*, 2000).

Diagnosis of Cryptosporidiosis is usually carried out by conventional method of examination of fecal smears or concentrates using acid-fast stains. These methods are tedious, time-consuming and require experienced personnel to identify the organism. The need for a test that is rapid, easy to perform and interpret, and cost-effective has led to the development of immunoassay techniques like immuno-fluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) for the diagnosis of Cryptosporidiosis (Garcia *et al.*, 1983). Commercial *Cryptosporidium* ELISA kits which are rapid, convenient and easy to use are now available as diagnostic tools for this disease. However, neither microscopy nor ELISA can differentiate *Cryptosporidium species* (Monis and Thompson, 2003) and genotypes/subtypes; *Cryptosporidium s*pecies can only be identified by polymerase chain reaction (PCR)-based techniques (Morgan *et al.,* 1998).

In spite of the limitations of the ELISA technique in the investigation of *Cryptosporidium parvum*, it is still relied on for epidemiological surveys in cattle due to higher sensitivity and ease of use compared to microscopy (Srijan *et al.*, 2005), especially in developing nations where molecular diagnostic tools are not readily available. In Nigeria, the few available reports of cryptosporidiosis in animals were based on microscopy of stained oocysts in feces (Ayeni *et al.*, 1985; Ibrahim *et al.*, 2007), with none utilizing the immunoassay method of investigation. The present study was conducted to assess the performance of a commercially

available *Cryptosporidium parvum* ELISA kit for the detection of *C. parvum* in some native cattle herds in Oyo State, Nigeria.

MATERIALS AND METHODS
Sampling
Stool specimens were collected from 406 cattle randomly selected from farm settlements and kraals in Ibadan, Iwajowa and Akinyele local government areas of Oyo State, Nigeria. Animals were classified based on their age range: less than 6 months of age (84), 7-12 months (96) and greater than 1 year (226). Fecal samples were taken from the rectum of each animal using disposable plastic bag. Each sample was divided into two parts (one part in 2.5% potassium dichromate and the other without preservative) and kept at 4°C until processed.

ELISA and Molecular typing techniques
Cryptosporidium sp. coproantigen was detected using a commercial ELISA kit for stool samples (RIDASCREEN *Cryptosporidium*, R-Biofarm, Germany) following the manufacturer's recommended procedures. The negative and positive controls contained in the kits were used. Optical density (OD) of each sample was measured at 450 nm utilizing a microplate reader (IRE 96, Germany). Samples were considered positive if their extinction is more than 10 % above the calculated cut-off respectively as specified in the formula provided by the manufacturer.

Following detection of *Cryptosporidium antigen* by ELISA, total DNA was extracted from 20 ELISA-positive fecal samples using the FastDNA® Spin Kit for Soil (MP Biologicals, Solon, OH, USA) and stored at - 20°C before being tested in a polymerase chain reaction (PCR) assay. The 826–864 bp fragment small subunit (18S) rRNA gene specific for *Cryptosporidium* spp. was detected in extracted DNA by nested PCR as described previously (Xiao *et al.*, 1999; Feng *et al.*, 2007). DNA of *C. meleagridis* was used as the positive control. PCR products were visualized on 1% agarose gel after ethidium bromide staining.

For *Cryptosporidium* genotyping, a restriction fragment length polymorphism (RFLP) analysis of secondary PCR products was conducted using the restriction enzymes *Ssp*I and *Mbo*II (New England Biolabs, Beverly, MA, USA) (*Feng et al.,*

2007). The restriction digestion products were separated by electrophoresis on 2% agarose gel and visualized under UV transillumination. *Cryptosporidium* species/genotypes were determined based on RFLP profiles described previously (Feng *et al.*, 2007).

PCR products of the SSU rRNA gene from representative RFLP patterns were sequenced to confirm the identification of *Cryptosporidium* genotypes. They were sequenced in both directions on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the secondary primers and the BigDye1 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequences obtained were aligned with reference sequences obtained from the GenBank using ClustalX software.

RESULTS
The results of the enzyme immunoassay (Table I) showed that 32.3% (131/406) of the fecal samples were positive for *Cryptosporidium* sp. antigen (O.D > 0.332). Cattle < 6 months old had the highest infection rate of 44 % (37/84) while those between 6 – 12 months and greater than 12 months had infection rates of 36.5% (35/96) and 26.1% (59/226) respectively. The prevalence of *Cryptosporidium* infection between the age groups were significantly different ($P < 0.05$), with the infection rate decreasing with increase in age.

Calves < 6 months were about twice and thrice at risk of being infected than those 6 – 12 months old (OR: 1.312; 95%CI: 0.721- 2.386) and > 12 months old (OR: 2.228; 95%CI: 1.327- 3.791) respectively. No significant difference ($P>0.05$) was observed in the infection rates between the sexes and between diarrhoeic and non-diarrhoeic cattle.

TABLE I: The result of ELISA examination for detection of *Cryptosporidium* sp in different age groups of cattle

Age range	No positive (O.D > 0.332) /No. examined	Infection rate (%)	95%CI
< 6months	37/84	44.0	0.3321 - 0.5489
6 - 12 months	35/96	36.5	0.2665 – 0.4626
> 12 months	59/226	26.1	0.2034 – 0.3188
Total	**131/406**	**32.3**	0.2770 – 0.3683

DNA from all the 20 fecal samples generated SSU rRNA PCR products with the expected band of 826 – 864 bp. RFLP analysis produced band patterns indicative of the presence of *C. bovis*, *C. ryanae*, and a mix of both in 13 (65%), 6(30%), and 1 (5.0%) of the samples respectively (Figure 1).

DNA sequencing of the SSU rRNA PCR products from six *C. bovis* and three *C. ryanae*-positive samples confirmed the identification of the *Cryptosporidium* species. The partial SSU rRNA gene sequences obtained had 100% similarity to reference sequences (accession numbers AY120911 and AY741305) obtained from the GenBank for *C. bovis*. It showed 96% identity with reference sequences (accession numbersAY587166 and EU410344) for *C. ryanae*.
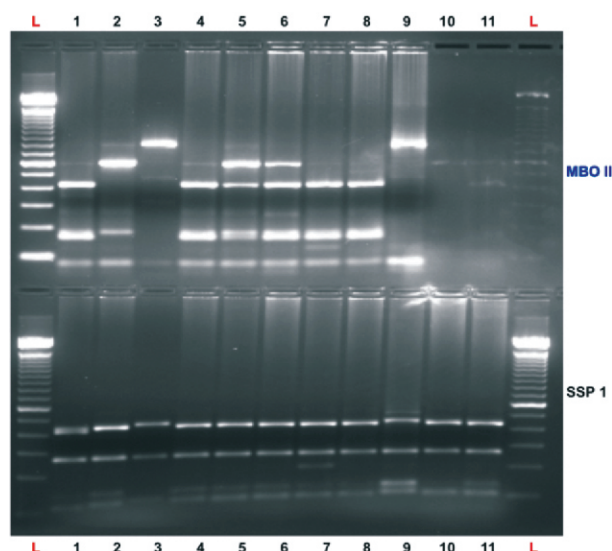
Figure 1. Genotyping of *Cryptosporidium* isolates by RFLP analysis based on digestion of 18S rRNA gene PCR product by SspI (Lower panel) and MboII (upper panel). Lanes 1, 4, 7 & 8: *C. bovis* (412, 185, 162, 76 bp); Lane 3: *C. meleagridis* (18S positive control); lanes 2 & 10: *C. ryanae* (574,185,76 bp); Lanes 5, 6 & 11: Mixed infection of *C. bovis* and *C. ryanae*; Lane 1 & 12: 100bp molecular marker.

DISCUSSION

The results of this study showed that *Cryptosporidium* sp. coproantigen was found in 32.3% (131/406; 95% CI: 27.4–36.8) of animals screened. This result is suggestive of the shedding of *Cryptosporidium* oocysts into the environment from bovine infection and the possibility of human infection in Oyo State, Nigeria. Although it should suffice to attribute the etiology of the infection in the sampled animals to *Cryptospordium parvum,* since the

commercial kit used was made from *Cryptosporidium parvum-specifi*c antibodies, genotypic studies carried out to confirm the species of the isolate did not identify *C. parvum* but *C. bovis* and *C. ryanae* which have not been reported to be of any zoonotic importance. These findings do not only emphasize the inability of the ELISA technique to detect the genotype, they also demonstrate cross-reactivity of *C. bovis* and *C. ryanae* with *C. parvum* antibodies.

Furthermore, while commercially available ELISA test kits are often relied on as diagnostic tools in the surveillance of several livestock diseases, this study showed that these kits are not reliable for assessing the prevalence of *C. parvum* infections in bovines and the result obtained using such kit cannot give any clue to possible risk of zoonosis in the study area. Although commercial ELISA kits have been used for the detection of *Cryptosporidium* coproantigens in faecal samples of canines (Rimhanen-finne *et al.,* 2007; Rinaldi *et al*., 2008) and bovines (Srijan *et al*., 2005; Duranti *et al*., 2009), they are manufactured mainly for diagnosis of *Cryptosporidiosis* in humans.

In conclusion, this study, which to the best of our knowledge, is the first to employ the use of *Cryptosporidium* coproantigen ELISA kit in the study of the prevalence of bovine cryptosporidiosis in Nigerian cattle, suggests that it is not suitable as a stand-alone surveillance tool for bovine cryptosporidiosis, but could be used as a primary screening technique before further analysis using molecular methods such as PCR-RFLP.

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